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Publication date
1997

Published in
Genetics

Large Scale Identification of Genes Involved in Cell Surface Biosynthesis and Architecture in *Saccharomyces cerevisiae*

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Manuscript received April 21, 1997
Accepted for publication June 20, 1997

ABSTRACT

The sequenced yeast genome offers a unique resource for the analysis of eukaryotic cell function and enables genome-wide screens for genes involved in cellular processes. We have identified genes involved in cell surface assembly by screening transposon-mutagenized cells for altered sensitivity to calcofluor white, followed by supplementary screens to further characterize mutant phenotypes. The mutated genes were directly retrieved from genomic DNA and then matched uniquely to a gene in the yeast genome database. Eighty-two genes with apparent perturbation of the cell surface were identified, with mutations in 65 of them displaying at least one further cell surface phenotype in addition to their modified sensitivity to calcofluor. Fifty of these genes were previously known, 17 encoded proteins whose function could be anticipated through sequence homology or previously recognized phenotypes and 15 genes had no previously known phenotype.

DETERMINATION of the *Saccharomyces cerevisiae* genome sequence focuses attention on how to make effective use of this unique resource to provide a global description of eukaryotic cell function (GOFFEAU *et al.* 1996). Strategies to determine the role of each of the approximately 6000 yeast genes, especially the 2400 of unknown function, remain unclear (DUJON 1996). Two main strategies have been proposed (OLIVER 1994, 1996). The ease of gene disruption in yeast has led to efforts to undertake the task of sequentially disrupting every gene in the genome. Such a comprehensive collection of mutants would complement the sequence and aid the study of gene function. A "genome-wide" disruption series has been started by the international yeast community and should be completed in 2–3 years (OLIVER 1996). The collection will be distributed among researchers, who will apply their own specialized phenotypic tests to the mutants.

The hierarchical classification of the many new and unknown yeast genes into families related by function constitutes a second approach (OLIVER 1994, 1996). A potential strength of this strategy is that classifying genes into functional subgroups avoids having to do detailed analysis on each and every gene in the genome. In the simplest case, only those genes within a subgroup are further analyzed by more specific tests. Here we have made an initial attempt to identify a broad functional class of genes: those involved with the biology of the cell surface.

The cell wall is composed of the major polymers, glucan, glucomannoproteins and mannoproteins and chitin, which are synthesized and elaborated into an extracellular matrix (FLEET 1991; BULAWA 1993; HERSCOVICS and ORLEAN 1993; KLIS 1994; LEHLE and TANNER 1995; VAN DER VAART *et al.* 1995). This extracellular matrix constitutes an organelle that is dynamically engaged with the plasma membrane and the underlying secretory organelles (PRYER *et al.* 1992) along with cytoskeletal and cytoplasmic components to maintain cell integrity during growth and morphogenesis (MULHOLLAND *et al.* 1994; CID *et al.* 1995). The cell surface varies in shape and composition throughout the life of a fungal cell; in the budding of vegetative cells, in mating projection formation, in cell fusion in haploid cell conjugation, in spore wall formation following meiosis and in the specialized cell surfaces and morphogenesis seen in pseudohyphal growth (MADDEN *et al.* 1992; FLESCHER *et al.* 1993; KRON *et al.* 1994; MULHOLLAND *et al.* 1994; CHANT and PRINGLE 1995; CID *et al.* 1995). In view of the complexity of this organelle, the number of genes directly or indirectly involved in cell wall synthesis and elaboration is expected to be large. However, only a relatively small fraction of these genes have been identi-

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fied and functionally characterized (KLIS 1994; RAM *et al.* 1994; CID *et al.* 1995). The aim of this study is to identify, phenotypically analyze and attempt to classify genes involved in these processes.

MATERIALS AND METHODS

Yeast strains, culture conditions and methods: All yeast manipulations were done in the AWM3CΔ630 (*MATa cir° leu2-3,2-112 ura3-639 his3-11,3-15*) (VERNET *et al.* 1987), PRY441 (*MATa cir° leu2-Δ1 ura3-52 his3-100 lys2-801a ade2-1° gal3*) or PRY442 (*MATα cir° leu2-Δ1 ura3-52 his3-100 lys2-801ª ade2-1° gal3*) backgrounds. Yeast cells were grown under standard conditions, (YEPD, YNB and Halvorson medium) as previously described (BROWN *et al.* 1994b). Calcofluor white solutions were either prepared fresh at 20 mg/ml and filter sterilized or were prepared at a stock concentration of 10 mg/ml in 50% ethanol and stored, in the dark, at −20° for a period of up to 1 mo. Calcofluor white containing plates were made as follows: calcofluor white solution was added to either pH 6.4 YNB agar (melted and kept at 70°) containing glucose and required supplements or to YEPD agar (melted and kept at 55°) containing glucose.

Generation of transposon-mutagenized yeast library: Haploid strains AWM3CΔ630 and PRY441 were mutagenized using transposon Tn3::*LEU2*::*lacZ* according to BURNS *et al.* (1994). Briefly, a yeast genomic library was mutagenized in *Escherichia coli* to generate a large number of independent gene-containing transposon insertions (kindly provided by Dr. MICHAEL SNYDER). The mutated yeast DNA was then released from vector DNA by digestion with *Not*I and was transformed into the appropriate strains using the LiAc/SS-DNA/PEG procedure (GIETZ *et al.* 1995) or the rapid transformation procedure of SONI *et al.* (1993). Yeast cells carrying the transposon as a recombinational replacement of the genomic copy with the transposon-mutagenized version were selected on synthetic minimal medium with auxotrophic supplements but lacking leucine.

Southern analysis of transposon insertions: In the Tn3::*lacZ*::*LEU2* transposon, the *lacZ* gene is flanked on its 3′ side by an *Eco*RI site. Mutant yeast genomic DNA was consequently digested with *Eco*RI, separated through a 0.8% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labeled-probe covering most of the *lacZ* sequence. The Tn *lacZ*-containing fragment detected after Southern analysis reflects a particular integration event since the other *Eco*RI site (5′ from the Tn *lacZ*) is within the flanking genomic sequence. Each band visualized after autoradiography corresponds to an individual integration event.

Isolation of calcofluor white mutants: Mutagenized AWM3CΔ630 yeast cells were replica plated on YNB plates without leucine containing 20 μg/ml calcofluor white and all mutants that showed calcofluor white hypersensitivity were reverified in a plate assay according to RAM *et al.* (1994). Briefly, mutant AWM3CΔ630 cells were grown to an OD_{600} value of 0.5 and 10^{-1}, 10^{-2}, and 10^{-3} cell dilutions were made. Three microliters of each dilution series were then spotted onto a series of YNB petri dishes containing varying amounts of calcofluor white up to 20 μg/ml. Final identification of mutants was made by scoring for growth after 48 hr at 30°. Mutagenized PRY441 yeast cells were picked and resuspended in YEPD liquid broth in a 96-well dish. Each 96-well dish contained three wells into which the parent strain (PRY441) and two predetermined mutants (one resistant and one hypersensitive) had been inoculated. The transformants were then replica plated, using a pronged manifold, to YEPD solid medium (in rectangular Nunc plates) and allowed to grow for 48 hr. The transformants were then serially diluted using a pronged manifold into 2 × 100 μl ddH_20. Each of the dilutions was then plated, using a pronged manifold onto rectangular plates containing 5, 10 or 15 μg/ml calcofluor white. For reverification, PRY441 mutants were grown overnight at 30° and then diluted to concentrations of ~1000, 100, 10, and 1 cell per μl. Five microliters of each dilution was then spotted onto plates containing 1–15 μg/ml calcofluor white. All mutants obtained showing hypersensitivity or resistance upon reverification were further analyzed.

