Universidade de Trás-os-Montes e Alto Douro

Genome-wide phenotypic analysis of *Saccharomyces cerevisiae* in response to chitosan

Dissertação de Mestrado em Biotecnologia e Qualidade Alimentar

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"A mente que se abre a uma nova ideia,

jamais volta ao seu tamanho inicial"

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ABSTRACT

In wine industry SO_2 has been widely used not only as an antioxidant and antioxidasic agent, but most importantly, due to its antimicrobial proprieties. The occurrence of spoilage yeasts that can tolerate high concentrations of SO_2 requires the use of levels near the European Union legal limit, constituting a threat to human health. Thus, there has been a great interest in looking for safer preservatives to replace or at least reduce the use of SO_2 as an antimicrobial agent. Chitosan, a natural nontoxic biopolymer, has been proposed as potential useful agent in food preservation due to their biological activities, such as antimicrobial activity. Chitosan addition, up to 0.1 g/L, has been accepted as a new oenological practice since July 2009 by the International Oenological Codex as a fining agent of wine. Additionally, it has been recognized its effectiveness in the control of the spoilage yeast such as Dekkera/*Brettanomyces* spp. Both the mode of action of chitosan and mechanisms of resistance in yeast are still poorly understood and subject to debate. In an effort to contribute to the elucidation of these questions, in this work we used the yeast *Saccharomyces cerevisiae* as a model. In this way, a genome-wide screen for altered susceptibility to chitosan was performed using the EUROSCARF haploid yeast deletion collection in order to identify new genes/pathways relevant in yeast resistance to this antimicrobial agent. In this study, we found that the maximum permissible concentration of chitosan for oenological use had no fungicide effect on the parental strain *Saccharomyces cerevisiae* BY4741. The use of a range of chitosan concentrations (0.25 - 1.0 g/L) allowed the identification of 252 genes whose deletion caused hypersensitivity to chitosan and 207 genes whose deletion conferred chitosan resistance, of which 29 mutants were classified as hyper resistant. Functional categories overrepresented with genes whose absence renders cells hypersensitivity to chitosan mainly include ribosomal proteins, cell cycle and DNA processing, regulation of C-compound and amino acid metabolism, cell wall, phospholipids metabolism, vacuolar/lysosomal transport and transcription. On the other hand, functional categories, such as intracellular transport routes (peroxisome, endoplasmatic reticulum and Golgi), ionic homeostasis, protein modification and cell aging, were overrepresented among the genes whose absence rendered mutants resistance. These findings shed light on the molecular basis of chitosan toxicity and will be helpful for future research on the application of chitosan as an effective and safer antimicrobial agent not only in wine, but also in other food industries.

Keywords: • Chitosan • *Saccharomyces cerevisiae* • Chemogenomics • Wine • Yeast Spoilage

RESUMO

O SO_2 tem sido amplamente utilizado na indústria vínica, não apenas como agente antioxidante e antioxidásico, mas mais importante, devido às suas propriedades antimicrobianas. A ocorrência de leveduras de contaminação que podem tolerar altas concentrações de SO_2 tem levado ao uso de níveis perto do limite legal estabelecido pela legislação da União Europeia, constituindo uma ameaça à saúde humana. Assim, existe um grande interesse na procura de outros conservantes mais seguros para substituir ou apenas reduzir a utilização de SO_2 como um agente antimicrobiano. A quitosana, um biopolímero natural não-tóxico, tem sido sugerida como um potencial agente útil na conservação de alimentos, devido às suas propriedades biológicas, tais como a sua atividade antimicrobiana. A adição de quitosana, até a um limite de 0.1 g/L foi aceite desde julho de 2009 pelo Codex Enológico Internacional como uma nova prática enológica, como agente de clarificação do vinho. Além disso, é também reconhecida a sua eficácia para eliminar microrganismos de contaminação, tais como leveduras, nomeadamente *Dekkera/ Brettanomyces* spp.

Tanto o modo de ação da quitosana como os mecanismos de resistência da levedura são ainda pouco compreendidos e sujeitos a debate. Na tentativa de contribuir para o esclarecimento destas questões, neste trabalho foi utilizada a levedura *Saccharomyces cerevisiae* como modelo. Assim, a coleção de mutantes haploides de *S. cerevisiae* deletados de genes individuais foi utilizada para identificar novos genes/vias importantes envolvidas na resistência da levedura a este antimicrobiano. Neste estudo, a concentração máxima de quitosana permitida para uso enológico não teve um efeito fungicida sobre a estirpe parental *Saccharomyces cerevisiae* BY4741. A utilização de uma gama de concentrações de quitosana (0.25 - 1.0 g/L) permitiu a dentificação de 252 genes cuja deleção conduz a um fenótipo de hipersensibilidade à quitosana e 207 genes cuja deleção conferiu resistência, dos quais 29 mutantes foram classificados como hiper-resistentes. A distribuição funcional dos genes cuja deleção conferiu hipersensibilidade à quitosana, inclui Proteínas ribossomais, Ciclo celular e processamento de DNA, Regulação do metabolismo de compostos de carbono e aminoácidos, Parede celular, Metabolismo dos fosfolípidos, Transporte vacuolar/lisossomal e Transcrição. Por outro lado, categorias funcionais como Transporte intracelular (Peroxissoma, Retículo endolasmatico e Golgi), Homeostasia iónica, Modificação de proteínas e Envelhecimento celular foram as mais representativas entre os genes cuja ausência conduziu a um fenótipo de resistência. Estas descobertas elucidam a base molecular da toxicidade da quitosana e serão úteis para pesquisas futuras sobre a

aplicação da quitosana como um agente antimicrobiano eficaz e seguro não só no vinho, mas também noutras indústrias de alimentos.

Palavras-chaves: Quitosana • *Saccharomyces cerevisiae* • Quimiogenómica • Vinho • Leveduras contaminantes.

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ABBREVIATIONS

AUC Area under of OD/time curve

CFUs Colony forming units

COS Chitooligosaccharide

DD Deacetylation degree

ER Endoplasmatic reticulum

ESCRT Endosomal sorting complexes required for intracellular transport

fa Fractional area

FDA Food and drug administration

GARP Golgi-associated retrograde protein

GRAS Generally recognized as safe

HOG High osmolarity glycerol

HOPS Homotypic fusion and vacuole protein sorting

i.e id est

IC_{50} Concentration causing 50% of growth inhibition

MIC Minimum inhibitory concentration

MMB Minimal medium base

MTPs 96-well microplates

MVB Multivesicular body

MW Molecular weight

NCR Nitrogen catabolite repression

NIC Non-inhibitory concentration

OD Optical density

ORF Open reading frame

RAVE Regulator of the H^+-ATPase of Vacuolar and Endosomal Membranes

ROS Reactive oxygen species

SAGA Spt-Ada-Gcn5-acetyltransferase

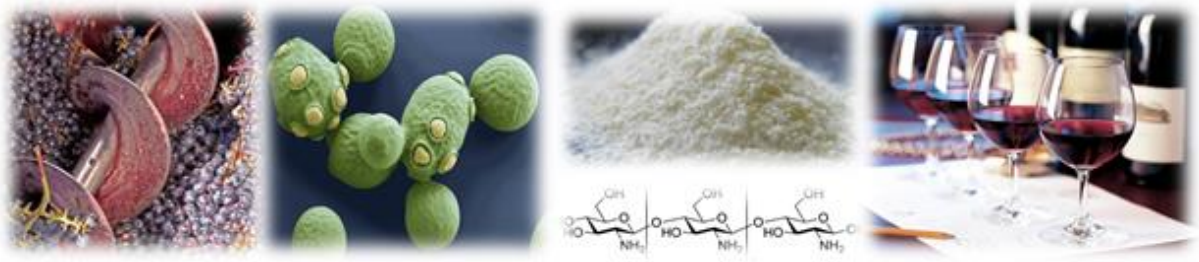
SGD *Saccharomyces* genome database

TFs Transcription factors

V-ATPase Vacuolar proton-translocating ATPase

YNB Yeast nitrogen base

YPD Yeast peptone dextrose



1. Literature Review

1.1. Microbiological spoilage of wine

Microbial spoilage of wine refers to the development of any microorganism that is unwanted at a particular place or time. This includes, the microorganisms that produce off-flavours, odours, colours or precipitates or that the ability to do it, under the conditions of vinification or later during the storage of wine (Boulton *et al.,* 1996). Spoilage or non-spoilage microorganisms come from the grapes and from the material and equipment used in the cellar. The so called microorganisms of wine include: filamentous fungi (mostly restricted to grapes), yeasts, and lactic and acetic acid bacteria. Microbiological instability of wines still is a problem in modern oenology, being the most severe problems of such instability caused by yeast and lactic acid bacteria, which are responsible for the undervaluation of the final product and consequently to high economic losses. Given the topic of this thesis we will focus on yeasts as spoilage microorganisms.

The wine is derived from the fermentation of grape-juice, being the result of a set of sequential biochemical reactions carried out by yeasts. Besides ethanol, the yeasts produce numerous other products, several alcohols, aldehydes, ketones, organic acids, volatile compounds, among others. These compounds are derived from grapes, from the metabolism of yeast and, in less extent, from the metabolic activity of other microorganisms, and from the chemical reactions that occur during storage and aging. The quality of wine is dependent on the concentrations and balance of all these compounds (Ribereau-Gayon *et al.,* 2006a).

In sound, healthy and intact berries we found a diversity of yeasts, including the so-called apiculate yeasts (*K. apiculata/ H. uvarum* that accounts for roughly 50 ± 75 % of the total yeast population) and species in other genera such as *Candida*, *Brettanomyces*, *Cryptococcus, Kluyveromyces, Metschnikowia*, *Pichia*, *Hansenula* and *Rhodotorula,* among others (Bisson and Kunkee, 1991; Fleet, 2003; Fleet and Heard, 1993; Pretorius, 2000). The non-*Saccharomyces* yeasts species initiate grape must fermentation but their activity, is generally limited to the first two or three days of fermentation or, in certain cases, can persist longer periods either in spontaneous or inoculated fermentations (Ciani *et al.,* 2010; Fleet, 2003; Fleet, 2008; Fleet and Heard, 1993; Jolly *et al.,* 2006). For a long time, the activity of the non-*Saccharomyces* yeasts in grape-juice fermentation was considered undesirable due to their potential to overproduce acetic acid and off-odours in the final wines (Amerine *et al.,* 1972; Fleet, 2008). Thus, inoculation with selected active dry yeasts has been a routine practice in most wine producing countries to guarantee a more rapid onset of fermentation, to reduce the risk of slow or premature fermentations arrest and to obtain a more reliable and

uniform quality. However, even when the alcoholic fermentation takes place under very well controlled conditions, the wine requires special attention after fermentation, to reduce the risk of microbial spoilage. Therefore, to avoid the microbial spoilage, the wine is removed from the contact with the lees, sulfur dioxide is added, storage proceeds under limited-aerobic conditions and low temperature, approximately 18 ^{o}C. The microbial growth is detrimental for the quality of the wine not only because microorganisms can use some compounds that can be essential for that quality or just by the production of unpleasant ones. Moreover, the wine at the point of consumption should be clean, bright and without cloudiness and deposits to meet the increase consumer's requirements (Boulton *et al.,* 1996; Ribereau-Gayon *et al.,* 2006a).

Susceptibility of wines to microbial spoilage is dependent on its chemical composition: ethanol concentration, pH and free SO_2 content. The wines are not susceptible to microbiological hazards capable of inducing risks to public health, since most of wines have more than 11% of ethanol and low pH, below 4.0, which assure low microbial instability (Loureiro and Malfeito-Ferreira, 2003).

To prevent and control microbial spoilage during winemaking, there are several strategies: physical treatments – involving thermic treatments and sterile filtration – and addition of preservatives (Bartowsky, 2009; Ribereau-Gayon *et al.,* 2006b; Toit and Pretorius, 2000).

1.2. Wine spoilage yeasts

Spoilage yeasts can be, according to the definition presented above, any yeast which is unwanted at a particular place or time. In Table 1.1 are presented the species that occur in each step of winemaking process and the changes they may cause on the appearance and/or on the final composition of the wine. After alcoholic fermentation carried out by *S. cerevisae*, the number of CFUs gradually decreases and after settling, the wine becomes clear and bright. Under suitable winemaking conditions, characterized by a rapid and complete exhaustion of sugars, no other yeast species significantly appears at the end of fermentation. Only under unsuitable conditions, spoilage yeasts can contaminate the wine (Ribereau-Gayon *et al.,* 2006a).

Thus, the species *Saccharomyces cerevisiae*, the yeast by excellence responsible for the alcoholic fermentation, is considered spoilage yeast when detected in wine with residual sugars, provoking re-fermentations, which is particularly severe when the wine is already

bottled (Loureiro and Malfeito-Ferreira, 2003). Therefore, bottled wine must be either be fermented to complete dryness or some measures must be taken to prevent yeast growth in wines with residual sugars (Boulton *et al.,* 1996). Re-fermentation yeasts, such as some strains of *S. cerevisiae*, *Zygosaccharomyces bailii* and *Saccharomycodes ludwigii* can also develop in sweet or botrytized sweet wines during ageing or bottle storage due to their particular resistance to ethanol and sulphur dioxide (Ribereau-Gayon *et al.,* 2006a). Indeed, the specie *Zygosaccharomyces bailii* is an important spoilage yeast because is high resistant to sulphur dioxide, to sorbic acid and other preservatives. As it happens with *S. cerevisiae*, this specie may also be found in semi-dry bottled wine. In both cases, the recognized symptoms of spoilage are cloudiness, sediment formation and gas production in bottled wines (Loureiro and Malfeito-Ferreira, 2003). The problem of contamination of wine at bottling with *Zygosaccharomyces* is best solved, as it is with *Saccharomyces*, by using sterile filtration before bottling. *Saccharomycodes ludwigii* is very difficult to eliminate from the winery since it shows high resistance to ethanol and sulphur dioxide and it is a strong producer of acetaldehyde. Spoilage by *Saccharomycodes ludwigii* was reported once in bottled wine, where flocculent masses settle as consistent pieces (Boulton *et al.,* 1996).

The growth of species of the genera *Dekkera/Brettanomyces* can result in defects, more or less relevant, depending on the concentration of the volatile phenols produced (Kheir *et al.,* 2013). One of the most frequent and most dangerous contaminations in French wines is due to the development of *Brettanomyces intermedius*, which is responsible for serious off-odours. In Australian wines *Dekkera* species, especially *Dekkera bruxellensis*, which is highly adapted to growth in wine, is generally considered to be a spoilage yeast although some consider that it adds flavour complexity. These yeasts, *Brettanomyces* or *Dekkera*, can develop in anaerobiosis, consuming trace amounts of sugars that have been incompletely or not fermented by *S. cerevisiae* and contaminate either bulk or bottled wine (Ribéreau-Gayon *et al.,* 2006a).

The yeasts with intense oxidative activity, like those of the genera *Pichia* and *Candida* are usually recognized by the film formation on the surface of bulk wines when stored under aerobic conditions (Boulton *et al.,* 1996; Malfeito-Ferreira, 2010; Ribéreau-Gayon *et al.,* 2006a). In this case, yeasts oxidize ethanol into aldehyde in dry wines, particularly in those with low ethanol content (Ribéreau-Gayon *et al.,* 2006a). The contamination of wine by this kind of yeasts is more visible in bulk wine and it is not a serious problem in bottled wine once the wine has been properly filtered and sulphite added before bottling. These yeasts cause more aesthetic nature problem than substantial changes in wine composition, except the overproduction of acetaldehyde in such wines. These microorganisms can be largely avoided

by making convenient disinfection and efficient cleaning of the filling lines, the piping, the pipe bends, the filler and corking machine (Malfeito-Ferreira, 2010; Ribéreau-Gayon *et al.,* 2006a).

Understanding the characteristics of yeast spoilage, as well as the available control technologies, is vital to producing consistent and high-quality of wines. More established methods of microbial control include sulphur dioxide, dimethyl dicarbonate and filtration. Current research is focused on the use of chitosan, pulsed electric fields, low electric current, and ultrasonics as means to protect wine quality (Zuehlke *et al.,* 2013).

Table 1.1 - Origins of wine spoilage yeast and most common hazards due to off-flavours (adapted from Malfeito-Ferreira, 2010).

Origins	Yeasts	Hazard
Raw material	*Saccharomyces spp., Kloeckera spp*., *Metschnikowia sp*., *Candida sp*. and *Hansenula sp*., *Hanseniaspora spp*., *Kluyveromyces spp*., *Pichia spp*., and *Rhodotorula spp*.	Primary source of spoilage yeasts; The production of unwanted amounts of metabolites such as ethyl acetate (causing vinegar smell).
Fermentation	*Saccharomyces cerevisiae.*	Production of hydrogen sulphide; Re-fermentation of wine with residual sugars.
Post-fermentation - bulk or bottled wines	*Saccharomyces cerevisiae, Candida sp*., *Pichia spp*., *Schizosaccharomyces spp., Zygosaccharomyces spp.* and *Dekkera/Brettanomyces.*	Production of acetaldehyde by film-forming yeasts; High levels of acetic acid and its esters, and produces killer toxins.

1.2.1. *Saccharomyces cerevisiae*

The genus *Saccharomyces* is characterized, according to Kurtzman and Fell (1998), as globose, ellipsoidal or cylindrical shapes cells with asexually reproduction through multilateral budding, vegetative phase is predominantly diploid and vigorously fermentation of sugars. The genus includes a set of 14 species, which encompasses *Saccharomyces cerevisiae.* In the pioneer work of 1960, Ribéreau-Gayon and Peynaud considered that only two species *S. cerevisiae* (formerly called *ellipsoideus*) and *S. oviformis* (now a synonym of *S. cerevisiae*)

were more frequently found in wine while *S. bayanus* was rarely found there. At present, the *Saccharomyces sensu strict* group includes the most industrially exploited microorganism, the four variably related species: *S. paradoxus*, *S. bayanus*, *S. cerevisiae* and *S. pastorianus* (Kurtzman and Fell, 1998). These species cannot be differentiated from one another by physiological tests but can be delimited by measuring the degree of homology of their DNA (Ribéreau-Gayon *et al.,* 2006). According to the authors, this classification adds a lot of confusion in the language regarding to the epithet "bayanus": for taxonomists, *S. bayanus* is a species distinct from *S. cerevisiae* whereas for enologists it designates a physiological race of *S. cerevisiae* that not ferment galactose and is high resistant to ethanol. To overcome the confusion, oenologists usually add the varietal name to *S. cerevisiae* to designate wine yeasts: *S. cerevisiae* var. *cerevisiae*, var. *bayanus*, var. *uvarum*. In fact, Le Jeune *et al.* (2007) and Naumov *et al.* (2000) reported that *S. cerevisiae* was the most commonly encountered species whereas, in cool climate, *S. bayanus* (var. uvarum) occurs more frequently, due to its cold tolerance. Genomic analysis identified a high percentage of *S. paradoxus* in Croatian grape microbiome (Redžepović *et al.,* 2002).

1.2.2. *Zygosaccharomyces bailii*

The genus *Zygosaccharomyces* is characterized, according to Kurtzman and Fell (1998), by cells with spheroidal, ellipsoidal or elongate shapes, variable dimensions (3 - 9) x (3 - 13) µm, with asexually reproduction through multilateral budding, occasional pseudohyphae is formed, glucose is fermented but pellicles are not formed in liquid media (Edwards, 2005; James and Stratford, 2011, Thomas and Davenport, 1985). Currently, the genus consists of six species, *Zygosaccharomyces kombuchaenis, Zygosaccharomyces lentus, Zygosaccharomyces mellis, Zygosaccharomyces bailii, Zygosaccharomyces bisporous,* and *Zygosaccharomyces rouxii* (James and Stratford, 2011). More recently, *Z. gambellarensis, Z. machadoi, Z. parabailiii, Z. pseudobailii, Z. pseudorouxii, Z. sapae e Z. siamensis* were added to the group (Hulin and Wheals, 2014).

However, the three species, *Z. bailii, Z. bisporous,* and *Z. rouxii*, have been associated with the spoilage of grape must, grape juice concentrate and wine (Fugelsang and Edwards, 2007). As mentioned before the specie *Zygosaccharomyces bailii* is an important spoilage yeast because it shows the ability of growing in the presence of weak acids, such as sorbic acid, benzoic acid, acetic acid, propionic acid, and in the presence of high concentration of SO_2, commonly added to grape-juice prior to fermentation and to wine during storage.

Besides growing up in the conditions aforementioned, this contaminant yeast is also able to tolerate high concentrations of ethanol and other alcohols, as well as low pH, high sugar concentrations and high temperatures. Moreover, is known by its fructophilic character, moderate tolerance to osmotic stress and to oxygen-restrictive conditions (Stratford *et al.*, 2013). Consequently, this spoilage yeast is recognized by the formation of sediments and evolution of carbon dioxide in bottled wine (Stratford and James, 2003).

1.2.3. *Dekkera/Brettanomyces*

Dekkera is the sporogenous form (ascospore-forming) or sexual teleomorph of Brettanomyces. The genus *Dekkera*, is characterized, according to Kurtzman and Fell (1998), by cells with spheroidal, subglobose to ellipsoidal, frequently ogival, cylindrical to elongate shapes, variable dimensions (2 - 5.5) x (3 - 22) µm, with asexual reproduction by budding, and occasional pseudohyphae formation. Currently, the genus consists of 2 species: *Dekkera anomala* and *Dekkera bruxelensis*. Five individual species of *Brettanomyces* (teleomorph *Dekkera*) are now recognized: *Brettanomyces bruxellensis*, *Brettanomyces anomala*, *Brettanomyces custersiana*, *Brettanomyces naardensis*, and *Brettanomyces nanus* (Smith, 2011). Currently, only *B. bruxellensis* is thought to be associated with grape and wine contamination (Egli and Henick-Kling, 2001; Mitrakul *et al.,* 1999). Cultures are slow growing, but remain viable for long periods of time (Malfeito-Ferreira, 2010; Serpaggi *et al.,* 2012) and during that period they are still metabolically active (Cocolin and Ercolini, 2008; Serpaggi *et al.,* 2012). The yeast contamination is recognized by formation of volatiles phenols, which although can be considered to positively contribute to the aroma of some wines, are better known as off-flavours such “band-aid”, “barnyard” or “stable” (Bartowsky and Pretorius, 2009; Swiegers *et al.,* 2005), which negatively influence the wine quality.

The production of volatile phenols is related to the enzymatic conversion of free hydroxycinnamic acid precursors, particularly p-coumaric acid, ferulic acid and caffeic acid (Kheir *et al.,* 2013; Ribéreau-Gayon *et al.,* 2006b). Two sequential conversion steps facilitated by cinnamate decarboxylase and vinylphenol reductase are necessary in order to form volatile phenols (Kheir *et al.,* 2013): i) a cinnamate carboxylase enzyme or phenolic acid decarboxylase decarboxylates hydroxycinnamic acids to intermediate hydroxystyrenes, namely 4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol; ii) vinylphenol reductase reduce vinyl-derivative double bond, in order to form respective ethyl-derivatives (4-ethylphenol, 4-

ethylguaiacol and 4-ethylcatechol), according to Figure 1.1, obtained by Oelofse *et al.,* 2008. The prevention of this contamination was found to be in very thorough cleaning of the crushing equipment and of the piping or hoses from the reception area into the winery, including judicious use of sulphur dioxide to aid sanitizing (Boulton *et al.,* 1996).

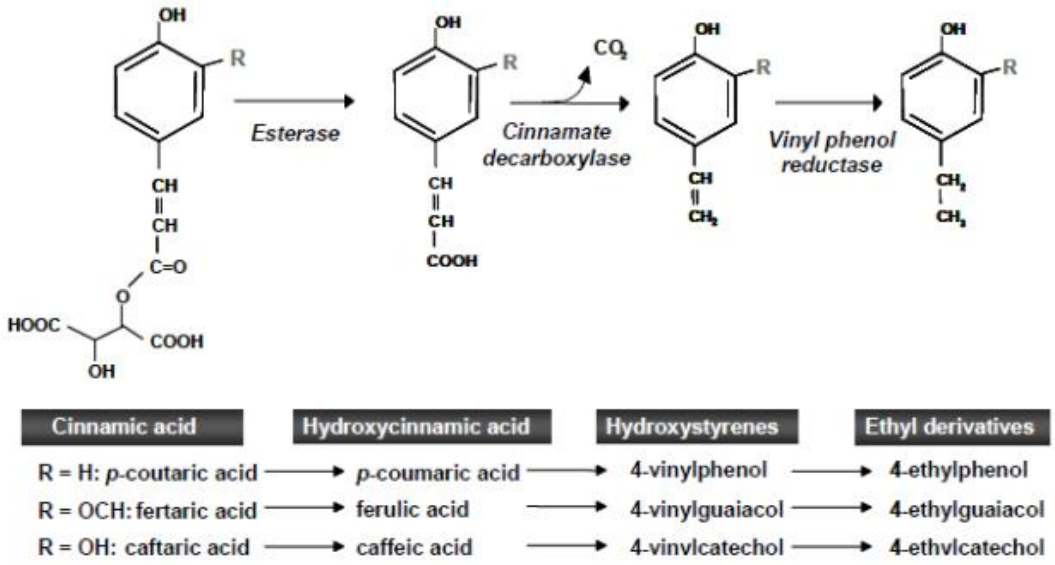


Figure 1.1 - Formation pathway of volatile phenols via the decarboxylation of hydroxycinnamic acids.

1.2.4. *Saccharomycodes ludwigii*

Saccharomycodes ludwigii is characterized by for its very large cell size (10 - 20) µm of lemon-shaped cells (Ribéreau-Gayon *et al.,* 2006a), frequently isolated from wine at the end of the fermentation and during wine storage (Romano *et al.,* 1999). *Saccharomycodes ludwigii* has been considered as a spoilage organism (Boulton *et al.,* 1996; Ribéreau-Gayon *et al.,* 2006a) and its growth is recognized by cloudiness, sediment formation and large flocculent masses settled in bottled wines (Boulton *et al.,* 1996). This species is not well seen in California where Boulton and colleagues reported that it was never isolated from the wines of that region. Although, based on its particularly high resistance to the stress conditions, found in fermentative environment, strains of this specie have been screened for their potential as adjunct of *S. cerevisiae* (Romano *et al.,* 1999; Bovo *et al.,* 2014). One strain of *Saccharomycodes ludwigii* produced a peculiar fermented beverage, although its high acetic

concentration was characterized by a fresh odour with a fruity, identified as apple-like or kiwi-like flavour (Romano *et al.,* 1999). More recently, Bovo *et al.,* (2014) using a strain of *Saccharomycodes ludwigii* for increasing varietal compounds, verified that the level of implantation of the strain was not sufficient to assure a clear beneficial effect on quality.

1.3. Wine treatment: the use of preservatives

Preservatives are defined as additives that are intentionally added to food during processing or storage, prevent the growth and proliferation of microorganisms which could cause food spoilage and lead to food poisoning. An ideal preservative is one that is efficient at low dosages, is innocuous to the consumer, and must be easily soluble and uniformly spread in the product. Ultimately, it can neither modify the product's organoleptic qualities nor mask any of its qualities (Untermann, 1998).

The most useful preservatives, to minimize infections caused by spoilage yeasts, such as *Z. bailii* and *B. bruxellensis*, two of the most dangerous yeasts to wine, are: sorbic acid and its K salts, dimethyl dicarbonate, sulfur dioxide and its derivatives, and chitosan. Other processing physical methods are presented in Table 1.2: Pulsed electric field is a non-thermal technology for pasteurization or sterilization of liquids, i.e. to reduce microbial contamination in wines (Santos *et al.,* 2012); low electric current can be applied throughout alcoholic fermentation or to finished bulk wine to prevent growth of spoilage microorganisms; also ultrasonic technologies have been used for removing tartrate deposits from barrels and, more recently, also to inactive of spoilage microbes (Jiranek *et al.,* 2008, Schmid *et al.,* 2011); finally, membrane filtration, are processes available that can prevent/remove microbial spoilage from wine and thus can be used by winemakers to preserve wine quality (Zuehlke *et al.,* 2013).

Considering the exorbitant cost of most of the equipment used in that process, chemical preservatives such as sulphur dioxide and sorbic acid are still widely used in the industry. However, we must increasingly meet the consumer’s demands who are increasingly looking for natural and healthy products without chemical additives. So the demand for natural substitutes as an alternative for the existing chemical preservatives should be a goal of researchers and technologists working in food science. Indeed, Toit and Pretorius (2000) anticipated that use of natural biological preservatives to satisfy consumers expectations would be in a near future a very interesting niche of the market.

Table 1.2 - Physical methods to limit yeast spoilage of wine (adapted from Zuehlke *et al.,* 2013).

Physical methods	Application	Relative lethality	
		Z. bailii	***B. bruxellensis***
Pulsed electric field	Grape must treatment	High	High
Low electric current	Pre/ post-fermentation	Unknown	Moderate
Utrasonics	Barrel sanitation	High	High
Filtration	Finished wine	Low to high	Low to high

1.3.1. Sulphur Dioxide

Sulphur Dioxide (SO_2) and its derivatives are particularly used as preservatives in food with low pH (Guerrero and Cantos-Villar, 2014). The utilization of SO_2 is due to its specific properties: i) it is an antioxidant, sulphite prevent enzymatic and non-enzymatic browning reactions; ii) it is an antimicrobial agent, prevents the growth of microorganisms, iii) in wine, inactivates certain enzymes, like tyrosinase and laccase, which are associated to the browning of wines (Ribéreau-Gayon *et al.,* 2006b). SO_2 also have a "dissolvent" action assisting on the extraction of several compounds, minerals, organic acids and phenolic compounds, from grape marc and facilitates the static clarification of wines (Pozo-Bayón *et al.,* 2012). SO_2 is used at different stages of winemaking and storage. More specifically, sulphur dioxide is added to grape-must, prior to alcoholic fermentation mainly to avoid the development of undesirable microorganisms and to restrict the extent of juice browning (Boulton *et al.,* 1996); later, after alcoholic fermentation, and when the malolactic conversion is not wanted, sulphur dioxide is again added to the wine for stabilization purposes. During storage, the concentration of SO_2, particularly free SO_2, is kept under an appropriate level again to prevent microbial instability (Ribéreau-Gayon *et al.,* 2006b). SO_2 can be applied in either one of several ways: as gas which is soluble in water; as a SO_2 prepared solution (6% w/v); and as potassium metabisulphite ($K_2S_2O_5$). SO_2 exists in wine two different states: free and bound SO_2. Free sulfite includes all unbound species of sulfurous acid whose relative

concentrations are dependent on pH (King *et al.,* 1981), i.e. molecular sulfite (SO_2), bisulfite (HSO_3^-) and sulfite (SO_3^{2-}). The equilibrium of the various free species is given below:

SO_2 (gas) + H_2O = H_2SO_3 sulfurous acid or "molecular sulfite"

H_2SO_3 = H^+ + HSO_3^- bisulfite ion, pKa1 = 1.77

HSO_3^- = H^+ + SO_3^{-2} sulfite ion, pKa2 = 7.2

All the chemical species of SO_2 present in this equilibrium are designated of free SO_2. At the pH of wine, pH 3.0 - 4.0, the most predominant species of SO_2 is the bisulphite ion (HSO_3^-) (Boulton *et al.,* 1996). Nonetheless, HSO_3^- and SO_3^{2-} are highly reactive, being able to react with several compounds present in wine, such as acetaldehyde, α-keto acids, anthocyanins, sugars, etc., and form the so-called “bound SO_2” (Beech and Thomas, 1985). The bound sulphite does not possess the antimicrobial and antioxidant properties of free sulfites species. The maintenance of adequate concentration of free SO_2 in the wine is critical because it is the most efficient form of sulfite that inhibits microbial growth. The so-called molecular form is the one with the highest antimicrobial activity, as it has no charge the molecule easily diffuses throughout the plasma membranes by simple diffusion. Once inside the cell, the less acidic cytoplasm promotes the dissociation of the molecule of SO_2 into bisulfite and sulfite, which, in turn, reduces its internal concentration and allows more SO_2 to enter into the cell. This chemical equilibrium encourages a concentration gradient that ultimately reduces intracellular pH. The mechanism of action of SO_2 is anticipated to be due to its interactions with ATP, NAD^+, and FAD; induction of mutations in genetic material through deamination of cytosine and uracil; and disruption of disulfide bridges in proteins (Hinze and Holzer, 1986; Pagano *et al.,* 1990; Schimz, 1980).

SO_2 is still the main antimicrobial agent used in winemaking for the protection of wine against contaminants. However, this is known to cause allergic reactions (Divol *et al.,* 2012). Thus, the EU legislation increasingly restricts the use of sulfite in wines. Thus the maximum legal limits of SO_2 permitted are: 0.16 g/L for red wines, with no more than 5 g/L of sugars; 0.21 g/L for dry white wines (with less than 5 g/L of sugars) and 0.26 g/L for white wines with more than 5 g/L of sugars (Reg. (UE) nº53/2011).

1.3.2. Sorbic Acid

Sorbic acid (2,4-hexadienoic acid), weak acid preservatives normally applied under the form of potassium sorbate, is an unsaturated fatty acid used as a fungicide in several foods and beverages with pH values below 4, including in juices and fruit purées waiting for future processing (Erich *et al.,* 1997). This preservative is utilised, at the legal limit concentration of 0.2 g/L in wines with residual sugars, for preventing the growth of yeasts. The inhibitory action of sorbic acid is greater at low pH when most of it is undissociated form (pKa = 4.75). As happens with SO_2 it is also more effective at low pH and in presence of high concentration of ethanol (Whiteley, 1979).

This potassium acid or salt is particularly efficient against microorganisms with an oxidative metabolism. Thus, whenever necessary, sorbic acid should be added immediately before bottling otherwise its spontaneous oxidation to dioxide and water can occur (Radler, 1986). Additionally, it should always be added simultaneously with sulphur dioxide (Webb, 1974) to prevent the growth of lactic acid bacteria and the possibility of being transformed by such bacteria into a compound that confers a geranium-like odour in wine (Radler, 1986). As a matter of fact Ribereau-Gayon *et al.,* (2006b) imply that in wines previously treated with sorbic acid, the concentration of free SO_2 must be maintained up 0.03 to 0.04 g/L to protect the wine against oxidations and to neutralize other substances that give aldehyde taste. Moreover, according to the authors, this concentration of SO_2 by itself is insufficient to avoid the growth of yeasts and the consequent re-fermentations.