Mating: To determine if the calcofluor white phenotype resulted from a transposon gene disruption, mutants obtained with strain PRY441 were crossed with PRY442 and the diploids were sporulated. All four spores were analyzed for calcofluor white resistance or hypersensitivity. All mutant phenotypes segregated with the transposon insertion.

Identification of genes causing calcofluor white phenotypes: Transposon-disrupted genes causing calcofluor white phenotypes were identified by plasmid rescue and DNA sequence analysis. Individual mutant yeast cells were transformed with 50–75 ng of *URA3*-based *Hpa*I-linearized pRSQ1 or *Puv*I-linearized YIp5 plasmids using the lithium acetate procedure with sheared, denatured carrier DNA (GIETZ *et al.* 1995) or electroporation (SIMON 1993). Transformants were selected on YNB plates lacking both leucine and uracil. Yeast genomic DNA from each rescued mutant was prepared by the DTAB lysis method as previously described (GUSTINCICH *et al.* 1991; BURNS *et al.* 1994). The recovered genomic DNA was digested overnight by *Eco*RI (pRSQ1) or *Nsi*I (YIp5) and afterwards ligated for 4 hr at 16°. The ligation mixture was transformed in *E. coli* strain DH10B and transformants were selected on ampicillin. Plasmid DNA was prepared from individual colonies and verified by restriction digesting with *Bam*HI plus *Eco*RI (pRSQ1) or *Eco*RI alone (YIp5). Rescued vector pRQS1 results in a 3-kilobase (kb) band with additional bands coming from genomic DNA. Correct rescue of mutant genes with vector YIp5 results after digestion in diagnostic bands of 1.0 and 1.3 kb. The identity of transposon-disrupted genes was made following the determination of the DNA sequence flanking the transposon insertion using an ABI sequencer (Applied Biosystems Inc., model 373A) or manually using the dideoxy chain-termination procedure (SANGER *et al.* 1977).

Computer analysis: DNA sequence and protein homology searches were conducted on the NCBI mail server using the BLAST program (ALTSCHUL *et al.* 1990). DNA and protein sequence analyses were performed using the GeneWorks (Intelligenetics, Mountain View, CA) and Gene Jockey (Biosoft, Cambridge, UK) software packages. Homology searches against GenBank and other major databases served to identify all known *Saccharomyces cerevisiae* genes and their homologues.

Gene disruptions: Deletional disruptions of a number of loci for verification of calcofluor white phenotypes in strain AWM3CΔ630 were made using a PCR-mediated approach (BAUDIN *et al.* 1993; WACH *et al.* 1994). In all, 15 genes suspected of causing calcofluor white phenotypes when mutated (see Tables 2–6) were entirely replaced with a DNA fragment containing a disruption module encoding the Green Fluorescent Protein and the *HIS3* gene (NIEDENTHAL *et al.* 1996). The tested genes/open reading frames (ORFs) include *YBR065c* (*ECM2*), *YKR076w* (*ECM4*), *YMR176w* (*ECM5*), *YEL030w* (*ECM10*), *YBL043w* (*ECM13*), *YHR132c* (*ECM14*), *YJR137c* (*ECM17*), *YDR125c* (*ECM18*), *YLR390w* (*ECM19*), *YBL101c* (*ECM21*), *YHL030w* (*ECM29*), *BUD8*, *HAL5*, *MRE11* and *TFC1*. The DNA fragments used for each disruption were prepared by PCR using plasmid pBM 2983 as a template. In all cases, oligonucleotides used for the production of the disruption DNA fragment contained two sections: a 5′ region

of ~50 nucleotides that corresponds either to the region immediately upstream to the start codon or to the region directly downstream of the stop codon and a 3′ portion (~20 nucleotides) identical to the DNA flanking the GFP-*HIS3* module. Haploid yeast cells were transformed with PCR products. *HIS3* integrants were selected on minimal medium lacking histidine and gene disruptions were confirmed by PCR analysis (BAUDIN *et al.* 1993; NIEDENTHAL *et al.* 1996) (data not shown). Deletional disruptants were checked for calcofluor white phenotypes.

Phenotypic tests: *Mannose:glucose ratios:* The sugar composition of stationary-phase mutant cells was determined as previously described (RAM *et al.* 1994).

Zymolyase sensitivity: Cultures of mutant yeast cells were grown overnight to stationary phase in YNB with all requirements or in YEPD. Cells were washed twice in water and resuspended in 10 mM Tris, pH 7.4. Approximately 1.5×10^7 cells were resuspended in the same buffer containing Zymolyase 20T (ICN, Montréal, Québec) at a concentration of 3 mg/ml. AWM3CΔ630 cell density was measured by OD_{600} at the start of the incubation and again after 1 hr. The decrease of the optical density reflects the proportion of cells that have lysed. A particular AWM3CΔ630 mutant was determined to be Zymolyase hypersensitive when the OD_{600} measured after 1 hr was <50% that of a wild type. In the case of PRY441, mutants were directly scored on plates for growth after treatment. Treated and untreated wild-type (as control) and mutant cells were serially diluted and a certain amount of each dilution series was then spotted onto YNB and YEPD petri dishes.

Hygromycin B/papulacandin B/caffeine sensitivity: Testing of mutants was similar for all three drugs. Hypersensitivity or resistance was determined in the same way as for calcofluor white sensitivity (RAM *et al.* 1994). Briefly, mutant cells were grown to stationary phase, diluted to an OD_{600} value of 0.5 and 10^{-1}, 10^{-2} and 10^{-3} cell dilutions were made. Three microliters of each dilution series were then spotted onto a series of YEPD petri dishes containing varying amounts of each drug, namely 50 and 100 mg/ml for hygromycin B; 1, 1.5 and 3 mg/ml for papulacandin B and 1 and 1.5 mg/ml for caffeine. Final identification of mutants was made by scoring for growth after 48 hr at 30°.

K1 killer toxin: Levels of sensitivity to K1 killer toxin were evaluated by a seeded plate assay using a modified medium consisting of 0.67% YNB, 0.0025% required amino acids, 1.0% Bacto agar, 0.001% methylene blue, 2% glucose and buffered to pH 4.7 with Halvorson minimal medium (BROWN *et al.* 1994b).

RESULTS

Transposon mutagenesis and screening for calcofluor white-hypersensitive and -resistant mutants: To identify genes involved in yeast cell surface assembly, we used transposon mutagenesis and a broad-based phenotypic screen to identify mutants. The mutated genes were retrieved from genomic DNA and identified by a short DNA sequence adjacent to the transposon tag. This procedure obviated the need for genetic complementation approaches to gene identification and enabled large numbers of new *S. cerevisiae* genes to be identified through their phenotypes. The yeast transposon library and the gene recovery and identification methodology were devised by BURNS *et al.* (1994). Similar and complementary approaches have been used by others (DANG 1994; CHUN and GOEBL 1996; MÖSCH and FINK 1997).