The mechanism of action of sorbic acid it is not completely elucidated. As a weak acid, the protonated form, at low extracellular pH, can easily diffuse through the plasma membrane and enter into the cell. Inside the cell, at the near-to-neutral cytosolic pH, the acid dissociates and generates protons and anions, which are charged and, therefore cannot simply diffuse back out. This causes both intracellular acidification and anion accumulation, which hamper the normal metabolic function. The stress sensed in cell by this weak acid provokes other consequences, such as oxidative damage and an inferred perturbation of the plasma membrane. Another major cause of growth inhibition by weak acids could be energy depletion, where weak acids likely inhibit glycolysis by acidification that should eventually lead to ATP depletion (Ullah *et al.,* 2012).

1.3.3. Dimethyl Dicarbonate

Similar to SO_2, Dimethyl Dicarbonate (DMDC) is added to fruit juice and wine to inactivate spoilage microorganisms (Costa *et al.,* 2008). This organic compound is an ester of carbonic acid with methylic acid, commercialized under the trade name of Velcorin (Anonymous, 2006). Velcorin addition, at maximum legal concentrations of 0.2 g/L, has been approved as a food additive by the Food and Drug Administration (FDA) in the United States since 1988 to be use in food products contaminated with fewer than 500 cells/ml of yeast, bacteria or molds (Zuehlke *et al.,* 2013). In Europe, the use of this additive has been approved later, in 1995, to be used in non-alcoholic flavoured drinks, alcohol-free wine and liquid-tea concentrate, at maximum legal concentrations of 0.25 g/L and residues cannot be detect in the final product (Reg. (UE) nº 53/2011). DMDC is more commonly added to finished wine at the filling bowl prior to bottling (Renouf *et al.,* 2008), and can only be added to semi-dry wines with more than 5 g/L of sugars (Reg. (UE) n º53/2011). The efficiency of DMDC against yeasts is dependent on the strain, on the initial cell concentration, temperature, ethanol content and pH. In fact, this is more efficient at 20 ºC, in wines with low pH and high ethanol content. Furthermore, at inoculum concentrations 10^6 cfu/mL, the maximum dosage legally allowed (0.2 g/L) is not efficient (Costa *et al.,* 2008).

The mode of action of DMDC against wine microorganisms appears to be largely related to inactivation of cellular enzymes, caused by irreversible reaction with the amino groups on active sites of enzymes (Bartowsky, 2009), like methoxycarbonylation of imidazoles, amines, and disruption of enzymes in glycolysis, alcohol-dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (Ough 1993; Renouf *et al.,* 2008).

1.3.4. Chitosan

Chitosan is a cationic heteropolysaccharide derived from chitin by deacetylation in concentrated alkaline solution (40-50% NaOH) at high temperature, such as shown in Figure 1.2 obtained from Raafat and Sahl (2009). Chitin is a natural polymer extracted from the exoskeletons of crustaceans, insects and molluscs, or from the cell wall of some fungi (45 and 25% from *Aspergillus niger* and *Penicillium notatum,* respectively). Cellulose, chitin, and chitosan have very similar structures. The difference among these three molecules is the functional group at C-2 position (Luo and Wang, 2013). Chitosan is composed of β(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (*N*-acetylglucosamine), by replacing hydroxyl group

at C-2 position in cellulose molecular chain with amino group (Hafdani and Sadeghinia, 2011; Luo and Wang, 2013).

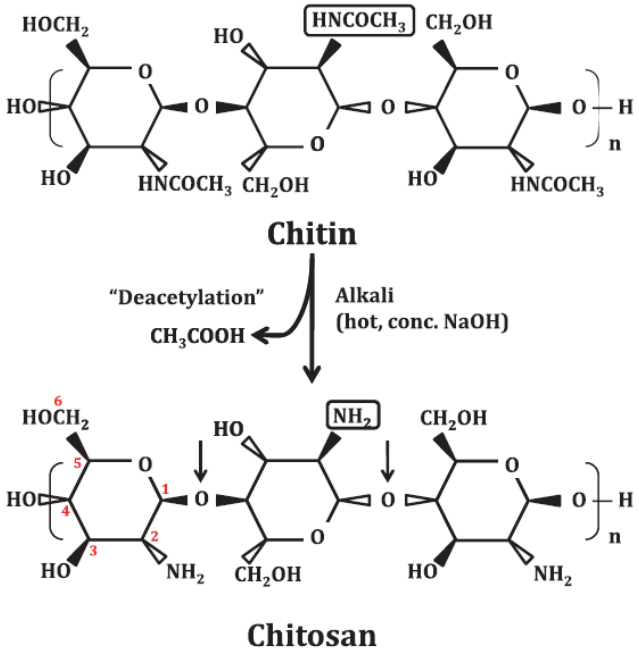


Figure 1.2 - Preparation of chitosan by deacetylation of chitin.

Chitosan is a weak base insoluble in water and organic solvents (Goy *et al.,* 2009; Hafdani and Sadeghinia, 2011; Pillai *et al.,* 2009). However, its solubility is dependent on the deacetylation level: chitosan with 50% of deacetylation is soluble in acidic aqueous solutions (pKa ~6.2), which are capable of converting the glucosamine moieties into its protonated soluble form ($R\text{-}NH_3^+$). After protonation, chitosan carries positive surface charges on its D-glucosamine repeat unit (Goy *et al.,* 2009; Hafdani and Sadeghinia, 2011; Luo and Wang, 2013; Pillai *et al.,* 2009).

The positive charge of chitosan confers unique physiological and biological properties on this polymer, which together with the status of GRAS – Generally Recognized as Safe – recognized encourage its application in areas as diverse as food, cosmetics, agriculture, and others (Hafdani and Sadeghinia, 2011). The antimicrobial activity of chitosan and derivatives has been tested against different groups of microorganisms, such as bacteria, yeasts and filamentous fungi (Ferreira *et al.,* 2013; Gómez-Rivas *et al.,* 2004; Rhoades and Roller, 2000; Roller and Covill, 1999). Although, chitosan can be recognized by some authors as a potent antimicrobial agent, others have obtained less satisfactory results. The discrepancies

between the results regarding the antimicrobial efficacy of chitosan can be due to the use of different types of chitosan or different experimental conditions. In fact, the efficiency of chitosan is dependent on intrinsic factors such as concentration, deacetylation degree (DD) and molecular weight (MW) of chitosan, as well as other extrinsic factors, as the pH of the environment and type of microorganism (Hafdani and Sadeghinia, 2011; Luo and Wang, 2013). Chitosan has a stronger inhibitory effect against microorganisms in medium with low pH (No *et al.,* 2002; Roller and Covill, 1999; Younes *et al.,* 2014) and high concentrations (Zheng and Zhu, 2003; Gómez-Rivas *et al.,* 2004;Taillandier *et al.,* 2014). In addition, the nutritional status of the environment largely also appears determines the antifungal effect of chitosan. Carbon and nitrogen limitation increase the antifungal activity of chitosan against *Neurospora crassa* and fungal human pathogens (Lopez-Moya *et al.,* 2014).

The DD and MW are the main parameters which defines solubility and physicochemical properties of this polymer. The DD also determines the charge density and the electrostatic interactions with microbial cell membranes (Luo and Wang, 2013). Usually, the higher the DD, the higher is the antimicrobial activity of chitosan (Mellegård *et al.,* 2011; Younes *et al.,* 2014). Regarding to the MW, there are also contradictory reports. Some studies show that the lower the MW of chitosan, the higher is the effect on reduction of microbial growth (Ferreira *et al.,* 2013; Li *et al.,* 2008), while others have shown exactly the opposite (Jeon *et al.,* 2001; No *et al.,* 2002; Qin *et al.,* 2006). As an example, Zheng and Zhu (2003) observed that in *E.coli*, Gram-negative bacteria, the antimicrobial activity was increased when chitosan with low MW was used, whereas in *S. aureus*, Gram-positive bacteria, the antimicrobial activity increased by increasing the MW of chitosan. Probably outer membrane of Gram-negative bacteria acts as a highly selective barrier, principally through the combined effect of a hydrophobic lipid bilayer together with pore-forming proteins of specific size-exclusion properties (Galdiero *et al.,* 2012). These results are also agreed with chitosan efficiency inhibitory also depend on the type of microorganisms. In addition to Gram-negative and Gram-positive bacteria, also fungi display different sensitivity to chitosan: in a comparative study using *Aspergillus niger, Fusarium oxysporum* and *Alternaria solani*, the first was the most sensitive species (Younes *et al.,* 2014). Probably the differences on cell walls composition may explain the different susceptibilities of these microorganisms (Goy, Britto and Assis, 2009).

The application of chitosan (only the one from fungi origin) has been very recently approved, in 2009, for use in wine at maximum legal concentrations of 0.1 g/L can be considered to be an emerging technology (OIV-oeno368). According to the UE Regulation 53/2011 of January 21 2011, chitosan is used in wines mainly for reduction of heavy metals, acting as an agent

of clarification and stabilization of wine. Although the application of chitosan as an antimicrobial agent in wine is very recent a few studies have provide evidence that it was effective on limiting growth of against some wine spoilage yeasts. For example, Gomez-Rivas *et al.* (2004) studied the antimicrobial action of chitosan against *S. cerevisiae* and the spoilage yeasts *Brettanomyces bruxellensis* and *B. intermedius* in culture medium fermentations. These authors found that the presence of chitosan above 1.0 g/L resulted in longer lag phases for the *B. bruxellensis* strain assayed. A similar effect was obtained for *B. intermedius* at 0.5 g/L and above. The exponential growth phase and the final population densities were not highly affected. Ferreira *et al.* (2013) showed that chitosan inhibits the growth of *Brettanomyces*/*Dekkera* at concentrations ranging from 0.2 to 0.5 g/L, depending on the molecular weight of the chitosan molecules (the lower the molecular weight, the lower the minimum inhibitory concentration values) and on the assayed strains. However, chitosan affected some physicochemical characteristics of wine, particularly the hue and colour intensity. Similar results were obtained by Bağder Elmaci *et al.* (2015) for *B. bruxellensis*. This species was among the most susceptible wine related microorganisms to chitosan being completely inactivated at 0.2 g/L.

The mode of action of chitosan still is not fully understood. In bacteria, two mechanisms have been proposed. The first, and mostly accepted, consider the antibacterial effect of chitosan a result from its cationic nature: is assumed that the electrostatic interaction between positive charge of chitosan ($R\text{-}NH_3^+$) and either negatively charged cell membrane components, such as phospholipids and/or proteins (Liu *et al.,* 2004); amino acids in the Gram-positive bacterial cell wall (Kumar *et al.,* 2005); or various lipopolysaccharides in the outer membrane of Gram-negative bacteria (Davydova *et al.,* 2000; Helander *et al.,* 2001), affecting membrane integrity and permeability, causing leakage of intracellular substances, and impairment of vital bacterial activities (Rabea *et al.,* 2003; Tripathi *et al.,* 2008). Electron microscopical examinations of various chitosan-treated microorganisms suggest that this compound affect microbial cell surface (Helander *et al.,* 2001; Savard *et al.,* 2002; Raafat *et al.,* 2008), for example, in a previous study, exposure of cells to chitosan resulted in altered outer membrane, which surface was covered by several vesicular structures (Helander *et al.,* 2001). Also in yeast, in particular *B. bruxellensis* was observed that chitosan is adsorbed to the cell wall, followed of the several changes on the plasma membrane. These changes lead to permeabilization and exit of essential molecules such as ATP and, consequently, a severe loss of cell viability (Taillandier *et al.,* 2014). The second mechanism consists on the inhibition of the mRNA synthesis and proteins by permeation of chitosan into the cell nucleus and binding/ interaction with microbial DNA, causing cell dysfunction and eventual cell death

(Goy *et al.,* 2009; Hadwiger *et al.,* 1986; Sudarshan *et al.,* 1992). However, as chitosan molecules itself are too large to enter cell membranes, may be hydrolyzed by host hydrolytic enzymes such as chitinase (Hadwiger *et al.,* 1986). In addition, other mechanisms have also been proposed, where the amino group present in chitosan may be responsible for the caption of metallic ions by chelation (Wang *et al.,* 2005). Thus, chitosan may inhibit microbial growth by acting as a metals chelating agent, trace elements or essential nutrients unavailable for the organism to grow at the normal rate (Goy *et al.,* 2009; Jia *et al.,* 2001; Rabea *et al.,* 2003).

Because mode of action of chitosan in yeasts is not yet fully understood, some genomic studies have been developed. Global scale genomic studies are used to identify new targets and to clarify the mode of action of several products, including anti-cancer, anti-malaria and antimicrobial drugs, and other bioactive compounds, allowing the study of the biological function of genes on the toxic effect of these compounds (Dos Santos *et al.,* 2012). The use of omics studies has a large potential to discover gene targets of chitosan in baker's yeast. This could be a fundamental step to develop chitosan as an antifungal. Actually, chitosan gene targets have been studied using two models: yeast *Saccharomyces cerevisiae* (Galván Márquez *et al.,* 2013; Jaime *et al.,* 2012; Zakrzewska *et al.,* 2005, 2007) and the filamentous fungus *Neurospora crassa* (Lopez-Moya *et al.,* 2016). Previous transcriptomic study has been performed with the aim of investigating the mode of action of chitosan in yeast (Zakrzewska *et al.,* 2005). This study reported that *S. cerevisiae* induces a specific transcriptional expression program comprising the environmental stress response and three more major transcriptional responses mediated by the transcription factors Cin5p, Crz1p, and Rlm1p, when is challenged with sublethal concentrations of chitosan. Cin5p is responsible for the response to multiple stresses and for the regulation of genes involved in the plasma membrane; Crz1p is responsible for the calcineurin pathway, activated in response to cell wall stress; and Rlm1p is under the sole control of the cell integrity signalling pathway. The authors, in order to investigate whether loss of the regulators leads to increase sensitivity to chitosan, were tested deletion mutants of these genes (Zakrzewska *et al.,* 2005). Indeed, deletion of *CIN5* and *CRZ1* mutant strains result in sensitivity to chitosan, but deletion of *RLM1* mutant strain offered a slight resistance to this compound when compared to the parental strain. The same authors, in another study, used of yeast deletion mutant collection to identify the genes and cellular processes involved in the sensitivity to this compound (Zakrzewska *et al.,* 2007). The deletion of genes encoding proteins that are involved (directly or indirectly) in maintaining plasma membrane integrity was found to increase the sensitivity to chitosan. The high osmolarity glycerol (HOG) pathway mutants were highly susceptible to

this compound, suggesting that activation of this pathway is required to offers protection against chitosan stress. More recently, another chemogenomic study was developed with the same purpose of the ones previously described (Galván Márquez *et al.,* 2013). The results corroborate the hypothesis that chitosan can interact with DNA and/or RNA, since those hypersensitive mutants are involved in protein biosynthesis, cell cycle and DNA processing. On the other hand, the combined results of new chemogenomic and transcriptomic studies gave insight on the mode of action and mechanisms of resistance in the response of *S. cerevisiae* to a chitooligosaccharide (COS) (Jaime *et al.,* 2012). The authors confirmed five genes (*ARL1*, *BCK2*, *ERG24*, *MSG5* and *RBA50*) which provide COS resistance when overexpressed or increased sensitivity when are deletion. These genes have important roles in signalling pathways, cell membrane integrity and transcription regulation. Deletion of *ARL1* strain results in sensitive to COS, but when this gene is overexpressed, becomes resistant to this compound. This protein could play a determinant role in the process of signalling during plasma membrane permeabilization of yeast by COS, since is a plasma membrane protein associated with signalling pathways acting as a sensor and modulating membrane homeostasis. Also a transcriptomic study revealed that chitosan induces changes in expression of *N. crassa* genes, namely genes involved in oxidative stress metabolism and in plasma membrane homeostasis. Deletion of glutathione transferase (NCU10521) gene resulted in an increase of sensitivity to chitosan, suggesting that this protein could play a determinant role in ROS detoxification (Lopez-Moya *et al.,* 2016).

1.4. Aims of the study

Recently, chitosan has attracted much attention due to its strong antimicrobial activity against a wide range of pathogenic and spoilage microorganisms, without having a significant effect on mammalian cells (Dutta *et al.,* 2012; Hafdani and Sadeghinia, 2011). In this context, there has been a great interest to use this natural polysaccharide as an alternative to food chemical preservatives, such as SO_2, that may be responsible for allergic reactions even when used within the limits imposed by the current legislation (Divol *et al.,* 2012).

In winemaking, the use of chitosan from a fungal source (*Aspergillus niger*), up to 0.1 g/L, has been approved, not only as a fining agent in the treatment of musts and for wine stabilization, but also in the control of the spoilage yeast such as Dekkera/*Brettanomyces* spp (OIV-Oeno368, 2009; Reg. (UE) nº53/2011). Indeed, several studies have shown that chitosan extracted from crab shells is effective in controlling yeast growth (Bağder Elmacı *et*

al., 2014; Ferreira *et al.,* 2013; Gómez-Rivas *et al.,* 2004; Rhoades and Roller, 2000; Roller and Covill, 1999). Nevertheless the effectiveness of fungal source chitosan for the control of food microbial contaminants, and of wine spoilage yeast in particular, has been poorly documented (Portugal *et al.,* 2013; Taillandier *et al.,* 2014).