To study cell wall elaboration, a primary screen was performed using calcofluor white hypersensitivity (RAM *et al.* 1994). Calcofluor white is a negatively charged fluorescent dye that binds to nascent chains of chitin and, to a lesser extent, glucan through hydrogen bonding and dipole interactions and, by preventing microfibril assembly, interferes directly with the supramolecular organization of the cell wall (ELORZA *et al.* 1983; MURGUI *et al.* 1985; RAM *et al.* 1994). A disturbed or weakened cell wall is not able to withstand drug concentrations that do not affect normal wild-type cells. Preliminary analyses using this screen identified 53 complementation groups affecting cell wall assembly (RAM *et al.* 1994), but only 17 genes have been identified because they must be cloned by complementation (see Table 1). Two different haploid yeast strains (strains AWM3CΔ630 and PRY441) showing significantly different levels of sensitivity to the drug were used. Wild-type cells with the AWM3CΔ630 background start to be affected at calcofluor white concentrations of 20 μg/ml and cannot grow at concentrations >30 μg/ml. Wild-type PRY441 cells are more sensitive as they cannot withstand concentrations of calcofluor white >7 μg/ml.

Approximately 9000 mutated *S. cerevisiae* cells were obtained after transformation of a yeast genomic library previously mutagenized in *E. coli* by transposon Tn3::*lacZ*::*LEU2* (BURNS *et al.* 1994). To assess the extent of the mutagenesis, several verifications were performed. The proportion of disrupted yeast cells producing β-galactosidase, which depends on an in-frame insertion of the transposon into the coding region of an expressed gene and reflects the randomness of the disruption mutagenesis, was ~12% (data not shown), similar to that previously obtained (BURNS *et al.* 1994). Only five of 99 tested mutants had two independently integrated transposons in their genomes based on Southern blot hybridization analysis (data not shown). Thus, the majority of mutants possess only one insertion, a result similar to that found previously (BURNS *et al.* 1994). The mutants were screened for calcofluor white hypersensitivity (see MATERIALS AND METHODS). Of 9000 mutants examined, 67 were stably hypersensitive to calcofluor white; 15 were more resistant to the drug than the parental wild type.

Identification of the genes causing calcofluor white phenotypes: To identify the disrupted genes, all mutants were transformed with a linearized *URA3*-containing "recovery" plasmid and the transposon-rescued mutants selected by their *LEU2* and *URA3* prototrophies. The DNA sequence adjacent to the *lacZ* gene of the transposon was obtained and the genes were identified by comparison to the complete yeast genome sequence. The transposon inserted directly in the ORF coding sequence in 79% of cases, 20% of cases in the immediate 5′ upstream presumed promoter region of

TABLE 1

Identified *CWH* genes

CWH no.	Gene/ ORF	Function	*CWH* no.	Gene/ ORF	Function
1	*MNN9*	Required for *N*-linked outer chain synthesis (R. ZUFFEREY and M. AEBI, personal communication)	26	*VMA1*	Vacuolar H^+-ATPase catalytic subunit (A. RAM, R. SANJUAN and F. KLIS, unpublished data)
2	*VRG1*	Involved in orthovanadate resistance and protein glycosylation (R. ZUFFEREY and M. AEBI, personal communication)	30	*NRK1*	Ser/Thr kinase that interacts with Cdc31p (J. VOSSEN and F. KLIS, unpublished data)
4	*GPI1*	Involved in GPI anchor synthesis (J. VOSSEN and F. KLIS, unpublished data)	32	*VPS16*	Required for vacuolar protein sorting (G. PARAVICINI, personal communication)
6	*GPI3*	*N*-acetylglucosaminyltransferase required for GPI anchor synthesis	36	*YCL007c*	Unknown; 130 aa (M. VAN BERKEL and F. KLIS, unpublished data)
8	*YGR036c*	Unknown; similarity to *T. denticola* phosphatase; 239 aa (M. VAN BERKEL and F. KLIS, unpublished data)	41	*CWH41*	ER protein involved in β1,6-glucan assembly
13	*ERD1*	Required for retention of ER proteins (A. RAM, R. SANJUAN and F. KLIS, unpublished data)	47	*PTC1*	Protein Ser/Thr phosphatase 2c
17	*URE2*	Nitrogen catabolite repression regulator (R. MONTIJN, S. BREKELMANS and F. KLIS, unpublished data)	48	*KRE6*	Golgi protein involved in β1,6-glucan synthesis
			50	*PLC1*	PI-specific phospholipase C (A. RAM, R. SANJUAN and F. KLIS, unpublished data)
			52	*GAS1*	Involved in β1,3-glucan crosslinking
			53	*FKS1*	β1,3-glucan synthase component

aa, amino acid.

a gene and once (*SLN1*; see Table 3) after a stop codon in the 3′ noncoding region. The mutants identified three classes of genes representing a broad spectrum of functional categories: (1) 50 genes of previously known function (61%); (2) 17 encoded proteins having homology to known proteins or possessing some known domain signature or phenotype (21%) and (3) 15 genes (18%) were novel and of unknown function. The genes of the latter categories were serially named *ECM,* for extra cellular mutants. The identified genes were classified according to their sequence similarities and possible roles inferred (see Tables 2–6).

The proportion of genes identified in this way are similar to the proportion of known and unknown genes in the yeast genome as a whole. Thus, importantly, we are revealing novel genes with this screen, not merely uncovering previously identified genes. Of a set of 59 genes from strain AWM3CΔ630, 54 were isolated once, two genes were isolated twice (*KRE6* and *ECM15*), two were isolated three times (*TFC1* and *ECM2*) and one was isolated four times (*MRE11*). Of a set of 25 genes from strain PRY441, 22 were isolated once and three were isolated twice (*TFC1, SLG1* and *ECM34*). Only two genes were identified in both strains (*TFC1* and *ACS1*) during this screen. Of the original set analyzed by RAM *et al.* (1994) only one, *KRE6,* was reisolated here. Thus, the calcofluor white screen is not saturated at this stage and should allow further identification of genes.

Verification of the association of calcofluor white phenotypes with identified genes: To demonstrate that the calcofluor white phenotypes were the result of the identified transposon insertions, a fraction of the genes identified in strain AWM3CΔ630 was entirely disrupted and the calcofluor white phenotype was examined. In all, 15 different genes were deleted in this way and all showed calcofluor white phenotypes similar to, or more severe than, those seen in the original mutants (see MATERIALS AND METHODS). In the case of the 25 mutants obtained in strain PRY441, phenotypic verification was carried out by meiotic co-segregation. Tn3*::lacZ::LEU2* disruption mutants were crossed with strain PRY442 and the resulting diploids sporulated. Tetrad analysis revealed a 2:2 segregation of leucine prototrophy correlating with calcofluor white hypersensitivity or resistance, demonstrating that the phenotype was caused by transposon insertion. It can therefore be concluded that, for the great majority of the mutants, the calcofluor white phenotypes are the direct result of a Tn disruption.

Functional characterization by additional phenotypic tests: The genes identified were considered to be candidates for involvement in cell surface biology. To further characterize the genes of unknown function and to better define the roles of the group of known genes in cell wall elaboration, additional phenotypic screens were performed.

Cell wall composition: The relative proportions of glucose, mannose and *N*-acetylglucosamine (GlcNAc), the three main cell wall hexoses, were determined in all mutants and 57% (47/82) of these showed an alteration in cell wall sugar ratios. These alterations could be grouped in a series from low mannose through normal

TABLE 2

Genes directly involved in cell surface assembly

Gene/ORF (synonyms)[a]	CFW phenotype[b]	Chromosome	Tn insertion[c]	Function/homology/domain[d]	Additional phenotypes[e]
KRE6 *CWH48* *YPR159w*	HS	XVI	CDS (2)	Golgi protein involved in β-1,6 glucan synthesis	M/G↑ Zymolyase-hs Killer-rs
KRE2 *MNT1* *YDR483w*	HS	IV	Promoter	Golgi α1,2-mannosyltransferase	Zymolyase-hs Papulacandin-hs Killer-rs
*CWP2** *YKL096w-a*	HS	XI	CDS	Cell wall mannoprotein	Zymolyase-hs Papulacandin-hs Caffeine-hs
DIT2 *CYO56* *CYP56* *YDR402c*	RS	IV	Promoter	Belongs to the cytochrome P450 family; involved in spore wall maturation	M/G↓

[a] Mutant genes were obtained in strain AWM3CΔ630, in strain PRY441, as indicated by an asterisk (*), mutant genes obtained in both strains, as indicated by a dagger (†, see Table 4).

[b] HS, hypersensitive; RS, resistant.

[c] CDS, coding sequence. Value in parentheses is the number of times a given gene was obtained; insertion was considered in promoter when Tn inserted in a 300-bp region just 5′ from ATG of coding sequence.

[d] For more information consult major on-line databases such as MIPS, SGD and YPD.

[e] M/G, mannose:glucose ratios.

wild-type ratios to low glucose, others had modified levels of GlcNAc (see Tables 2–7). Such an analysis suggests areas of function. For example, defects in genes known to affect glucose transport or glucan synthesis like *RGT2* (MARSHALL-CARLSON *et al.* 1991) (Table 3) and *KRE6* (ROEMER *et al.* 1993) (Table 2) cause reduced cell wall glucose; while mutations in the mannosyltranferase encoding genes *KTR6* (LUSSIER *et al.* 1997) and *ALG9* (BURDA *et al.* 1996) (Table 3) cause reduced proportions of mannose. The amount of chitin in the cell wall is low and mechanisms exist to overproduce it when the cell wall is stressed or through suppression of cell wall mutations (BULAWA 1993; RAM *et al.* 1994). A group of calcofluor white-hypersensitive mutations in 13 genes cause elevated *N*-acetylglucosamine levels, and there are likely to be many and differing reasons for this elevation. A smaller group of mutations in six genes cause reduced levels of GlcNAc and one of these (*lag2*) results in some resistance to calcofluor white, consistent with less chitin to bind the drug, whereas mutations in the other five genes cause hypersensitivity to calcofluor white. Two of the genes that resulted in high levels of GlcNAc when mutated (*MSN1* and *BUD8*) are involved in morphogenic processes (ESTRUCH and CARLSON 1990; ZAHNER *et al.* 1996).

Zymolyase sensitivity: Sensitivity of yeast cells to this β1,3-glucanase and protease-containing yeast lytic preparation was used to monitor changes in cell wall composition and arrangement (DE NOBEL *et al.* 1990; RAM *et al.* 1994). Possible explanations of a greater or diminished accessibility of the glucanase towards cell wall β1,3-glucans include (1) incomplete *N*- and *O*-linked polysaccharides; (2) defect in incorporation of cell wall proteins; (3) diminished levels of branched β1,3-glucan polymers. Using this assay, 26 of 82 mutants tested showed an altered sensitivity to Zymolyase digestion compared to wild-type cells, a phenotype consistent with cell wall defects.

Hygromycin B: Fungi show limited sensitivity to aminoglycoside antibiotics like hygromycin B but yeast cells showing marked *N*-glycosylation defects are rendered sensitive to these drugs (BALLOU *et al.* 1991; DEAN 1995), but the basis for this phenotype is unclear. Twenty-five mutants showed hypersensitivity and two were found to be resistant. *VAN1* (Tables 3 and 7) is a previously recognized gene giving a hygromycin B phenotype (BALLOU *et al.* 1991; DEAN 1995). However, most of the mutants that were obtained in this screen could not be directly attributed to *N*-glycosylation defects. To extend this, we examined a set of cell wall mutants for hypersensitivity to this drug. *kre5* (MEADEN *et al.* 1990), *pmt1* and *pmt2* mutants (LUSSIER *et al.* 1995b; GENTZSCH and TANNER 1996) were all more sensitive than their isogenic parental strain; thus defects in β1,6 glucan synthesis and *O*-mannosylation also lead to hygromycin hypersensitivity (data not shown). Such strains are not generally drug sensitive, as no sensitivity was found with other antibiotics, namely, fusidic acid, emetine, a fluoroquinolone, or viomycin. Thus, sensitivity to this antibiotic constitutes a new and broad screen for cell surface defects and a wide functional variety of genes were obtained.

TABLE 3

Genes plausibly related to the cell surface

Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
				Secretory Pathway	
KTR6 *YPL053c*	HS	XVI	CDS	Member of the *KRE2/MNT1* α1,2-mannosyltransferase gene family	M/G↓ Hygromycin-hs
MAS5 *YDJ1* *YNL064C*	HS	XIV	CDS	Involved in protein import into ER and mitochondria; highly similar to *E. coli* DnaJ	
*VAN1** *VRG7* *YML115c*	HS	XIII	CDS	Vanadate resistance protein; mutants show altered pattens of phosphoproteins and have defective glycosylation; may be involved in retention of enzymes in the ER or Golgi	M/G↓ Zymolyase-hs Hygromycin-hs
*ALG9** *YNL219c*	RS	XIV	CDS	Involved in the step-wise assembly of lipid-linked oligosaccharides in *N*-linked glycosylation; multiple transmembrane domain (TMD) protein	M/G↓ Killer-rs Caffeine-hs
				Morphogenesis	
MSN1 *FUP1* *PHD2* *YOL116w*	HS	XV	CDS	Transcriptional activator for genes regulated through Snf1p (a multicopy suppressor of invertase defect in *SNF1* mutants); required for pseudohyphal form	M/G↑ GlcNAc↑ Hygromycin-hs Papulacandin-hs Caffeine-rs
BUD8 *YLR353w*	HS	XII	Promoter	Required for bipolar budding	GlcNAc↑ Zymolyase-hs Caffeine-rs
*BUD4** *YJR092w*	RS	X	CDS	Required for formation of axial but not bipolar budding	
PPH22 *YDL188c*	RS	V	CDS	Protein serine/threonine phosphatase; involved in cell integrity and morphogenesis	
*DFG16** *YOR030w*	RS	XV	CDS	Involved in invasive growth upon nitrogen starvation; 619 aa; probable multiple TMD protein	M/G↓
				Regulatory/Signal Transduction	
HAL5 *YJL165c*	HS	X	CDS	Protein kinase involved in salt tolerance and pH sensitivity; high homology to C-terminus of Ykl168p	M/G↓ Hygromycin-hs
*YCK2** *YNL154c*	HS	XIV	Promoter	Casein kinase I isoform; mutants have increased salt sensitivity and show defects in morphogenesis; TMD	M/G↓ Papulacandin-hs
*SLN1** *YPD2* *YIL147c*	HS	IX	67 bp after stop codon	Two-component signal transducer; functions in the high osmolarity signal transduction pathway	Hygromycin-hs Caffeine-hs
*SSK2** *YNR031c*	RS	XIV	CDS	MAP kinase kinase kinase of the high osmolarity signal transduction pathway	Caffeine-rs
MKS1 *YNL076w*	HS	XIV	CDS	Negative regulator of RAS-cAMP pathway; involved in carbohydrate utilization regulation	M/G↑ GlcNAc↑ Killer-hs Caffeine-rs
*SNF3** *YDL194w*	HS	IV	CDS	High-affinity glucose transporter; can play a positive or negative role in glucose transport; multiple TMD protein	Zymolyase-hs Hygromycin-hs Papulacandin-hs Caffeine-hs