Previous studies conducted in our laboratory evaluated yeast susceptibility to fungal source chitosan (Arantes *et al.,* 2015) and SO_2 (Costa *et al.,* 2014) using several *Saccharomyces cerevisiae* commercial wine strains (Figure 1.3). The results obtained showed a great variability in yeast tolerance to both preservatives. Interestingly, while for a limited number of strain both preservatives were effective in controlling their growth, for some strains a differential effect was found. For instance, the highly SO_2 sensitive yeast strain 532 was the most resistant to chitosan. Also it was noted that the majority of yeast strains tested were able to grow in media with chitosan concentrations above the maximum concentration permitted. Indeed, other studies reported that *S. cerevisiae* was resistant to chitosan concentration (from crab shells) greater than 2.0 g/L (Elmacı *et al.,* 2014), 5.0 g/L and 6.0 g/L (Gomez-Rivas *et al.,* 2004). Altogether, these results underscored the need of further studies to evaluate the possibility of the use chitosan as an alternative to the chemical preservative SO_2.

In this line, the **aim of this study** was to contribute to the elucidation of the mechanisms of action of fungal source chitosan, using a chemogenomic approach where the EUROSCARF *S. cerevisiae* haploid yeast deletion collection was screened for targets and determinants of yeast resistance to this antimicrobial agent.

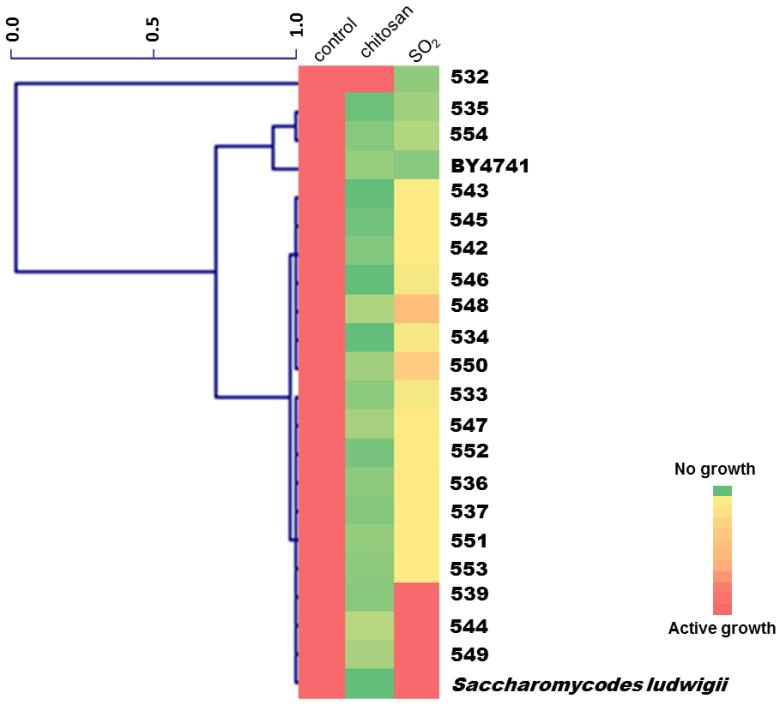
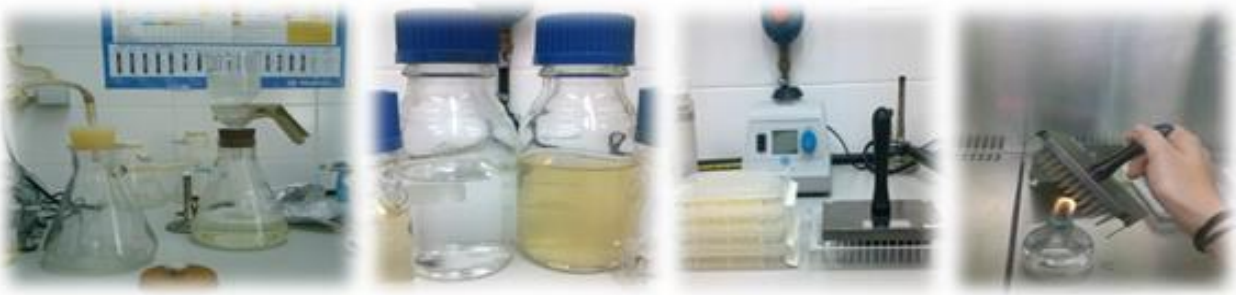


Figure 1.3 - Phenotypic diversity of commercial wine yeast strains. Laboratory yeast strain BY4741 and *Saccharomycodes ludwigii* were used as control. The yeast strains and growth conditions are organized through hierarchical clustering based on growth variation obtained by spot dilution assay, using YPD medium agar plates containing a range of chitosan (Arantes *et al*., 2015) and SO_2 concentrations (Costa *et al.,* 2014).



2. Experimental Procedure

2.1. Strains and growth media

The haploid parental strain of *Saccharomyces cerevisiae* BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and the EUROSCARF yeast culture collection of BY4741-derived haploid mutant strains, with all nonessential open reading frames (ORFs) individually deleted, were used in this study. The collection arrayed in a 96-well plates is maintained at -80 ºC in yeast peptone dextrose (YPD) medium, containing, per liter, 20 g glucose, 10 g bactopeptone and 5 g yeast extract, supplemented with 40% of glycerol.

Chitosan susceptibility assays were performed using minimal medium base (MMB), containing, per liter, 1.7 g yeast nitrogen base (YNB) without amino acids or ammonium sulphate, 20 g glucose, 2.65 g $(NH_4)_2HPO_4$, 20 mg methionine, 30 mg lysine, 60 mg leucine, 40 mg tryptophan, 20 mg histidine, 20 mg uracil (Dos Santos and Sá-Correia, 2011), acidified to pH 3.5 with HCL. Solid MMB medium was prepared by addition of 20 g/L of agarose.

Chitosan susceptibility was tested using No Brett inside® obtained from Lallemand. This commercial product, recommended for use in winemaking, consists in chitosan extracted from *Aspergillus niger* with a degree of acetylation < 30%. Stock solutions, in a range of 0.04 - 2.00 g/L, were prepared in water acidified to pH 3.5 with HCL, and sterilized at 121 °C for 15 min.

2.2. Chitosan susceptibility assays

2.2.1. Growth curves

To determine suitable experimental conditions for the evaluation of the susceptibility of the yeast mutants to chitosan, the wild-type strain BY4741 was grown in MMB liquid media containing 0.0 - 1.8 g/L of chitosan. Chitosan was included in media by incorporating equal amounts of freshly prepared stock solutions of chitosan No-Brett Inside®. Cells suspension used to prepare the inoculum were grown overnight in MMB medium (pH 3.5) at 30 ºC in an orbital shaker (250 rpm) and then diluted to an initial suspension of standardized OD_{600nm} = 0.2 (corresponding to a starting cell number of ~ 10^6 cells/ml) in a final volume of 200 µl MMB (pH 3.5) in 96-well microplates (MTPs). The MTPs were sealed with Breathe Easy gas permeable membranes and incubated for 24 h at 30 ºC. Yeast growth was followed by measuring the optical density (OD) at 630 nm every 15 min using a microplate reader

Multiskan Ascent spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). All experiments were carried out in at least triplicate.

2.2.2. Estimation of the NIC, IC_{50} and MIC parameters

To determine the effect of chitosan concentration on yeast growth, three key parameters were calculated: the Minimum Inhibitory Concentration (MIC) and Non-Inhibitory Concentration (NIC) and the concentration causing 50% of growth inhibition (IC_{50}). For the determination of these three parameters, the area under of the OD/time curve (AUC) of the growth curves in control (absence of chitosan) and in presence of increasing chitosan concentrations were used as a measure of overall yeast growth, as described by Arroyo-López *et al.,* (2009). The relative amount of yeast growth in each chitosan concentration, denoted as the fractional area (*fa*), was obtained using the ratios of the AUCs of chitosan treatments to that of the control condition. The plot of the *fa* versus $\log_{10}$ chitosan concentration produces a sigmoid-shape curve that is be well-fitted with the modified Gompertz function for decay (Lambert and Pearson, 2000). The values of the three growth parameters were obtained by a non-linear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, using the GraphPad Prism 5 software.

2.2.3. Spot-assay

The susceptibility of the parental strain BY4741 to chitosan was further determined by spot-assay on solid media. In this way, cells were grown overnight in MMB liquid medium at 30 ºC with orbital agitation (250 rpm), and diluted to a standardized OD_{600nm}= 1 (corresponding to a starting cell number of $\sim 10^7$ cells/ml). These cell suspensions and tree subsequent dilutions (1:10; 1:100 and 1:1000) were spotted (4 µl) onto the surface of solid MMB media (pH 3.5) unsuplemented or supplemented with adequate chitosan concentrations (0.00; 0.04; 0.08; 0.10; 0.25; 0.50; 0.75; 1.00; 1.25; 1.50; 1.75 and 2.00 g/L). Plates were incubated at 30 ºC for 48 to 72 h.

2.3. Genome-wide screening to identify yeast genes required for maximal tolerance to chitosan

2.3.1. Screening of the deletion mutant collection for chitosan susceptibility

The screen of chitosan susceptibility of the full set of haploid yeast deletion strains was performed as depicted in Figure 2.1. Each 96-well plate was replica-inoculated from the frozen stock using a 96-pin tool into selective MMB pH 3.5 medium and incubated at 30 ºC with orbital agitation (250 rpm) during 24 h. Five replicates of the wild type strain were included in each plate to minimize inter and intra experimental condition. After that period, the cellular suspensions were spotted with a 96-pin replica platter onto the surface of MMB agar medium supplemented or not with chitosan to a final concentration of 0.00, 0.25, 0.50 and 1.00 g/L and incubated at 30 ºC during 3 days. Slow growing strains that showed a growth defect in control plates were removed from further analysis.

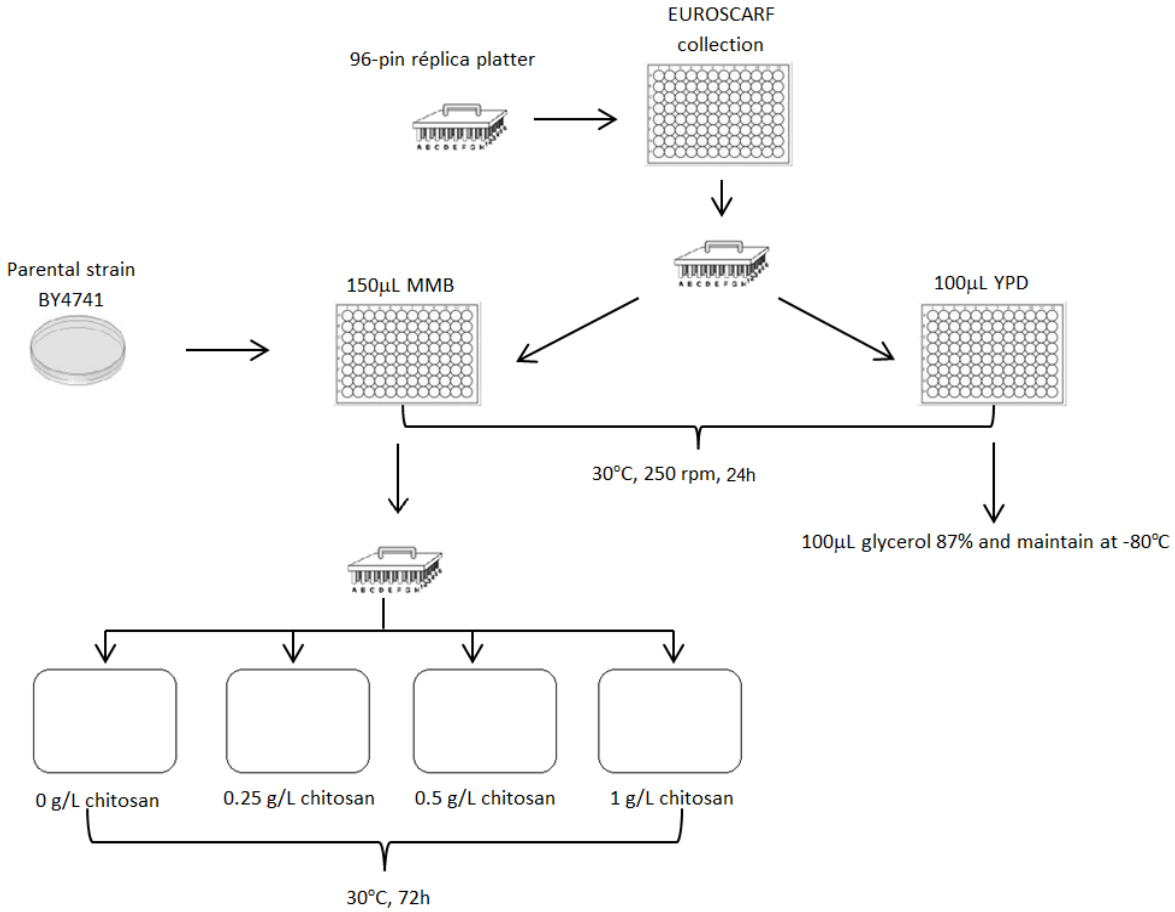
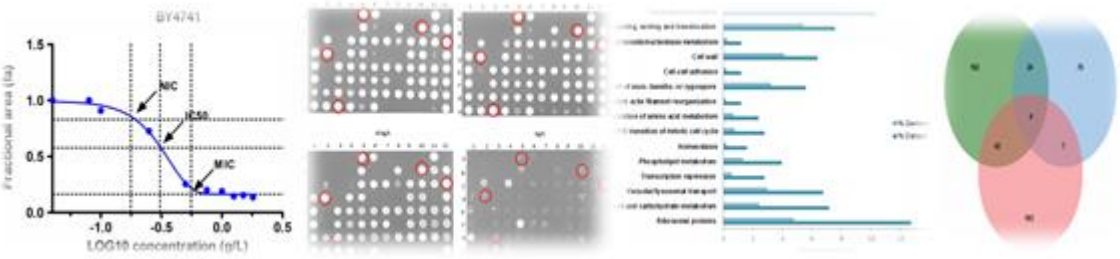


Figure 2.1- Scheme of the procedure used to perform the genome-wide phenotypic screening of EUROSCARF for the identification of determinants of resistance and sensitivity to chitosan.

Growth susceptibility phenotypes were scored by comparing the growth of each mutant to that of the parental strain, based on visual inspection of the plates. Three levels of susceptibility were considered. Mutants that did not grow in the lower concentration tested were labelled as hypersensitive (++) and those that did not grow on plates with the 0.5 g/L of chitosan were classified as sensitive (+). Also a higher concentration that inhibits the growth of wild type strain was used to potentially identify resistant mutants (R).

The different set of genes identified was assigned to functional categories using MIPS database and the *Saccharomyces* Genome Database (SGD) included in FunSpec tool (http://funspec.med.utoronto.ca/). The description of gene function was complemented using the information available in SGD (http://www.yeastgenome.org).



3. Results and Discussion

3.1. Determination of experimental conditions for screening of chitosan-susceptibility strains

3.1.1. Determination of the NIC, IC_{50} and MIC

To determine the most suitable experimental conditions for evaluating the susceptibility of the yeast mutants collection to chitosan, first the parental strain BY4741 was cultivated in MMB liquid media (pH 3.5) containing a range of concentrations of 0.0 - 1.8 g/L chitosan. A total of 33 growth curves (11 levels of chitosan × 1 strain × 3 replicates) were obtained in an automated spectrophotometer (Figure 3.1). The area under OD/time curve (AUC) has been considered as an appropriate indicator of the overall yeast growth due to the fact that this value showed a clear proportionality (direct or inverse) with the kinetic growth parameters, there is, AUC is inversely related to the lag phase, but directly correlated to both the maximum population level and maximum specific growth rate of yeast (Arroyo-Lopez *et al.,* 2010). In this study, the higher the chitosan concentration used the higher its effect on the growth of *S. cerevisiae* with the corresponding reduction of the AUC relatively to no treatment control (Figure 3.2a).

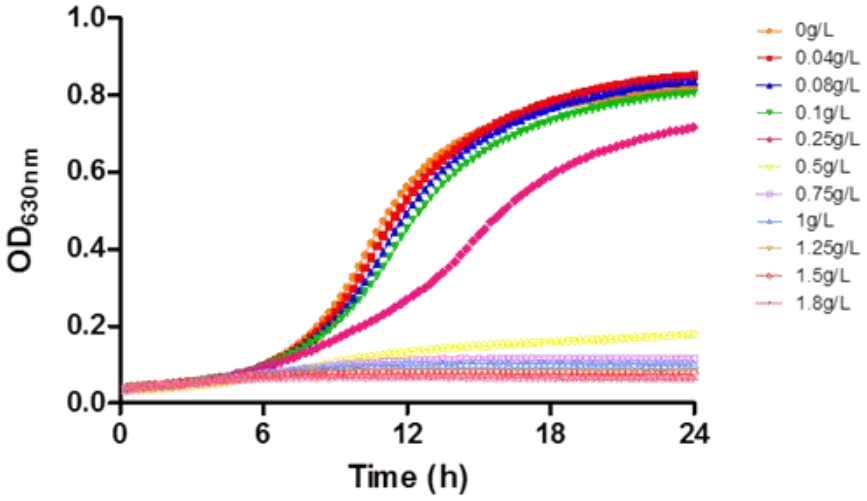


Figure 3.1 - The effects of chitosan addition on the growth of *S. cerevisiae* BY4741. Growth curves of *Saccharomyces cerevisiae* strain BY4741 in MMB (pH 3.5) medium supplemented with different concentrations of chitosan. Cell growth was monitored by measurement of optical density at 630mn for 24h.

From the analysis of area under of the OD/time curve (AUC) of the growth curves in control (absence of chitosan) and in presence of increasing chitosan concentrations,

three basic parameters was determined. In this way, the smallest concentration of chitosan found to reduce yeast growth (NIC) was 0.18 g/L; the concentration of chitosan that was required for 50% inhibition of the yeast cell viability (IC_{50}) was 0.31 g/L; and the smallest concentration of chitosan that completely inhibits yeast growth (MIC) was 0.55 g/L (Figure 3.2b). The values obtained in this study greatly differed from those previously reported by Zakrzewska *et al.* (2007) and Jaime *et al.* (2012) using the yeast strain BY4743 (Table 3.1). In fact the inhibitory values reported in both these studies, expressed in mg/L, are well below those most reported effective chitosan concentrations which are around 1.0 g/L (Rabea *et al.,* 2003).

Besides the difference in the yeast strain tested, the differences seen in our results and between them are likely due to a number of other different experimental conditions, as it is known that the physiochemical properties of chitosan such as, its origin, degree of deacetylation and molecular weight affect chitosan biological activity (Goy *et al.,* 2009; Kong *et al.,* 2010). Also differences in the chitosan dissolution method could account for such differences since the use of soluble and homogeneously acetylated chitosan is essential to draw conclusions on biological properties of chitosan (Younes *et al.,* 2014). Also, discrepancies in these results can also be attributable to the different medium composition and different pH.

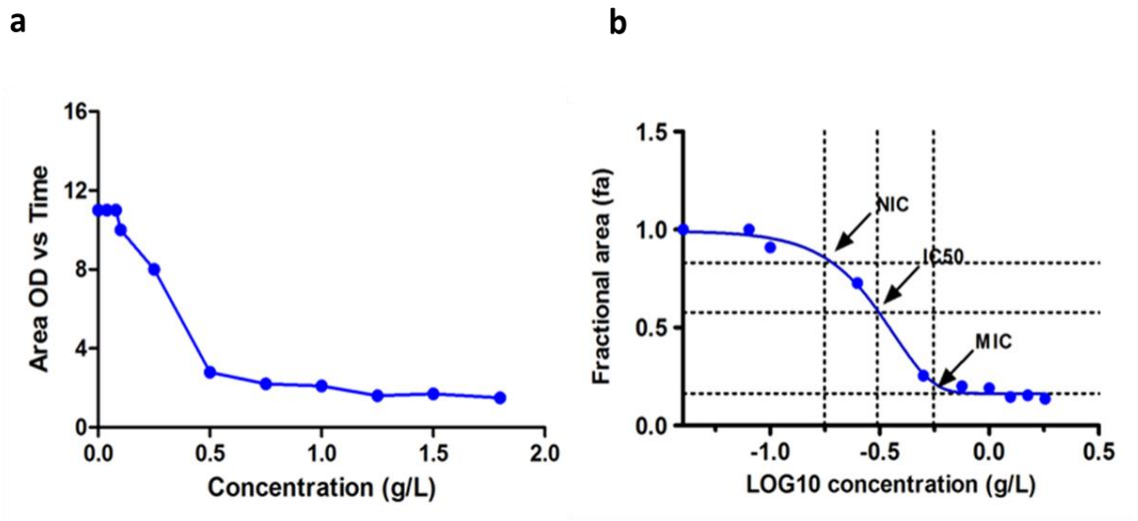


Figure 3.2 - Determination of the growth parameters (NIC, MIC and IC_{50}). (**a**) Graph displaying growth inhibition of *Saccharomyces cerevisiae* strain BY4741 constructed with the values of the AUCs calculated from the growth curves. (**b**) The NIC, IC_{50} and MIC values determined by calculating the area under the curve (i.e., defined as the fraction of the area under the curve of the negative control and the area under the curve of the positive control) versus the $\log_{10}$ of the concentration of positive control.

Table 3.1 - Experimental conditions and inhibitory concentrations found in different screenings of *S. cerevisiae* susceptibility to chitosan.

Strains	Chitosan				Medium	pH	Inhibitory concentration	Reference
	Origin	MW (kDa)	DD (%)	Dissolution				
BY4741	*Aspergillus niger*	-	> 70	H_2O, pH 3.5	MMB	3.5	IC_{50} 0.31 (g/L)	This study
S288C	Low Molecular Weight -SIGMA	150	75-85	1% acetic acid	YPD	5.5	IC_{50} 1.5 (g/L)	Galván Marquez *et al.,* 2013
BY4743	Crab shells	≥ 600	85	10% acetic acid	SC	5.5	nd (25 mg/L)	Zakrzewska *et al.,* 2007
BY4743	Chitosan (T8s) - Marine BioProducts GmbH	70	80	DMSO + HCl, pH 5.7	0.5X YPD	5	nd (mg/L)	Jaime *et al.,* 2012
BY4743	Chitosan oligosaccharide (COS)	5.44	97	DMSO + HCl, pH 5.7	0.5X YPD	5	IC_{70} 112.5 (mg/L)	Jaime *et al.,* 2012

MW – Molecular Weight; DD – Degree of Deacetylation; nd – not determined

MMB – Minimal Medium Base; YPD – Yeast Peptone Dextrose; SC – Synthetic Complete

IC_{50} and IC_{70}- concentration causing 50% and 70%, respectively, of growth inhibition

3.1.2. Spot-assay

Given that the goal of this study was to screen the yeast deletion mutant collection by evaluating their ability to growth on solid media supplemented with chitosan, the susceptibility of the parental strain BY4741 to chitosan was further determined by spot-assay, using MMB (pH 3.5) agarose plates containing a range of concentrations of chitosan, 0.0 – 2.0 g/L. As it can be seen in Figure 3.3, the effect was clearly depended on the amount of cells. At the higher cell concentration, the cells were able to grow up to 0.75 g/L of chitosan, a value above that previously determined in liquid media. Only when 1 g/L of chitosan was used, a clear growth inhibition was observed for all cell densities tested. The inferior effectiveness of chitosan in reducing yeast growth on solid medium was probably due to the lower chitosan-yeast cell contact in these conditions compared to growth in liquid medium. Higher inhibitory chitosan concentration (1.5 g/L) was found by Galván Márquez *et al.* (2013) using the same strain and the same methodology, spot-assay. Again, differences on the experimental conditions (pH, media), the origin of chitosan used as well as the preparation of stock solution, could account for such discrepancy (Table 3.1). Indeed, Peña *et al.* (2013), evaluating the antifungal activity of chitosan on the pathogenic yeast *Candida albicans* found that rather higher concentrations of chitosan were needed to inhibit yeast growth in YPD medium than in 10 mM MES-TEA buffer, pH 6.0. The authors concluded that such observation was most probably due higher salt (anions and cations) concentrations of YPD.

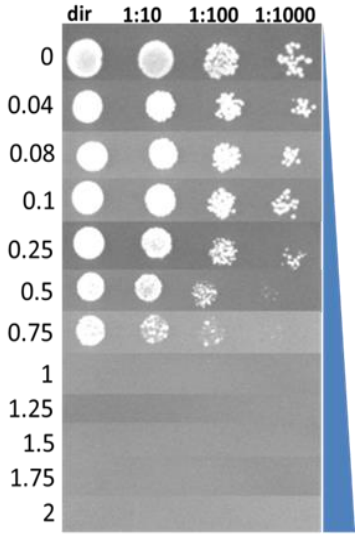


Figure 3.3 - Spot dilution assay of wild type strain BY4741 were spotted on MMB medium agar plates containing various concentrations of chitosan (between 0.0 and 2.0 g/L). The cell dilution series started at 10^7 cells/ mL followed by 10^6, 10^5, 10^4 cells/ml where 4 µl per spotted. Growth was observed after 2-3 days incubation at 30 ^{o}C.

3.2. Genome-wide identification of deletion strains with altered susceptibility to chitosan

The identification of the genes underlying *S. cerevisiae* tolerance to chitosan was based on the comparison of the susceptibility to chitosan of the mutants of the EUROSCARF haploid knockout strain collection (approximately 5200 deletion mutants) with the parental strain BY4741. Based on the results obtained on experiments described above three levels of susceptibility were considered. Hypersensitive and sensitive strains were identified in plates containing a moderately inhibitory chitosan concentration (0.25 g/L and 0.5 g/L, respectively), while resistant strains were identified in plates containing 1.0 g/L of chitosan which proved to be an inhibitory concentration for the parental strain (Figure 3.4). Hypersensitivity and/ or sensitive phenotype to chitosan theoretically suggest that the deleted gene is important for conferring resistance in the parental cell, while resistant phenotypes to chitosan possibly suggest targets or genes that are involved in modifications or pathways that enable the cytotoxic action of compound. Through this screening we identified 745 mutants with an altered susceptibility profile to chitosan, of which 252 strains hypersensitive, 287 strains sensitive and 207 strains displayed enhanced resistance to chitosan. In the Supplementary files Table S1 and Table S2 are presented showing the standard gene name (if applicable) or the systematic name, along with a brief description of the function of each gene.

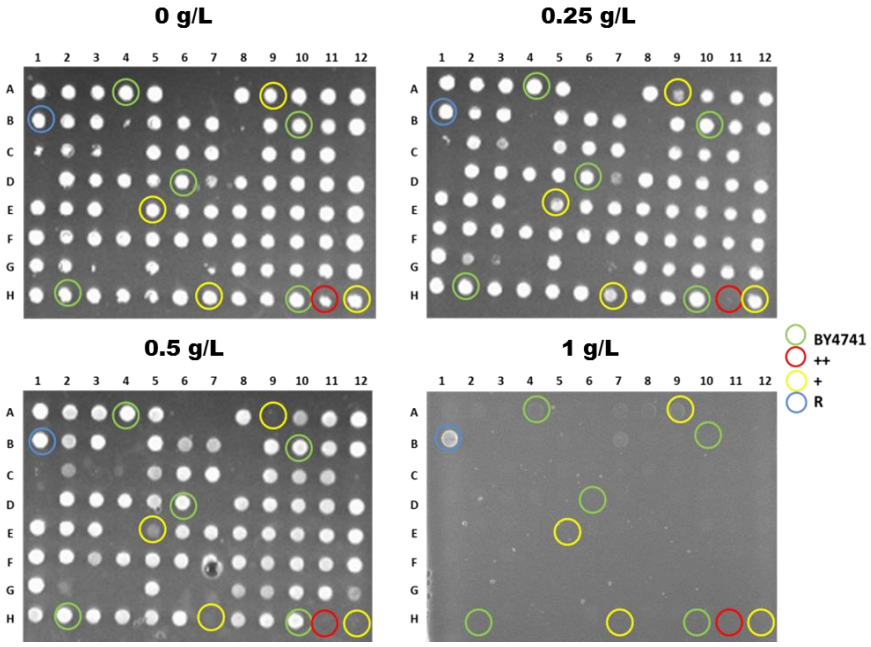


Figure 3.4 - Illustrative example of deletion strains exhibiting different susceptibility profiles. Two levels of susceptibility were considered, based on growth deficiency in the presence of increasing levels of chitosan of the deletion mutants tested, compared to the parental strain (BY4741). Mutant strains displaying growth in the presence of 1.0 g/L (chitosan) were labelled as resistant. Legend code: (+) sensitive phenotype; (++) hypersensitive phenotype; (R) resistant phenotype; BY4741, wild type.

3.2.1. Identification of genes conferring sensitivity to chitosan

Two independent genome-wide phenotypic studies have previously screened yeast gene deletion strains collections growing under different chitosan concentration aiming the elucidation of the mechanisms underlying *S. cerevisiae* tolerance to this natural compound (Zakrzewska *et al.,* 2007; Galván Márquez *et al.,* 2013). In the first study the authors used both homozygous and heterozygous tagged deletion mutant collections of strain BY4743 and collected data after 5 and 9 h of yeast growth in liquid medium in the presence of chitosan (Zakrzewska *et al.,* 2007). The second study was more similar to ours, using the haploid yeast deletion mutants of strain BY4741 spotted onto solid medium, and colony size reduction due to chitosan exposure was monitored after 24 - 48 h (Galván Márquez *et al.,* 2013). It was carried out the comparison between the genes identified in our study as conferring hypersensitivity and sensitivity to fungal origin chitosan with those previously identified in both studies (Figure 3.6). It was somehow surprising the higher number of genes identified in our study as the methodologies used by those studies, an in particular by Zakrewska *et al.* (2007), are more likely to detect growth defects that may be overlooked in our approach. The contrasting choice of doses as well as other conditions described above may also account for the differences in chitosan sensitive strains identified by each screen.

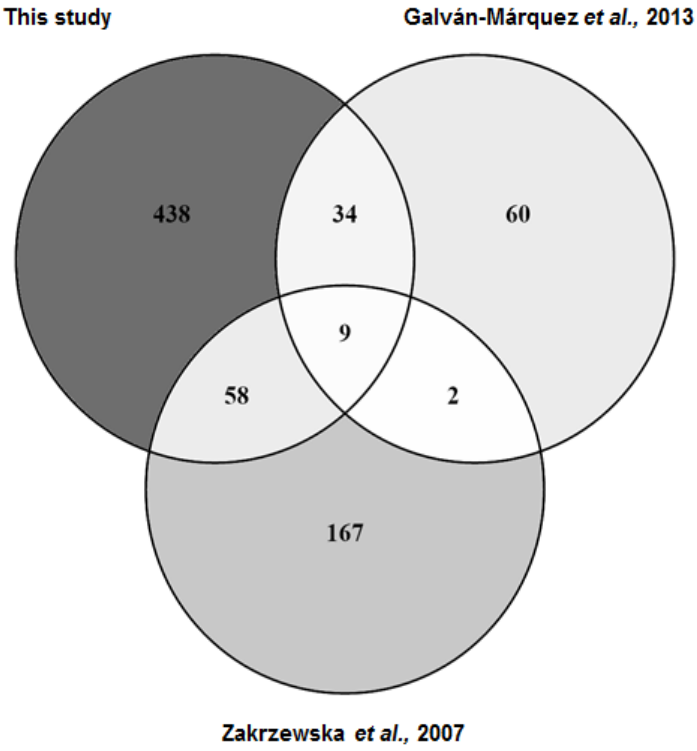


Figure 3.1 - Venn diagram indicating the number of overlapping genes whose deletion was found to confer a sensitive phenotype in three independent studies.

This analysis revealed that there are only nine genes commonly (Table 3.2), underlying the importance of these genes on the yeast resistance to chitosan. Only one of these genes, ***SNF8*** encoding a subunit of the endosomal sorting complexes required for intracellular transport (ESCRT-II), has been also pointed out has being sensitive to COS - chitosan oligosaccharide (Jaime *et al.,* 2012). This derivative of chitin acid hydrolysis, like chitosan, has shorter chains of N-glucosamide turning it more water-soluble and is described as having higher antimicrobial proprieties than chitosan (Xia *et al.,* 2011)**.**

Table 3.2 - Commonly genes whose deletion was found to confer a sensitive phenotype in three independent studies and their corresponding description

Gene*	Function**
Protein modification	
LAS21	Integral plasma membrane protein involved in the synthesis of the glycosylphosphatidylinositol (GPI) core structure
LDB7	Component of the RSC chromatin remodeling complex
OST6	Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes protein asparagine-linked glycosylation
Transcription	
SIN3	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes
SNF8	Component of the ESCRT-II complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome
Celular sensing and response	
SCP160	Essential RNA-binding G protein effector of mating response pathway, mainly associated with nuclear envelope and ER
Stress response	
LTV1	Component of the GSE complex, which is required for proper sorting of amino acid permease Gap1p
Unknown/ Dobious	
API2	Dubious open reading frame, unlikely to encode a protein
YLR374C	Dubious open reading frame, unlikely to encode a protein

*Genes marked in **bold** represent the genes whose deletions resulted in hypersensitive to chitosan in our screen.
**Biological function is based on the information available in SGD (www.yeastgenome.org).

In order to obtain a more complete understanding of the which biological functions that are important for tolerance to chitosan, the genes identified in this study were clustered into functional categories using MIPS database included in FunSpec tool. The two sensitive phenotypes data sets were first analyzed together and separately. Functional analysis of both data sets individually revealed the genes are mainly involved in similar categories. Nevertheless, some functional categories identified when only hypersensitive set was considered (Figure 3.6) were less enriched. Thus, during our analysis besides hypersensitive genes marked in bold, sensitive genes belonging to the same functional category were also included. The *p*-values calculated by FunSpec represent the probability that the intersection of a given list with any functional category occurs by chance. Note that many genes are contained in many categories, especially in the MIPS database (which are hierarchical) and that this can create biases.