TABLE 3

Continued

Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
RGT2 *YDL138w*	HS	IV	CDS	Involved in regulation of glucose transport; homology with glucose transport proteins (*e.g.*, Snf3p); multiple TMD protein	M/G↑ GlcNAc↑
EFF2 *YML048w*	HS	XIII	Promoter	Defective in glucose repression; TMD	M/G↓ GlcNAc↑ Hygromycin-hs
*ROM2** *YLR371w*	HS	XII	CDS	Putative GDP-GTP exchange protein for Rho1p which is involved in regulation of β1,3-glucan synthesis	Caffeine-hs
SWI6 *YLR182w*	HS	XII	CDS	Involved in cell cycle regulation and in controlling the expression of some genes involved in cell wall biosynthesis	

aa, amino acid. * and †, for explanation, see Table 2.

Papulacandin B: The glycolipid papulacandin B is thought to be an inhibitor of β1,3-glucan synthesis. It has been postulated that it may directly hinder some components of the β1,3-glucan synthase complex (BAGULEY *et al.* 1979; KOPECKA 1984; RAM *et al.* 1994) or inhibit incorporation of β1,3-glucans into the molecular organization of the extracellular matrix (FONT DE MORA *et al.* 1993). Mutations in 22 genes resulted in papulacandin B-hypersensitivity phenotypes, though these showed no correlation with altered levels of glucose, mannose or GlcNac as was previously found (RAM *et al.* 1994). Consistent with this, hypersensitivity to this drug could not be specifically linked to cell wall β1,3-glucan defects (RAM *et al.* 1994). In the RAM *et al.* (1994) calcofluor mutant collection, three mutated yeast strains (*cwh26*, *cwh32* and *cwh53-1*) additionally showing papulacandin B hypersensitivity had their causative gene isolated (see Table 1): *FKS1* encodes a subunit of the 1,3-β-D-glucan synthase (RAM *et al.* 1995) and can obviously be directly linked to this type of defect. However, the two other identified genes (*VMA1* and *VPS16*; see Table 1) encode vacuolar proteins (CONIBEAR and STEVENS 1995). Both our screen and that of RAM *et al.* (1994) indicate that papulacandin B hypersensitivity is not β1,3-glucan specific, but permits detection of a broad range of cell wall defects.

K1 killer toxin: K1 killer yeast strains secrete a small pore-forming toxin that requires a cell wall receptor for function (BUSSEY 1991). Killer resistant mutants have been found to be defective in β1,6-glucan and in *O*-mannosylation, indicating that the *in vivo* receptor includes these polymers (BOONE *et al.* 1990; HILL *et al.* 1992; ROEMER *et al.* 1993; GENTZSCH and TANNER 1996). Five genes, all known, were identified here with resistance phenotypes, three in expected classes (*ALG9*, *KRE2*, *KRE6*), two unexpected, *IMP2'* (DONNINI *et al.* 1992) and *PAS8* (VOORN-BROUWER *et al.* 1993), with other wall phenotypes. A larger class of eight mutants led to killer toxin hypersensitivity and these have never been previously screened for in a systematic way. Mutations leading to wall defects that retain a wall receptor can lead to hypersensitivity. Disruption of *PKC1* (ERREDE and LEVIN 1993) with a wall with reduced amounts of all polymers or *PBS2* lead to hypersensitivity (ROEMER *et al.* 1994; JIANG *et al.* 1995); this may be a good indicator of wall changes. Three known genes associated with this phenotype are regulatory, the five novel ones are associated with a range of additional wall phenotypes.

Caffeine: This drug is an inhibitor of cAMP phosphodiesterases (PARSONS *et al.* 1988). Several mutants involved in growth control and in the *PKC1-MPK1* signal transduction pathway show increased sensitivity to caffeine (COSTIGAN *et al.* 1992; PARAVICINI *et al.* 1992; POSAS *et al.* 1993; RAM *et al.* 1994). This phenotype is loosely indicative of a defect in regulation/signal transduction, and all mutants were tested for growth in the presence of this drug. Caffeine sensitivity is a common phenotype among this calcofluor collection (24%), with five resistant and 15 hypersensitive mutants. Among the 15 known genes identified, nine can reasonably be termed regulatory. *MKS1*, which acts as a negative regulator of the RAS-cAMP pathway (MATSUURA and ANRAKU 1993), was picked up in the screen.

Morphology: Because the cell wall determines cellular architecture, cells of the different mutants were evaluated for altered morphology. Four mutants were found to possess an abnormal morphology when compared to the wild type. All four mutants (*ecm2*, *ecm5*, *ecm19* and *ecm20*) showed a similar morphology: cells were enlarged, having a cellular volume of up to four times greater than wild-type cells, and had large drooping

TABLE 4

Genes not previously related to the cell surface

Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
			Metabolism		
URA7 *YBL039c*	HS	II	CDS	CTP synthase 1; last step in pyrimidine biosynthesis pathway; activated by GTP and inhibited by CTP; TMD	M/G↓
ACS1† *YAL054c*	HS	I	CDS	Acetyl-CoA synthetase (acetate-CoA ligase)	Zymolyase-hs
GDH3 *YAL062w*	HS	I	CDS	NADP-glutamate dehydrogenase 3	
ORD1 *YKL184w*	HS	XI	Promoter	Ornithine decarboxylyase	M/G↑ Zymolyase-hs
FOX2 *YKR009c*	HS	XI	Promoter	Bifunctional β-oxidation peroxisomal protein	M/G↑ Hygromycin-hs
*SPE3** *YPR069c*	RS	XVI	CDS	Putrescine aminopropyltransferase (spermidine synthase)	
*CIS2** *YLR299w*	RS	XII	CDS	Gamma-glutamyltransferase homologue	
ARG7 *YMR062c*	RS	XIII	CDS	Acetylornithine acetyltransferase	
			Mitochondria related		
*IMP2'** *YIL154c*	HS	IX	CDS	Nuclear gene controlling the mitochondrial dependence of galactose, raffinose and maltose utilization; TMD	Zymolyase-rs Hygromycin-hs Killer-rs Caffeine-hs
IFM1 *YOL023w*	HS	XV	CDS	Mitochondrial translation initiation factor 2	Zymolyase-hs Papulacandin-hs
SMP2 *YMR165c*	HS	XIII	CDS	Null mutant has increased plasmid stability and respiration-deficient phenotype	M/G↑ GlcNAc↑ Killer-hs
COX11 *YPL132w*	HS	XVI	CDS	Required for cytochrome oxidase assembly	
PEL1 *YCL004w*	HS	III	Promoter	CDP diacylglycerol-serine *O*-phosphatidyltransferase; required for survival of petite mutants	GlcNAC↓ Zymolyase-hs Papulacandin-hs Caffeine-hs
			Nucleic acids		
MRE11 *YMR224*	HS	XIII	CDS (4)	DNA repair protein; member of the *RAD52* epistasis group; required for double-strand repair and meiotic recombination	M/G↑ GlcNAc↓ Papulacandin-hs Killer-hs
HEL1 *YER176w*	HS	V	Promoter	DNA helicase I	M/G↑ Hygromycin-hs Papulacandin-hs
*RAD23** *YEL037c*	RS	V	CDS	Nucleotide excision repair protein	
MEC3 *YLR228c*	HS	XII	Promoter	Checkpoint protein required for arrest in G2 after DNA damage	M/G↓ Caffeine-hs