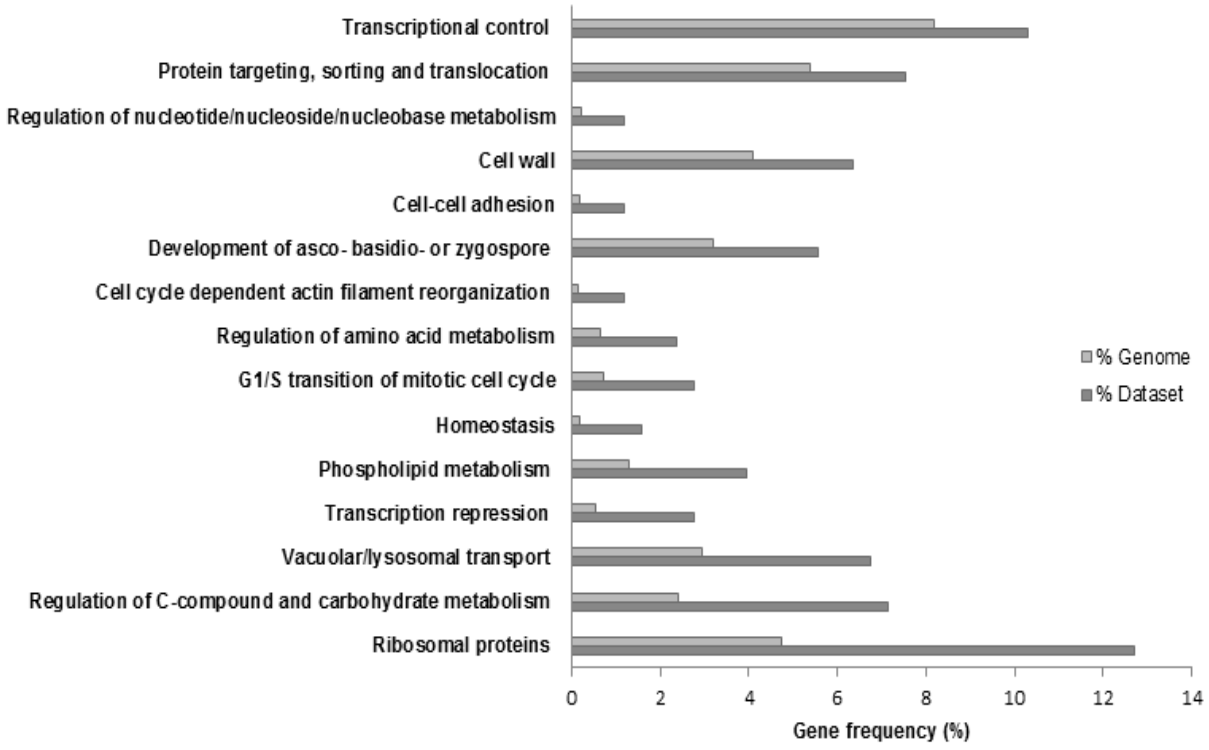


Figure 3.6 - Functional categorization of genes whose deletion led to either increased sensitivity to chitosan. Genes identified by the genome-wide screen with altered susceptibility to chitosan were clustered into functional categories that were significantly enriched (p-value below 0.01) based on the MIPS resource. Dark grey bars: gene frequency within each class in the chitosan dataset; light grey bars: frequency registered for the whole genome.

A significant enrichment of sensitive mutants had deletions of genes involved in growth related functions. Accordingly, Galván Márquez *et al*. (2013), has observed that almost 50% of the chitosan sensitive deletion mutants identified had deletions of genes involved in protein synthesis, cell cycle and DNA processing. In fact, the overlapping genes between both studies (Figure 3.6) include 12 genes involved in ribosomal proteins **(*RPS18A*,** *RPS23A,* ***NSR1, RPS4B****, RPS24B,* ***RPS30A****,* ***RPS17A****,* ***RPS18B****, RPL13B, RPS16A,* ***RPS10B*** and ***RPS19A***). Our study added further 33 genes related with **ribosomal proteins**. These observations could be explained by the age of the cells culture. In this study, cells were grown to mid-exponential phase.

The functional class **cell cycle and DNA processing** contains genes involved in the mitotic cell cycle transition, for example genes encoding subunits of casein kinase 2 (*CKA1*, ***CKA2***, ***CKB1*** and ***CKB2***). In addition, it was also observed a high number of genes involved in DNA conformation modification (e.g. chromatin), namely genes belonging to the SWR complex (***SWC3***, ***SWC5***, ***SWR1***, ***ARP6***, *VPS71*, *VPS72* and ***YAF9***) required for ATP-dependent exchange of histone H2A for the H2AZ variant in *S. cerevisiae* (Nguyen *et al.,* 2013), as well as the H2AZ gene itself (HTZ1); and components of components of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (*SGF29,* ***GCN5***, ***ADA2, CHD1*** and *SUS1*). The SAGA complex is involved in transcriptional regulation of approximately 10% of the genes in yeast, one of the essential processes by which the cell can respond to environmental signals (Huisinga and Pugh, 2004). Since these complexes are involved in both the positive and negative transcriptional regulation of numerous genes, particularly under conditions of cellular stress, they may have a role important in chitosan tolerance. Indeed, previous studies showed that electrostatic interactions can occur between positively charged amino groups from the N-glucosamine, forming-monomers of chitosan and negatively charged phosphate groups on DNA and RNA (Ma *et al.,* 2009; Souza *et al.,* 2009). Taken together, ours and Galvan Marquez *et al.* (2013) results suggest that cationic chitosan can interact with DNA of S. cerevisiae cells, inhibited mRNA synthesis and proteins, and causing cell dysfunction and eventual death (Hadwiger et al., 1986). Furthermore, cells exposed to chitosan appear respond to DNA damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate DNA repair.

Another functional class emerged from this study, included a high number of genes involved in **regulation of C-compound and carbohydrate metabolism**. In this category are ***HOG1***, ***PBS2*** and *SSK2* genes, encoding members of the mitogen-activated protein kinase (MAPK), MAP kinase kinase (MAPKK) and MAP kinase kinase kinase (MAPKKK) family, respectively, involved in high osmolarity glycerol (HOG) signaling pathway. Interestingly, a similar

behavior of deletion mutants in HOG pathway was observed by Zakrzewska *et al*. (2007) indicating that this pathway is required to offer protection in yeast against chitosan. In addition to the osmotic stress response (Schüller *et al.,* 1994), several works indicated that the HOG pathway might also be activated in response to cytosolic acidification resulting from other stresses (Kawahata *et al.,* 2006; Lawrence *et al.,* 2004; Mollapour and Piper, 2006). Thus, this pathway might be activated, directly or indirectly by chitosan stress, in response to cytosolic acidification.

In addition to the signaling pathway described above, *S. cerevisiae* contain a dedicated signal transduction pathway that is activated under pH changes: the so-called RIM101 pathway. In this study several genes involved in RIM101 pathway (***RIM8***, ***RIM9***, ***RIM13***, ***RIM20***, ***RIM21, RIM101*** and ***DFG16***) were identified as conferring resistance to chitosan. Although the role of RIM101 pathway in *S. cerevisiae* is well established in the literature, recent studies suggest that this pathway may have other roles beyond alkaline pH-induced responses (Lamb and Mitchell, 2003; Peñalva and Arst, 2004; Su and Mitchell, 1993), for example in cell wall construction (Castrejon *et al.,* 2006), in mediating tolerance to high concentrations of sodium and lithium (Parsons *et al.,* 2003) and in adaptation and resistance to weak acids (Mira *et al.,* 2009). Others studies demonstrating that the RIM101 pathway is also activated by changes in lipid composition and in physicochemical properties of the plasma membrane (Ikeda *et al.,* 2008; Mattiazzi *et al.,* 2010).

The yeast cell wall structure and membrane composition appears to be important processes for the increase resistance to chitosan. Subsequently, a set of genes involved in **cell wall** were identified as determinants of resistance to chitosan, including genes related with synthesis (***SMI1***, *CWH43*, *FKS1,* ***ROT2*** and ***KRE1)*** and maintenance (***BGL2***, ***GAS1,*** *GAS2* and *CCW12*). The cell wall is a complex structure (strong, but elastic) essential, not only for the maintenance of cell shape, prevention of lysis, and protection against harmful environmental conditions, but also for progression through the cell cycle (Lagorce *et al.,* 2003; Levin, 2011). This layered structure is composed principally of mannoproteins, chitins and glucans. Chitin and glucan components should be good drug targets because they are unique and essential to fungi (Georgopapadakou and Tkacz, 1995). **Smi1p** and Fks1p are essential for the synthesis of *β*-1,3-glucan, a major component in the fungal cell wall, as well as **Rot2p** and **Kre1p** are necessary for normal levels of *β*-1,6-glucan synthesis in the yeast *S. cerevisiae* (Lesage and Bussey, 2006). Cell wall stress require remodeling of the crosslinking of *β*-1,3- and *β*-1,6-glucans to themselves and to other cell wall components and ***BGL2***, ***GAS1*** and *GAS2* genes encode cell surface proteins which are known to be able to remodel *β*-1,3-glucan (Dickinson and Schweizer 2004). These observations indicate that cell

wall structure, namely the contents of *β*-1,3- and *β*-1,6-glucans and mannans are required to withstand chitosan stress. Additionally, a number of genes related to **phospholipid metabolism** (***CST26***, ***CHO1***, ***CHO2***, ***OPI1***, ***OPI3***, ***TSC3*** and ***SCS7***) were also identified as conferring resistance to chitosan. The phospholipid composition of the plasma membrane is complex, the inner of the *S. cerevisiae* plasma membrane is composed for phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), while the external is composed for phosphatidylcholine (PC) (Rest *et al.,* 1995). Mitochondria and ER are the subcellular compartments that contribute most to phospholipid biosynthesis (Daum *et al.,* 1998). ***CHO1*****,** ***CHO2*** and ***OPI3*** encode enzymes involved in biosynthesis of PE and PC, two of the most abundant phospholipids in the plasma membrane (Figure 3.7a). Interestingly, in this functional category there are included genes that are involved in **sphingolipid biosynthesis** (***SUR1*** and ***IPT1***). Sphingolipids are involved in numerous cellular processes, such as protein anchoring, stress responses, apoptosis and autophagy (Hannun *et al.,* 1995; 1996; Yamagata *et al.,* 2011). In *S. cerevisiae*, the sphingolipids constitute about 40% of the inositol-containing lipids in the plasma membrane (Patton and Lester, 1991). There are three species of *S. cerevisiae* sphingolipids differ by polar head group composition, and they are inositolphosphoryl-ceramide (IPC), mannosyl-inositolphosphoryl-ceramide (MIPC) and mannosyl-diinositolphosphoryl-ceramide ($M(IP)_2C$). ***SUR1*** encodes the enzyme that catalyzes the conversion of IPC into MIPC and the gene product of the ***IPT1*** catalyzes the conversion of MIPC into $M(IP)_2C$, the major sphingolipid in membranes of *S. cerevisiae* (Daum *et al.,* 1998; Dickson and Lester, 2002). The observations that deletion mutants strains involved in phospholipid and sphingolipid biosynthesis are highly sensitive to chitosan, suggests that chitosan affects membrane fluidity, probably due to its cationic nature. The involvement of chitosan-induced loss of plasma membrane integrity has been previously pointed out by Zakrzewska *et al.* (2005). In that study, the authors performed a genome-wide transcriptomic analysis of *S. cerevisiae* treated with chitosan and showed that a significant number of genes related with plasma membrane are induced under chitosan stress. Additionally, Palma-Guerrero *et al.* (2009) using *Neurospora crassa,* reported that membrane fluidity, and in particular the level of fatty acid unsaturation, determines filamentous fungi sensitivity to chitosan. Recently, also a study in *N. crassa* confirmed that membrane homeostasis is a main function in response to chitosan (Lopez-moya *et al.,* 2016).

We have chosen a set of mutants strains within phospholipid metabolism category to be tested individually, and in this way, the susceptibility of the deletion mutants involved in phospholipid (***Δcho1*****,** ***Δcho2*****,** ***Δopi1*** **and** ***Δopi3***) and sphingolipid (***Δsur1*** and ***Δipt1***)

biosynthesis pathways, all identified as having a higher susceptibility to chitosan in the disruptome screening, was assessed by spot assays or by comparison of the growth curves of these strains in MMB liquid growth medium (at pH 3.5) either or not supplemented with chitosan (Figure 3.7b and 3.7c). All six tested deletion strains exhibited complete growth suppression by 0.25 g/L of chitosan, confirming their strong sensitivity.

The enriched class of ***vacuolar/lysossomal transport*** is essentially composed by genes involved in proteins targeted, sorted and translocated to the Golgi, for example components of the multimeric membrane-associated retromer complex (***VPS29***, *VPS5* and *PEP8)*, components of the Golgi-associated retrograde protein (GARP) complex (***VPS51***, ***VPS52***, *VPS53* and ***VPS54***), components of t-SNARE (*TLG2*) and v-SNARE (***TVP38*** and *VAM7*), and components of the homotypic fusion and vacuole protein sorting (HOPS) complex (***VAM6*** and ***VPS41***). Also previous studies reported that deletions strains encoding components of GARP complex and HOPS complex cause sensitivity to cationic drugs (Barreto *et al.,* 2011; Wagner *et al.,* 2006). In addition, it were also identified more genes encoding components of the ESCRT complex – ESCRT I (**STP22**, *SRN2* and *VPS28*), ESCRT-II (**SNF8**, ***VPS36, VPS25***) and ESCRT-III (***SNF7***) – which are involved in lysosomal catabolism of transmembrane proteins through the multivesicular body (MVB) pathway. MVBs are formed by invagination of the endosomal membrane to receive the transmembrane proteins (Katzmann *et al.,* 2002). A strong perturbation in the plasma membrane might produce defective invaginations impeding the proper trafficking and therefore inefficient endosomal transport (McMahon and Gallop, 2005). Several studies reported that some of ESCRT subunits are also required for activation of RIM101 pathway (Bowers *et al.,* 2004; Hayashi *et al.,* 2005; Ito *et al.,* 2001; Xu *et al.,* 2004). Indeed, ESCRT deletion strains exhibiting susceptibility in the presence of chitosan are genes encoding proteins known to participate in the induction of the RIM101 pathway. Thus, this pathway is again pointed in the protection of *S. cerevisiae* against damage inflicted by chitosan.

3. Results and Discussion

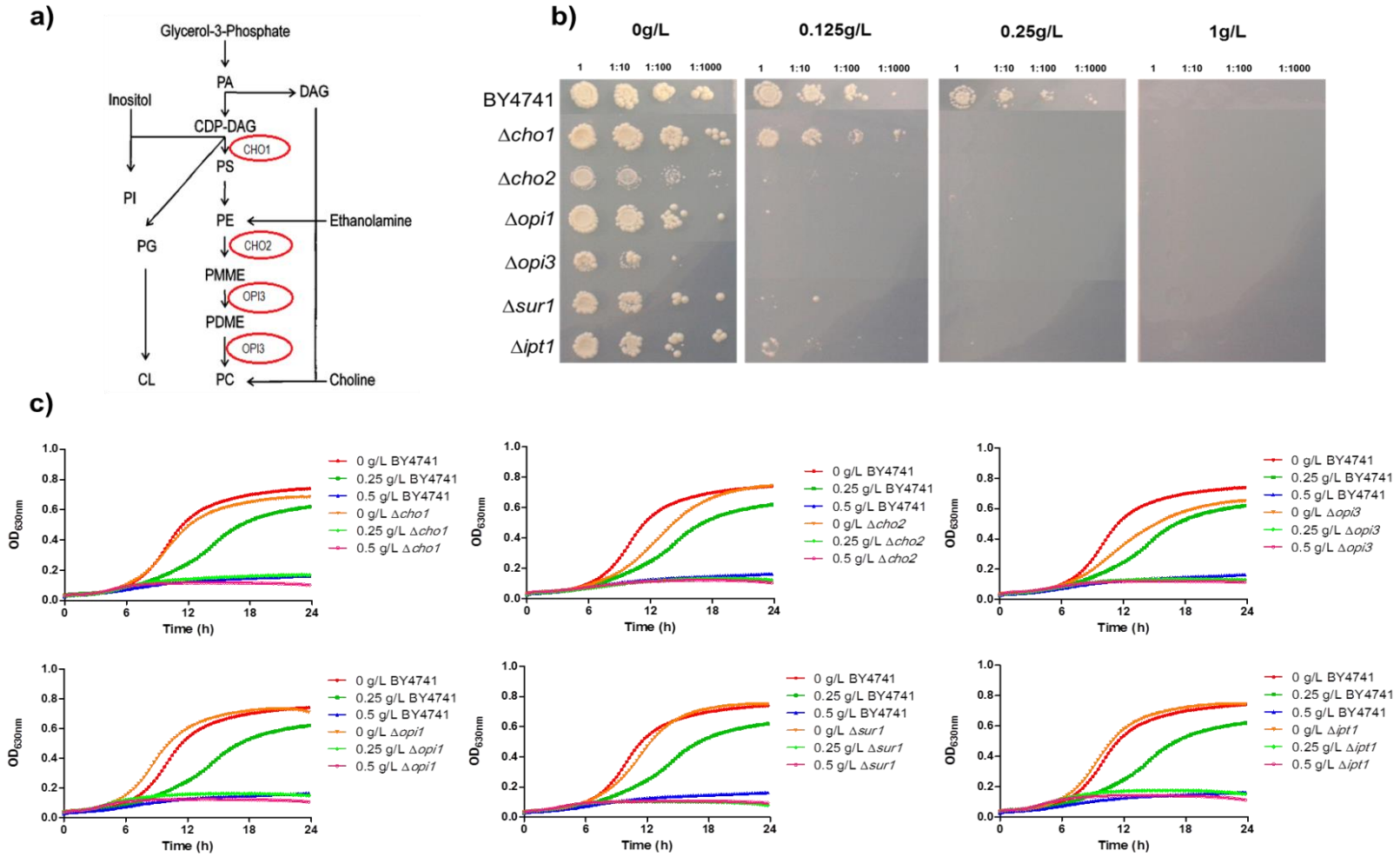


Figure 3.7 - The phospholipid biosynthetic pathway in *S. cerevisiae* (a) and phenotypic confirmation of representative deletion mutant strains with deficient growth after exposure to chitosan in solid (b) and liquid medium (c). Spot-assay (b) and growth curves (c) for strains with deletions in the genes *CHO1*, *CHO2*, *OPI1*, *OPI3*, *SUR1* and *IPT1* grown in MMB 3.5 (control) and chitosan supplementation (0.25 and 0.5 g/L). Growth of BY4741 wild type is included as the reference. All strains exhibited extensive sensitivity to treatment.

Among our set of determinants of yeast resistance to chitosan were found genes encoding the RNA polymerase II mediator complex (***NUT1***, ***CSE2*** and ***SIN4***), a multi-subunit protein complex that plays diverse roles at multiple stages of transcription, including elongation, termination, mRNA processing and epigenetic regulation (Yin and Wang, 2014). The mediator complex seems to be the central binding interface between gene-specific transcription factors and the RNA polymerase II machinery (Borggrefe and Yue, 2011). Transcription factors (TFs) habitually control gene transcription through binding to specific DNA-binding sites, which can either promote (activate) or repress (inhibit) the recruitment of the transcription initiation machinery (Hahn and Young, 2011). Consequently, the identification of genes encoding TFs are of particular interest because their deletion may affect a set of chitosan resistance genes under their control. In this study, we have found twenty genes whose deletion altered susceptibility to chitosan encoding TFs, of which ten appeared as chitosan-hypersensitive and more ten appeared as chitosan-sensitive (Table 3.3). The genes whose deletion confers susceptibility to chitosan (hypersensitive and sensitivity data) were also clustered based on their regulatory associations with these transcription factors, using the YEASTRACT database ("rank genes by TF"). The computational tools available in this database make it possible cluster genes according to the TFs which are known to regulate them. So, this clustering method allowed the identification of **Gcn4p**, the master regulator of amino acid biosynthesis (Natarajan *et al.,* 2001), as regulator of approximately 40% of the susceptibility genes during the yeast response to chitosan, most of these genes encoding ribosomal proteins. Indeed, a recent study reported that this TF is involved in negative regulation of ribosomal protein gene transcription from RNA polymerase II promoter in response to amino acid starvation (Joo *et al.,* 2011). **Cbf1p, Dal81p and Met31p**, which were suggested to regulating about 22% and 6% of the chitosan-sensitive genes, respectively, are also involved in regulation of the amino acid metabolism. Furthermore, approximately 20% of the chitosan-susceptibility genes was documented target of **Gln3p** a transcriptional activator that is involved in positively regulating genes that are subject to nitrogen catabolite repression (NCR) (Godard *et al.,* 2007), responsible for poor nitrogen source import and metabolism (Crespo and Hall, 2002). The action of chitosan in yeast plasma membrane may result in loss of nutrient and consequently in response to energy and nutrient limitation. Recently, Lopez-Moya *et al.* (2014) have showed that the nutritional limitation, either carbon or nitrogen, affects the sensitivity of filamentous fungi and yeast pathogens by increasing the antifungal activity of chitosan. The authors suggested plasma membrane permeabilization due to the interaction between the

positively charged chitosan molecules and negatively charged microbial cell membranes, together with nutrient limitation, could result in lack of energy necessary for the cell wall and for plasma membrane repair after chitosan damage.

Table 3.3 - Transcriptional factors identified in this study as determinants of yeast resistance to chitosan and their corresponding description

	TF*	% in user set	Description**
Hypersensitive	Gcn4p	39.37	Activator of amino acid biosynthetic genes
	Cbf1p	21.46	Activates genes involved in inositol biosynthetic process and sulfate assimilation; represses genes involved in ceramide biosynthetic process
	Gln3p	19.96	Activator of genes regulated by nitrogen catabolite repression
	Swi4p	16.79	DNA binding component of the SBF complex (Swi4p-Swi6p)
	Zap1p	15.30	Involved in response to zinc ion starvation
	Pho2p	14.74	Regulatory targets include genes involved in phosphate metabolism
	Ume6p	12.13	Regulation of pseudohyphal growth, mitosis, meiosis, sporulation, lipid metabolism and nitrogen catabolite repression
	Rim101p	8.77	Involved in meiosis, spore formation, cell wall biosynthesis, and the cellular responses to anoxia and alkaline pH
	Dal81p	6.16	Positive regulator of genes in multiple nitrogen degradation pathways
	Sfl1p	2.05	Repression of flocculation-related genes, and activation of stress responsive genes
Sensitive	Swi5p	30.22	Regulation of the mitotic cell cycle and of mating-type switching
	Gcr2p	20.34	Activator of genes involved in glycolysis and ribosomal protein
	Aft1p	14.55	Regulates chromatid cohesion, chromosome segregation, and cellular iron homeostasis
	Sko1p	13.62	Forms a complex with Tup1p and Cyc8p to both activate and repress transcription; involved in osmotic and oxidative stress responses
	Rox1p	13.25	Represses expression of hypoxia-induced genes in the presence of oxygen and represses target genes during osmotic stress
	Rfx1p	10.07	Repressor of DNA-damage-regulated genes
	Crz1p	7.84	Activates transcription of stress response genes
	Dig1p	6.34	Regulation of mating-specific genes and the invasive growth pathway
	Met31p	6.16	Regulation of the methionine biosynthetic genes
	Stp3p	-	Unknown function; possibly involved in pre-tRNA splicing and in uptake of branched-chain amino acids

*Only the transcription factors that were found to exert protection against chitosan were considered in this analysis.
**Description of TFs is adapted from SGD (www.yeastgenome.org).

It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation such as iron, copper, cadmium and magnesium (Helander *et al.,* 2001). Included in our dataset were found *Aft1p* a transcription factor involved in iron utilization (Rutherford and Bird, 2004). The requirement for these genes could be an indication of a disturbance in iron homeostasis in cells treated with chitosan. Additionally, mutants deleted of the Aft1p target genes, *FET3* and *FTR1,* required for high affinity iron uptake, displayed increased susceptibility to chitosan. This suggests that chitosan treatment may have an indirect effect on intracellular iron pools by inducing iron deficiency, probably due to the chelating ability of chitosan.

In addition, Crzp1p may be involved in regulation of about 8% of the chitosan susceptibility genes. The Crz1p dependent response is activated by many forms of stress, including cell wall stress, and is regulated by calcineurin, a Ca^{2+} /calmodulin-dependent protein phosphatase (Lagorce *et al.,* 2003). A previously study reported that Crz1p-controlled response offers protection against chitosan, suggesting that induction of cell leakage by chitosan stress could lead to activation of the calcineurin-dependent pathway in order to deal with ion fluctuations (Zakrzewska *et al.,* 2005).

3.2.1. Identification of genes conferring resistance to chitosan

Considering the genes whose deletion caused resistance to chitosan it was observed that the functional categories most significantly enriched (p-value below 0.01) with the highest percentage (11.6%) play a role in “Transport routes” (Figure 3.8). This category is essentially composed of genes encoding for proteins involved in peroxisomal transport, ER to Golgi transport, Vesicular transport and Endocytosis. A considerable percentage (2.9%) of genes is involved in "Ionic homeostasis", "Nucleotide metabolism" and "Protein modification". Finally, the lowest percentage (1.9%) was found in “Cell aging”.

It is noteworthy that most of the genes whose deletion led to increased resistance to chitosan are located in membrane, especially in the ER membrane and in the Golgi apparatus membrane, suggesting that these cellular components are the main targets in response to chitosan stress.

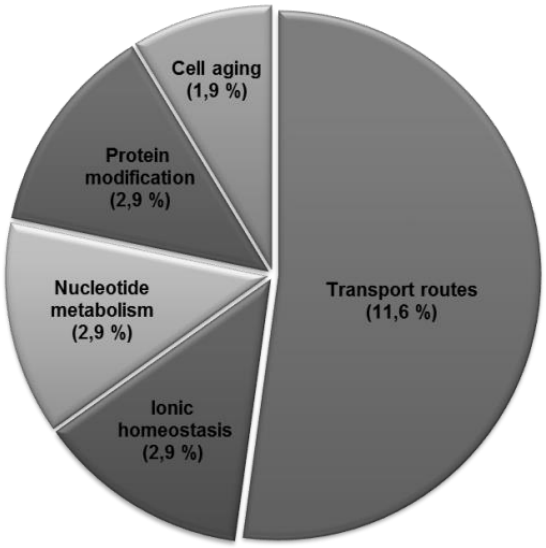


Figure 3.8 - Functional categorization of genes whose deletion led to either increased resistance to chitosan. Genes identified by the genome-wide screen with altered susceptibility to chitosan were clustered into functional categories that were significantly enriched (p-value below 0.01) based on the MIPS Functional catalogue database (FunCatDB). The relative percentages of each category to the data set are indicated.

Subsequent testing of these resistant mutants on higher concentrations of chitosan confirmed 29 mutants that could grow in the presence of chitosan up to 1.75 g/L, which is almost 2-fold higher concentration than lethal dose (1.0 g/L). Five of these twenty nine corresponds to ORFs with poorly known functions or dubious. It was observed that the functional categories of genes whose deletion cause hyper resistance to chitosan (marked with asterisk along the text) include **Transport routes** and **Ionic homeostasis** (Table 3.4), raising the hypothesis that chitosan action might be directly related with these proteins.

Table 3.4 - Genes whose deletion confers hyper-resistance to chitosan and their corresponding description

Gene	Function*
Transport routes	
APL6	Beta3-like subunit of the yeast AP-3 complex; functions in transport of alkaline phosphatase to the vacuole via the alternate pathway
APM3	Mu3-like subunit of the clathrin associated protein complex (AP-3); functions in transport of alkaline phosphatase to the vacuole via the alternate pathway
APS3	Small subunit of the clathrin-associated adaptor complex AP-3, which is involved in vacuolar protein sorting
CDC50	Endosomal protein that interacts with phospholipid flippase Drs2p; interaction with Cdc50p is essential for Drs2p catalytic activity
FPS1	Plasma membrane channel, involved in efflux of glycerol and in uptake of acetic acid and the trivalent metalloids arsenite and antimonite

Table 3.4 – Continued

Gene	Function*
GET3	Subunit of the GET complex, involved in Golgi to ER trafficking and insertion of proteins into the ER membrane
GLO3	ADP-ribosylation factor GTPase activating protein (ARF GAP), involved in ER-Golgi transport
ERV14	Protein localized to COPII-coated vesicles, involved in vesicle formation and incorporation of specific secretory cargo
PMR1	High affinity Ca^{2+}/Mn^{2+} P-type ATPase required for Ca^{2+} and Mn^{2+} transport into Golgi
RCY1	F-box protein involved in recycling plasma membrane proteins internalized by endocytosis
Ionic homeostasis	
PKR1	V-ATPase assembly factor, functions with other V-ATPase assembly factors in the ER to efficiently assemble the V-ATPase membrane sector (V0)
RAV1	Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p), which promotes assembly of the V-ATPase holoenzyme
RAV2	Subunit of RAVE (Rav1p, Rav2p, Skp1p), which promotes assembly and reassembly of the V-ATPase holoenzyme
VMA21	Integral membrane protein that is required for assembly of the V-ATPase function, although not an actual component of the V-ATPase complex
VPH1	Subunit a of V-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p)
Transcription	
IST3	Component of the U2 snRNP, required for the first catalytic step of splicing and for spliceosomal assembly
LEA1	Component of U2 snRNP; disruption causes reduced U2 snRNP levels
RTT103	Protein that interacts with exonuclease Rat1p and Rai1p and plays a role in transcription termination by RNA polymerase II
SNT309	Member of the NineTeen Complex (NTC) that contains Prp19p and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs
TOP1	Nuclear enzyme that relieves torsional strain in DNA by cleaving and re-sealing the phosphodiester backbone
Translation	
TEF4	Gamma subunit of translational elongation factor eEF1B, stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing eEF1A (Tef1p/Tef2p)
FES1	Hsp70 (Ssa1p) nucleotide exchange factor
Unknown/ Dubious	
EMP65	Putative protein of unknown function; genetic interactions suggest a role in folding of ER membrane proteins

Table 3.4 – Continued

Gene	Function*
HUR1	Protein of unknown function
YDR203W	Dubious open reading frame unlikely to encode a functional protein, based on available experimental and comparative sequence data
YGL007W	Dubious ORF located in the upstream region of *PMA1*
YLR338W	Dubious open reading frame unlikely to encode a protein, based on available experimental and comparative sequence data
YML095C-A	unknown
YMR010W	Putative protein of unknown function

* Biological function is based on the information available in SGD (www.yeastgenome.org)

Deletion of *FPS1** was found to play a role in yeast resistance to chitosan. This gene encodes an aquaglyceroporin which are integral membrane channels that facilitate transport of small molecules, such as glycerol and often facilitate the entry/exit of small toxic compounds to/from the cell (Hohmann, 2002; Mollapour and Piper, 2007; Nozawa *et al.,* 2006; Tamás *et al.,* 1999). Nozawa and colleagues analyzed the mutants strains tolerance to boric acid, reporting that *Δfps1* cells reveal high resistance compared with wild-type cells. The authors suggests that this gene has a important role in transport boron (Nozawa *et al.,* 2006). Furthermore, this plasma membrane channel, at slightly acid pH (pH 4.5), also appears facilitate the entry of undissociated acetic acid into the cell (Mollapour and Piper, 2007). In this line, our result suggests *FPS1** may have a role on chitosan entrance in yeast cell and thus be responsible for enhancing its toxicity.

Also, deletion of a number of Pex genes (*PEX4, PEX13, PEX15, PEX19* and *PEX22*), encoding peroxins involved in peroxisomal import, was found as resistant in this study. These peroxins are required for the biogenesis of peroxisomes, acting in the transport of matrix proteins from the cytosol into the peroxisome lumen or insertion of membrane proteins at the organelle membrane (Hiltunen *et al.,* 2003). Peroxisomes perform various oxidative reactions to adapt to the changing needs of the cell and varying external environments (Smith and Aitchison, 2013). In *S. cerevisiae* this organelle appear to be the sole site of β-oxidation, the main pathway for degrading fatty acids, namely those that are removed from membrane phospholipids (Lockshon *et al.,* 2006). Since yeast cells deficient in peroxisomal functions are unable to effectively control the fatty acid composition of membrane phospholipids and the composition of membrane

phospholipids is a key feature of the chitosan stress response (mentioned above), peroxisomal function could be required for the synthesis or degradation of membrane phospholipids for remodeling cell membranes. However, the individual deletion of genes encoding fatty acid β-oxidation enzymes did not result in sensitive to chitosan stress. Additionally, the peroxisomes are known to be involved in the metabolism of peroxides and other reactive oxygen species by enzyme catalase (Schrader and Fahimi, 2004). Deletion of these genes creates peroxisomes unable to decompose these compounds, which are harmful into the cell, and even then yeast cells were resistant. Thus, it may be suggested that chitosan can offer protection against oxidative stress. Indeed, antioxidant activity is one of the well-known functions of chitosan. As mentioned above, chitosan can chelate metal ions (Helander *et al.,* 2001), as well as scavenge free radicals (Xie *et al.,* 2001). Several studies reported that chitosan could significantly inhibit lipid oxidation in fish products (Amiza and Kang, 2013; López-Caballero *et al.,* 2005; Mao and Wu, 2007) and burgers (Georgantelisa *et al.,* 2007), and prevent formation of carbonyl and hydroperoxide groups in human serum albumin exposed to peroxyl radicals (Anrakua *et al.,* 2008). Accordingly, it was found a resistance phenotype of Δyap1 as compared to the parental BY4741. Yap1p is required for transcriptional activation of antioxidant genes in response to oxidative stress (Temple *et al.,* 2005; Toone and Jones, 1999). Nevertheless, gene deletion of superoxide dismutase, catalases or thioredoxins showed no significant differences of sensitivity with the parental strain. Similar results have been obtained in cells treated with the cell-penetrating antifungal peptide PAF26 (Carmona *et al.,* 2012). Moreover, in our study, deletion of the *GSH1*, regulated by Yap1p, encoding a gamma glutamylcysteine synthetase and catalyzes the first step in glutathione biosynthesis (Wo and Moye-Rowley, 1994) also resulted in increased.

Additionally, Erg mutants, namely *ERG3*, *ERG4* and *ERG6*, encodes proteins that catalyze the final five steps in ergosterol biosynthesis, were resistant to chitosan in our study, contrary to the results previously observed by Jaime *et al.* (2012) and Zakrzewska *et al.* (2007). This resistance phenotype may be probably due to a lower affinity of this drug for episterol, ergosta-5,7,24(28)-trienol and zymosterol, which are accumulated in *Δerg3, Δerg4* and *Δerg6,* respectively. Also resistance phenotype has been observed in *S. cerevisiae* (Mukhopadhyay *et al.,* 2002) and *C. albicans* (Hitchcock *et al.,*1986; Sanglard *et al.,* 2003) to azole stress. Ergosterol is the main component responsible for the regulation of membrane rigidity, fluidity and permeability, and for regulating the activity of membrane transporters (Daum *et al.,* 1998). Since these mutants have altered sterol compositions, due defective ergosterol

biosynthesis and the accumulation of various intermediates, it is likely that sterol interactions with other membrane lipids and transporters could be more relevant to the higher drug susceptibilities observed. Indeed, changes in membrane lipid composition, for example phospholipid and ergosterol, its permeability and fluidity, and asymmetry have been shown to be important determinants in the drug susceptibilities of yeast cells (Kodedová and Sychrová, 2015; Mukhopadhyay *et al.,* 2002).

Eight mutant strains, involved in peroxisome (***Δpex4*, *Δpex13*, *Δpex15, Δpex19* and *Δpex22***) transport and ergosterol (***Δerg3, Δerg4*** and ***Δerg6***) synthesis, all identified as having increased fitness, were also tested individually for growth in the presence of chitosan (Figure 3.9). Except *Δerg6*, the remaining mutants confirmed their resistance to chitosan. Previously, it has been observed that *Δerg6* strain exhibit the slowest growth compared to the other erg strains, suggesting that the product of *ERG6* (facosterol) represents a weak spot in ergosterol biosynthesis (Young *et al.,* 2003; Kodedová and Sychrová, 2015).