TABLE 4

Continued

Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
RNH1 *YMR234w*	HS	XIII	Promoter	Ribonuclease H; degrades specifically RNA-DNA hybrids	M/G↑ Hygromycin-hs Papulacandin-hs Caffeine-hs
PAN3 *YKL025c*	RS	XI	CDS	Subunit of the Pab1p-dependent poly (A) nuclease; similarity to *C. elegans* hypothetical protein ZK632.7	
SRD2 *YPL021w*	HS	XVI	CDS	Homologue of Srd1p that affects pre-rRNA processing	M/G↓
HCA4 *YJL033w*	HS	X	CDS	DEAD and DEAH box families ATP-dependent RNA helicase	M/G↓ Zymolyase-hs
			RNA polymerase III		
TFC1† *YBR123c*	HS	II	Promoter (1) CDS (4)	95-kD subunit of TFIIIC (TAU); mediates tRNA and 5s RNA gene activation by binding to intragenic promoter elements	M/G↓ Zymolyase-hs
RPC34 *YNR003c*	HS	XIV	CDS	DNA-directed RNA polymerase (III) chain	M/G↓ Hygromycin-hs
			Others		
YEF3 *TEF3* *EFC1* *YLR249w*	HS	XII	CDS	Translation elongation factor eF-3; stimulates EF-1 α-dependent binding of aminoacyl-tRNA by the ribosome; requirement for EF-3 is unique to fungi; member of ATP-binding cassette (ABC) family	GlcNAc↑ Zymolyase-hs
STF2 *YGR008c*	HS	VII	Promoter	ATPase stabilizing factor	M/G↓
*PAS8** *YNL329c*	RS	XIV	CDS	Peroxisomal assembly protein	Papulacandin-hs Killer-rs
LAG2 *YOL025w*	RS	XV	CDS	Determines yeast longevity; TMD	GlcNAc↓

* and †, for explanation, see Table 2.

buds with an elongated neck (data not shown). Interestingly, these mutants have been found to be pleiotropic, possessing many of the tested phenotypes.

DISCUSSION

The mapping and sequencing phase of the yeast genome is complete, and work now focuses on functional analysis of the component genes. Systematic functional analysis of this magnitude breaks new ground in genomics. For yeast genes of unknown function, and for many of the already known genes, definitive roles remain to be determined. Here we show that broad "genome-wide" screens are possible and constitute an immediate approach to functional analysis. Transposon mutagenesis/gene recovery technology coupled with a calcofluor white screen is an efficient approach to identifying genes with mutations that cause defects in all the major cell surface polymers.

Classification by sequence similarity to genes of known function: The genes sampled represent a rich cross section of the yeast genome and include known genes, some not previously suspected to be involved in cell surface biology, and genes of unknown function found by systematic sequencing. Initial characterizations of the mutant gene collection by sequence similarity, from which function can often be inferred, has permitted their categorization into broad and sometimes overlapping classes that are outlined illustratively in Tables 2–6.

Known genes related to cell wall assembly: In some cases, the transposon integrated in genes of known function with a clear role in cell wall elaboration or structure (see Table 2). Other genes can reasonably be related

TABLE 5

Genes of unknown function having a recognized signature, sequence similarity or a previously known phenotype

ECM no.	Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
	DCG1 *YIR030c*	HS	IX	CDS	Unknown; transcript level sensitive to nitrogen-catabolite repression; TMD	GlcNAc↓ Zymolyase-hs Caffeine-hs
	*SLG1** *YOR008c*	HS	XV	CDS (2)	Unknown; weak similarity to Ynl283p and *Leishmania mexicana* Imsap2 gene (secreted acid phosphatase 2); 378 aa	M/G↓ Hygromycin-hs Papulacandin-hs Caffeine-hs
10	*YEL030w*	HS	V	CDS	Heat shock protein signature; 644 aa (70 kD); similarity to Pmr1p and Ens1p	M/G↑ Hygromycin-hs
14	*YHR132c*	HS	VIII	CDS	Similarity to zinc carboxypeptidase family; 430 aa; TMD	M/G↑ Hygromycin-hs Papulacandin-hs
15	*YBL001c*	HS	II	CDS (2)	Unknown, 104 aa, partial homology to *Staphylococcus xylosus* glucose kinase	Hygromycin-hs
17	*YJR137c*	HS	X	CDS	Putative sulfite reductase (ferredoxin); 1442 aa; homology to yo72h02.s1 *Homo sapiens* cDNA clone 1	Zymolyase-hs Hygromycin-hs
18	*YDR125c*	HS	IV	CDS	Unknown; 453 aa; some similarity to aromatic hydrocarbon catabolism esterase; similarity to hypothetical protein Ylr099p (Ch XII)	
31	*YBR176w**	HS	II	CDS	Strong similarity to *E. coli* 3-methyl-2-oxobutanoate hydroxymethyltransferase; 312 aa	M/G↑ Hygromycin-hs Papulacandin-hs
16	*YMR128w*	HS	XIII	CDS	DEAD and DEAH box families ATP-dependent helicase signature; 1267 aa	GlcNAc↑ Zymolyase-hs Hygromycin-hs Papulacandin-hs
20	*YGR195w*	HS	VII	CDS	Unknown; 256 aa; TMD; 22% identity to *E. coli* ribonuclease over 195 aa	M/G↑ GlcNAc↑ Hygromycin-hs Papulacandin-hs Killer-hs Caffeine-hs Abnormal morphology
25	*YJL201w*	HS	X	CDS	Unknown; 599 aa; promoter has a consensus sequence for factor Abf1p	
30	*YLR436c**	HS	XII	CDS	Unknown; 1274 aa; probable multiple TMD protein; has phosphopantetheine attachment site	M/G↓
27	*YJR106w*	HS	X	CDS	Unknown; 725 aa; weak similarity to Na^+/H^+ antiporter; probable multiple TMD protein	Papulacandin-hs

TABLE 5

Continued

ECM no.	Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
39	*YNR030w**	RS	XIV	CDS	Unknown; 551 aa; probable multiple TMD protein; weak similarity to SMP3p	Papulacandin-hs
33	*YBR078w**	HS	II	CDS	Unknown; strong similarity to sporulation specific Sps2p and to Ydr055p; 468 aa	M/G↓ Zymolyase-hs Hygromycin-hs Killer-hs
5	*YMR176w*	HS	XIII	CDS	Unknown; 1411 aa; some similarity to SW:X169_Human; contains ATP/GTP-binding site motif A	M/G↑ GlcNAc↑ Hygromycin-hs Caffeine-hs Abnormal morphology
8	*YBR076w*	HS	II	CDS	Unknown; 339 aa; similarity to surface antigens from trophoblast endothelial-activated lymphocytes	Killer-hs Hygromycin-rs

aa, amino acid. * and †, for explanation, see Table 2.

to processes impinging on the cell surface (see Table 3). These include genes that function in the secretory pathway or that are involved in maintaining cellular integrity or required in morphogenesis or for the pseudohyphal cell type.

Known genes with an unanticipated involvement in the cell surface: The unexpected association of some known cellular genes with cell wall phenotypes (Table 4) emphasizes the value of genome-wide screens to define function and to examine global aspects of regulation in the yeast cell. Such genes perform a wide range of roles, ranging from involvement in metabolism, mitochondrial function, transcription, translation and DNA repair. Many of the effects seen in these mutants are likely indirect. For example, a yeast cell that transcribes or translates incorrectly because of enzymatic machinery problems may produce defective proteins and ultimately the cell wall and other cellular organelles will have a modified composition. However, one must not overlook possible regulatory associations between cellular pathways and cell wall synthesis and assembly. A prime candidate for such an association is *MRE11* (Table 4); it classically encodes a DNA repair protein (JOHZUKA and OGAWA 1995) and appears incongruous here. Mutations in the gene were isolated independently four times and have a range of strong cell surface phenotypes. While we do not understand what is happening in *mre11* mutants, we can draw on our knowledge of prokaryotes for precedents. DNA synthesis and repair and the SOS response are known to lead to cell surface morphological changes, osmoresistance and filamentous growth in *E. coli* and *Bacillus subtilis* (ENNIS *et al.* 1993; RUZAL *et al.* 1994) Further work will be required to establish if we have uncovered an analogous "global" response in yeast.

Another possible example of a global regulatory response is illustrated by mitochondrial defects that appear to perturb the yeast cell surface. *IFM1, SMP2* and *COX11* are all nuclear petite genes (VAMBUTAS *et al.* 1991; IRIE *et al.* 1993; TZAGOLOFF *et al.* 1993) with cell surface phenotypes. Again this relationship seems unexpected. However, there is an earlier literature on this theme that, perhaps because of an underlying lack of an explanatory paradigm, has been overlooked (EVANS *et al.* 1980; WILKIE *et al.* 1983). Our results independently suggest that there may be some regulatory link between mitochondrial function and the cell surface.

These results are surprising and can be viewed in two ways. One can dismiss them as nonspecific or indirect or indicative of the bluntness of the primary calcofluor screen. Alternatively, mutations in these genes, which do give strong cell wall phenotypes consistent with the screen working, are identifying unanticipated interactions of these genes. Finding new roles for established genes is bound to be a controversial activity but is likely to be an important and general outcome of genome-wide functional screens.

ORFs for which there is limited functional information or no known function: Some ORFs gave a match defining the biochemical class but not the specific function of the gene (Table 5). In a limited number of cases, the biochemical role of the gene is unknown but some superficial information about function has been reported. ORFs of totally unknown function that were discovered through genome sequencing, the so-called single Orphan genes (DUJON 1996), are listed in Table 6, some

TABLE 6

Genes of totally unknown function

ECM no.	Gene/ORF (synonyms)	CFW phenotype	Chromosome	Tn Insertion	Function/homology/domain	Additional phenotypes
			With homology to other unknown genes			
3	*YOR3165w*	HS	XV	CDS	Unknown; 614 aa; probable multiple TMD protein; highly similar to Ynl095p	
4	*YKR076w*	HS	XI	Promoter	Unknown; 370 aa; homology to hypothetical protein Ygr154p	GlcNAc↑
21	*YBL101c*	HS	II	CDS	Unknown; 1077 aa; similarity to Ypr030p	GlcNA↓
29	*YHL030w*	HS	VIII	CDS	Unknown; 1868 aa; probable multiple TMD protein; similarity to *C. elegans* unknown protein D2045.2	Zymolyase-hs Hygromycin-rs
34	*YHL043w**	HS	VIII	CDS (1) Promoter (1)	Unknown; similarity to subtelomeric-encoded proteins such as Ykl219p, Ybr302p, Ycr007p, Yhl048p, Ynl336p; two putative TMDs; 170 aa	
			Orphan genes			
1	*YAL059w*	HS	I	CDS	Unknown; 212 aa; TMD	
9	*YRK004c*	HS	XI	CDS	Unknown; 292 aa; TMD	Papulacandin-hs
12	*YHR021w-a*	HS	VIII	Promoter	Unknown; 151 aa; TMD	Zymolyase-hs
19	*YLR390w*	HS	XII	CDS	Unknown; 112 aa; TMD	M/G↑ GlcNAc↑ Zymolyase-hs Hygromycin-hs Killer-hs Abnormal morphology
37	*YIL146c*	RS	IX	CDS	Unknown; 529 aa; TMD	M/G↓ Zymolyase-hs Caffeine-rs
7	*YLR443w*	HS	XII	CDS	Unknown; 448 aa; probable multiple TMD protein	GlcNAc↓ Zymolyase-hs
2	*YBR065c*	HS	II	CDS (3)	Unknown; 364 aa	Zymolyase-hs Hygromycin-hs Papulacandin-hs Killer-hs Caffeine-hs Abnormal morphology
11	*YDR446w*	HS	IV	CDS	Unknown; 302 aa	Zymolyase-hs Hygromycin-hs Papulacandin-hs
13	*YBL043w*	HS	II	CDS	Unknown; 257 aa	GlcNAc↑ Zymolyase-hs Papulacandin-hs Caffeine-hs
26		HS	VIII	Promoter	Unknown; 30 bp from ATG of previously unidentified small ORF (51 aa) between *SCH9* and *SKN7*	

aa, amino acid. * and †, for explanation, see Table 2.

TABLE 7

Classification of mutants by phenotype

$\frac{\text{Mannose}}{\text{Glucose}}\uparrow$	*FOX2, HEL1, KRE6, MKS1, MRE11, MSN1, ORD1, RGT2, RNH1, SMP2, ECM5, ECM10, ECM14, ECM19, ECM20, ECM31*
$\frac{\text{Mannose}}{\text{Glucose}}\downarrow$	*ALG9, DIT2, DFG16, EFF2, HAL5, HCA4, KTR6, MEC3, RPC34, SLG1, SRD2, STF2, TFC1, URA7, VAN1, YCK2, ECM30, ECM33, ECM37*
GlcNAc↑	*BUD8, EFF2, MKS1, MSN1, RGT2, SMP2, YEF3, ECM4, ECM5, ECM13, ECM16, ECM19, ECM20*
GlcNAc↓	*DCG1, LAG2, MRE11, PEL1, ECM7, ECM21*
Zymolyase hypersensitivity	*ACS1, BUD8, CWP2, DCG1, HCA4, IFM1, KRE2, KRE6, ORD1, PEL1, SNF3, TFC1, VAN1, YEF3, ECM2, ECM7, ECM11, ECM12, ECM13, ECM16, ECM17, ECM19, ECM29, ECM33, ECM37*
Zymolyase resistance	*IMP2′*
Hygromycin B hypersensitivity	*EFF2, FOX2, HAL5, HEL1, IMP2′, KTR6, MSN1, RNH1, RPC34, SLG1, SLN1, SNF3, VAN1, ECM2, ECM5, ECM10, ECM11, ECM14, ECM15, ECM16, ECM17, ECM19, ECM20, ECM31, ECM33*
Hygromycin B resistance	*ECM8, ECM29*
Papulacandin B hypersensitivity	*CWP2, HEL1, IFM1, KRE2, MRE11, MSN1, PAS8, PEL1, RNH1, SLG1, SNF3, YCK2, ECM2, ECM9, ECM11, ECM13, ECM14, ECM16, ECM20, ECM27, ECM31, ECM39*
K1 killer toxin resistance	*ALG9, IMP2′, KRE2, KRE6, PAS8*
K1 killer toxin hypersensitivity	*MKS1, MRE11, SMP2, ECM2, ECM8, ECM19, ECM20, ECM33*
Caffeine hypersensitivity	*ALG9, CWP2, DCG1, IMP2′, MEC3, PEL1, RNH1, ROM2, SLG1, SLN1, SNF3, ECM2, ECM5, ECM13, ECM20*
Caffeine resistance	*BUD8, MKS1, MSN1, SSK2, ECM37*
Abnormal morphology	*ECM2, ECM5, ECM19, ECM20*

of these have homology with another yeast hypothetical protein or with an ORF from some other organism.

Attempts at a hierarchical classification through cell surface phenotypes: To try to classify further the calcofluor white collection, a number of additional screens were undertaken. Simple wall-related phenotypes were scored to aid a progression to specific analysis of function in extracellular matrix assembly.

Cell wall hexose levels: Examination of the amounts of cell wall polymer sugars offers a powerful way to sort mutants, as a reduced level of a component sugar likely indicates a defect leading to reduced synthesis of the relevant polymer. Such a classification formed the primary basis for classifying the original calcofluor white-hypersensitive collection (RAM *et al.* 1994). However, there are some caveats and limitations in this approach that would have to be further examined in working with individual mutants. These reflect the fact that only sugar ratios and not absolute amounts of polymers have been determined. Thus if the level of all polymers falls, the ratio of the sugars may not change. A significant number of mutants (43%) do not show an obvious change in monomer ratios, but some may have suffered pleiotropic effects leading to a global reduction in polymer levels or may have more modest defects in the cell wall (like the Kre2p mannosyltransferase (HÄUSLER and ROBBINS 1992; HÄUSLER *et al.* 1992; HILL *et al.* 1992; LUSSIER *et al.* 1995a) or may have no wall defect at all. If the level of the mannose or glucose polymers rise, this will be interpreted using the ratio method as a fall in the level of the other polymer. This formal possibility seems less likely, simply because of the large amounts of mannose and glucose already present in the wild-type cell wall and the consequent difficulty of their overproduction to an extent required to significantly distort the mannose:glucose ratio.

Further screens for wall phenotypes: A range of additional phenotypic screens using drugs or proteins/enzymes that affect the cell surface or its regulation have been made on the mutant collection. These attempted to confirm or extend the data from the calcofluor and sugar ratio screens into more specific functional subclassifications. The results are shown in Tables 2–7. This hierarchical classification was only of limited success. A major shortcoming that emerged was the empirical nature of many of the tests for cell surface defects, with many of the drugs identifying a broad range of genes affecting many cell surface processes and limiting a useful hierarchical classification.

Of the original calcofluor mutations in 82 genes, 47 caused some obvious change in polymer sugar ratios. Mutations in a further 18 genes resulted in a phenotype with respect to at least one of these additional tests for a wall phenotype. Thus mutations in 65 of 82 genes

(79%) caused some additional wall phenotype beyond that of altered calcofluor sensitivity. This high proportion attests to the value of calcofluor white as a reliable primary cell surface screen and is consistent with the earlier work (RAM *et al.* 1994). However, the full extent of calcofluor white toxicity is not known, and it is possible that the mutations in the 17 remaining genes do not result in cell surface defects. For example, one could imagine that not all of the drug remains extracellular and that defects in removing intracellular calcofluor white could lead to hypersensitivity.

Perspectives: This large scale screen has certain inherent limitations; some have been raised, two others are worth mentioning: lethal phenotypes will be missed as haploid strains were used and a proportion of mutated genes may not cause phenotypes because they are members of one of the large number of yeast gene families (DUJON 1996; GOFFEAU *et al.* 1996). In some cases individual members of a group of related genes have been identified, for example, *KRE2* and *KTR6* (LUSSIER *et al.* 1996, 1997). In other cases, this would be unlikely, for example, the seven members of the Pmtp protein:*O*-mannosyltransferase are highly redundant and calcofluor phenotypes were only seen in cells bearing at least two disruptions (GENTZSCH and TANNER 1996).

The genes found have been categorized according to their respective phenotypes (Table 7). The wide range of gene categories and phenotypes obtained reemphasize that cell surface synthesis and its integration with cellular growth and division is complex, with regulation of the individual polymers and likely some global overall sensing and control. Extracellular matrix synthesis has constraints, temporal in the cell cycle and spatial in cell architecture (KLIS 1994; CID *et al.* 1995; IGUAL *et al.* 1996). In addition there is much physiological evidence that environmental conditions such as nutrient and carbon source, temperature and the medium osmoticum affect the composition of the cell wall (KLIS 1994; CID *et al.* 1995). Recently several major signal transduction cascades have been found to regulate the cell surface. Known components include the protein kinase C cascade (PARAVICINI *et al.* 1992; ERREDE and LEVIN 1993; IGUAL *et al.* 1996), the osmotic sensing *HOG* pathway (SCHULLER *et al.* 1994; JIANG *et al.* 1995), a two component regulatory system with the *SKN7* transcription factor as a receiver module (BROWN *et al.* 1994a), and the calcium-modulated protein phosphatase, calcineurin, has been implicated in β-glucan synthesis (GARRET-ENGELE *et al.* 1995). Other protein kinases and phosphatases identified here also have wall effects. It is likely that these different pathways are coordinated and that cell surface biosynthesis and assembly is controlled at many levels, from transcriptional regulation to the cell wall itself.

Large-scale functional studies on sequenced genomes are in their infancy. Exploratory studies like this one are informative but point up both strengths and weaknesses in the approach. Detailed functional analysis of the identified genes will be longer term and will involve the participation of yeast specialists. This will require access by the community to the large body of data on the genes. To assist this process we will place our information on the identified genes in the major databases.

We thank MIKE SNYDER, PETRA ROSS-MACDONALD and NANCY BURNS for advice, discussions and materials necessary for transposon mutagenesis and mutant rescue; TROY KETELA and PETER DIJKGRAAF for helpful discussions; MALCOLM WHITEWAY for providing strain AWM3CΔ630 and DIANE OKI for assistance with manuscript preparation. This work was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada and by the Canadian Genome Analysis and Technology program to H.B. and by National Institutes of Health grants 31318 and 45188 to P.W.R.

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Communicating editor: M. JOHNSTON