In addition, it was found genes required for transport from the ER to the Golgi apparatus and retrograde transport from the Golgi to the ER, namely, genes encoding proteins involved in COPI and COPII-coated vesicle formation (*ERV14** and *SEC28*), in retention of membrane proteins in the ER (*RER1*), in protein insertion into ER membrane (*GET1*, *GET2*, *GET3** and *GET4*) and in cargo exit from the ER (*EMP24*, *TED1* and *GLO3**). Also deletion of genes involved in clathrin/ adaptor proteins (AP) complexes, in particular associated with AP3 complex (*APL5*, *APL6**, *APM3** and *APS3**), responsible for protein transport from the Golgi directly to the vacuole without proceeding through an endosome intermediate (Cowles *et al.,* 1997), resulted in resistant to chitosan, as well as genes that are defective in endocytosis (*EDE1*, *SAC6*, *VRP1*, *END3*, *INP53* and *RCY1**), other pathway of protein delivery to various cellular compartments. Yeast cells depend of vesicle formation, transport and recycling for maintaining cellular organization and homeostasis, and for buffering its response to environmental changes (Giaever and Nislow, 2014). In this study, the importance of proper membrane traffic was demonstrated by deletion strains of this process than results in sensitive to chitosan. Moreover, the importance of the protein transportation system in the toxicity of *S. cerevisiae* to chitosan compounds is also evident by the fact that many deletion strains of these transport routes were found resistant to chitosan. However, the relationship between the toxicity of these yeast cells and the resistance of these deletion strains to chitosan stress is not clear.

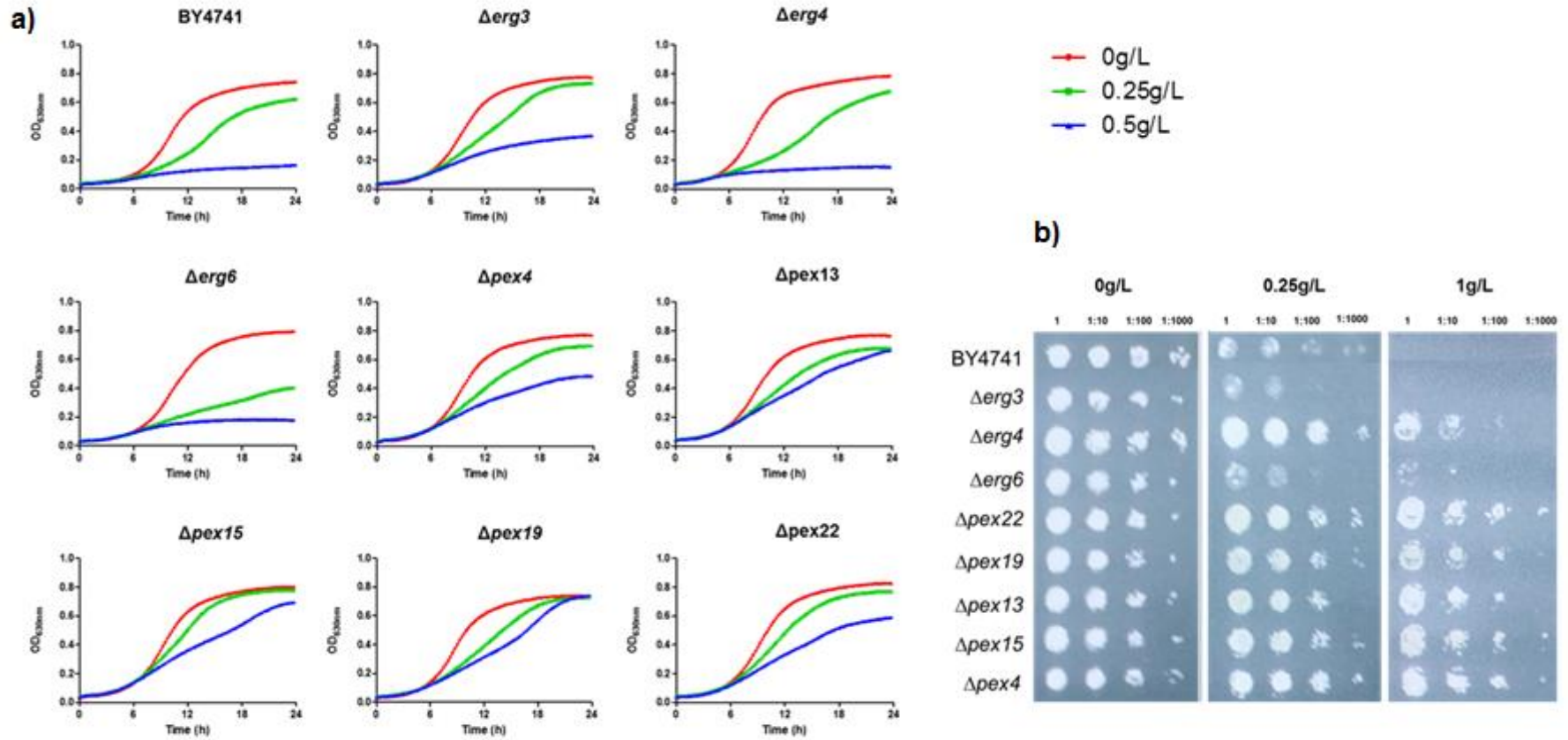


Figure 3.9 - Phenotypic confirmation of representative deletion mutant strains with improved growth after exposure to chitosan. Spot-assay (a) and growth curves (b) for strains with deletions in the genes *ERG3*, *ERG4*, *ERG6*, *PEX4, PEX13, PEX15, PEX19* and *PEX22* grown in MMB 3.5 (control) and chitosan supplementation (0.25 -1 g/L). Growth of BY4741 wild type is included as the reference. All strains exhibited improved growth ability.

Membrane traffic and dynamics are intimately connected with the vacuole. This multifunctional organelle is essential for protein sorting, organelle acidification, ion homeostasis, autophagy, response to environmental stresses, and provides the cell several options for dealing with drugs (Li and Kane, 2009). For example, several studies have reported the important role of vacuolar proton-translocating ATPase (V-ATPase) in yeast tolerance to stress (Hillenmeyer *et al*. 2008; Parsons *et al.,* 2004; Dos Santos and Sá-Correia, 2011; Teixeira *et al*., 2009). In our study, deletion of genes involved in V-ATPase results in resistant to chitosan, namely, genes encoding multi-subunit proteins: a peripheral V1 (*VMA8*) and an integral membrane V0 (*VMA11*, *VMA16* and *VPH1*); as well as assembly factors (*VMA21** and *PKR1**). Additionally, deletion of genes encoding a subunit of Regulator of the H^+-ATPase of Vacuolar and Endosomal Membranes (RAVE) complex (*RAV1** and *RAV2**) were also more resistant to chitosan than the parental strain. RAVE complex, plays a role in regulatory assembly and disassembly of the V-ATPase, and in the absence of this complex, V1 and V0 subunits are unstable and there is very little ATPase activity in isolated vacuole. These results suggest that proton pump V-ATPase is a strong target of chitosan in yeast. In vacuoles, acidification is achieved through the action of this proton pump V-ATPase, responsible for the coupling the free energy of ATP hydrolysis to proton transport from the cytosol to the organelle lumen (Kane, 2006). Thus, deletion of genes involved in molecular structure and regulation of this multi-subunit can affect its physiological roles, for example appears to abolish organelle acidification. However, it has been proposed that yeasts lacking V-ATPase activity (deletion of Vma) are viable when grown at low pH, suggesting alternative methods of organelle acidification independent of the V-ATPase (Nelson and Nelson 1990; Plant et al. 1999). The role of chitosan stress (indirectly or directly), at low pH, in acidification of the vacuole can only be hypothesized. As mentioned above, there is a possibility that this cationic drug may induce cytosolic acidification. Thus, acidification of the vacuole may result simply from the passive leakage of these acids from the cytosol to the vacuolar lumen.

3.3. Cross-susceptibility between chitosan and SO_2

As already mentioned, in addition to excessive doses of SO_2 to be avoided, mostly because of health reasons, but also because it can have a negative impact on aromas and flavors in wine, the consumer desire is to acquire “natural products”. Therefore, there is a great interest to use chitosan as an alternative to SO_2. In this context, in an attempt to understand

differences in terms of the toxicity of chitosan and sulphur dioxide, it was carried out the comparison between the genes identified in our study as conferring sensitivity and resistance to chitosan with those identified as conferring sensitivity and resistance to SO_2, performed recently in the UTAD lab using a similar analysis. This analysis revealed that there are 174 strains sensitivity in common and none strains resistance in common (Figure 3.10). It should be noted that there is the higher number of genes identified as conferring sensitivity to SO_2 and, contrary, the higher number of genes identified as conferring resistance to chitosan.

Although a number of functional categories were shared by these compounds, the majority of identified genes fall into several distinct functional groups (data not shown). These data suggest that yeast ability to counteract SO_2 and chitosan damage involves distinct pathways, allowing us to anticipate that chitosan may not be an alternative to SO_2, however may be used as an adjuvant, allowing thereby reducing SO_2 levels to be applied to wine and make this product more natural. Moreover, a particular combination of these two compounds should be tested as an important strategy to overcome resistance.

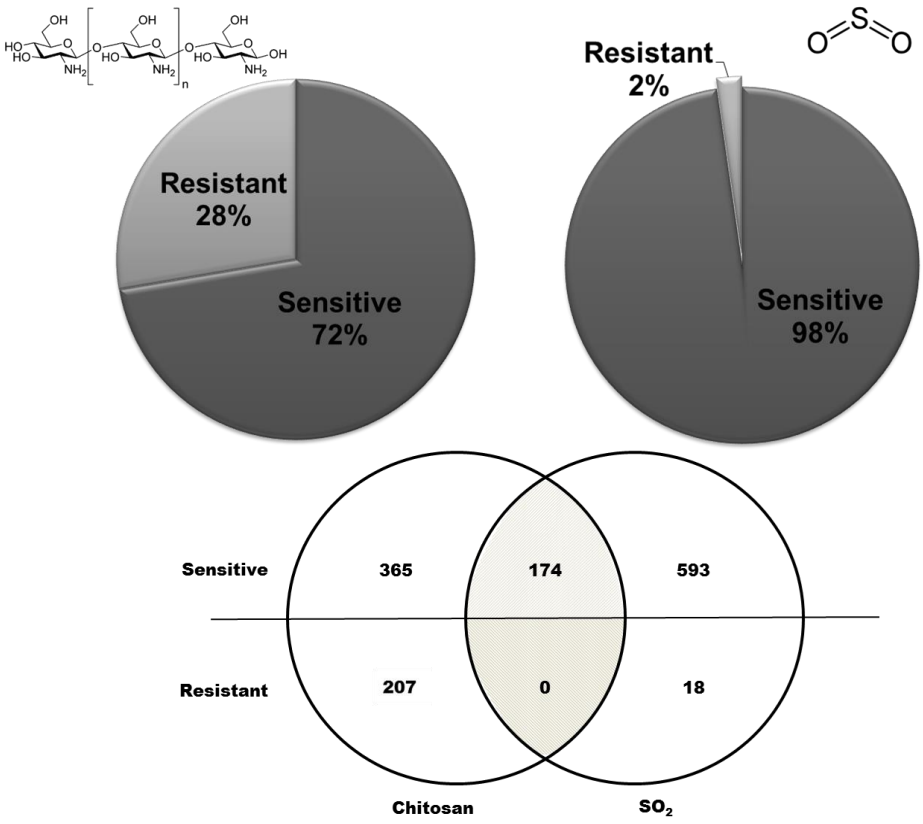


Figure 3.10 - Diagrammatic representation of the distribution of mutant strains identified as conferring sensitivity and resistance to chitosan (in this study) and SO_2 (performed recently in the UTAD lab).

4. Conclusions and future perspectives

- The use of chitosan up to 0.1 g/L in the laboratory *S. cerevisiae* strain BY4741 was not effective. Previous studies conducted in our lab using commercial *S. cerevisiae* strains have also shown that the maximum legal concentration approved by OIV is not effective in controlling yeast growth although a great variability in the susceptibility among strains have been observed. Further studies are required to evaluate the antimicrobial activity of chitosan in other relevant spoilage yeasts, such as *Brettanomyces/Dekkera*, *Zygosaccharomyces bailii* and *Saccharomycodes ludwigii*, under the same conditions.

- In this study we have used a commercial product approved to be used in winemaking, No-Brett Inside®, to uncover the mode of action of chitosan using a chemeogenomic approach. Our results suggest that inhibitory effect is due to its interference in growth functions (protein synthesis, cell cycle and DNA processing), cell wall structure (remodeling and synthesis) and in membrane lipid composition (ergosterol, phospholipid and sphingolipid biosynthesis);

- The comparison of our susceptible-chitosan with previous studies using chitosan extracted from crab-shells and distinct experimental conditions, revealed only nine genes in common. This lack of overlapping suggests underlies the importance of the nature of chitosan and of the experimental conditions to be used that should be as close as possible to the real conditions.

- Our screen has identified a substantial number of genes which were not previously described to play a direct or indirect role in chitosan susceptibility. . For instance, the high susceptibility of the *Δrim101* strain, along with several members of the RIM101 signaling pathway and other target genes involved in cell wall structure and MVB transport suggest the involvement of this pathway on yeast adaptation to chitosan treatment. Further experimental work should be performed to confirm these results.

- Deletion of genes involved in biosynthesis phospholipids and ergosterol, resulted in contrary phenotypes, suggesting that lipid membrane components are determinant for yeast adaption to chitosan. It would be interesting to screen yeast strains with different susceptibility profiles for their lipid membrane composition.

- The small overlap of our data set with that obtained with SO_2, using the same experimental conditions, suggests that these two compounds have distinct modes of action.

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ATTACHMENTS

Supplemental Tables

ATTACHMENTS Supplemental Tables

Table S1- Genes whose deletion confers hypersensitivity and sensitivity to chitosan.

Gene/ORF*	Function**
	METABOLISM
C and N-compounds and carbohydrate metabolism	
DAL5	Allantoate permease; ureidosuccinate permease; also transports dipeptides, though with lower affinity than for allantoate and ureidosuccinate
DGA1	Diacylglycerol acyltransferase, catalyzes the terminal step of triacylglycerol (TAG) formation
GPD2	NAD-dependent glycerol 3-phosphate dehydrogenase; located in cytosol and mitochondria
GPM2	Homolog of Gpm1p phosphoglycerate mutase, which converts 3-phosphoglycerate to 2-phosphoglycerate in glycolysis
HXK2	Hexokinase isoenzyme 2 that catalyzes phosphorylation of glucose in the cytosol
ICL2	2-methylisocitrate lyase of the mitochondrial matrix, functions in the methylcitrate cycle to catalyze the conversion of 2-methylisocitrate to succinate and pyruvate
KGD1	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the tricarboxylic acid (TCA) cycle
MPD1	Member of the protein disulfide isomerase (PDI) family; interacts with and inhibits the chaperone activity of Cne1p
MTD1	NAD-dependent 5,10-methylenetetrahydrafolate dehydrogenase, plays a catalytic role in oxidation of cytoplasmic one-carbon units
PPG1	Putative serine/threonine protein phosphatase of the type 2A-like phosphatase family, required for glycogen accumulation
PYC1	Pyruvate carboxylase isoform, cytoplasmic enzyme that converts pyruvate to oxaloacetate
RPE1	D-ribulose-5-phosphate 3-epimerase, catalyzes a reaction in the non-oxidative part of the pentose-phosphate pathway
SAM1	S-adenosylmethionine synthetase, catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine
TPS1	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose
ZWF1	Glucose-6-phosphate dehydrogenase (G6PD), catalyzes the first step of the pentose phosphate pathway
Amino acid metabolism	
ARG7	Mitochondrial ornithine acetyltransferase, catalyzes the fifth step in arginine biosynthesis; also possesses acetylglutamate synthase activity
CPA1	Small subunit of carbamoyl phosphate synthetase, which catalyzes a step in the synthesis of citrulline, an arginine precursor
DPH5	Methyltransferase required for synthesis of diphthamide, which is a modified histidine residue of translation elongation factor 2 (Eft1p or Eft2p)
GCV3	H subunit of the mitochondrial glycine decarboxylase complex, required for the catabolism of glycine to 5,10-methylene-THF; also required for all protein lipoylation
HIS6	Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase, catalyzes the fourth step in histidine biosynthesis
MET6	Cobalamin-independent methionine synthase, involved in methionine biosynthesis and regeneration
MUP1	High affinity methionine permease, integral membrane protein with 13 putative membrane-spanning regions
ODC2	Mitochondrial inner membrane transporter, exports 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix to the cytosol for use in lysine and glutamate biosynthesis
PAA1	Polyamine acetyltransferase; acetylates polyamines (e.g. putrescine, spermidine, spermine) and also aralkylamines (e.g. tryptamine, phenylethylamine)

Table S1 – continued.

Gene/ORF*	Function**
PFA4	Palmitoyltransferase with autoacylation activity, required for palmitoylation of amino acid permeases containing a C-terminal Phe-Trp-Cys site; required for modification of Chs3p
PSD2	Phosphatidylserine decarboxylase of the Golgi and vacuolar membranes, converts phosphatidylserine to phosphatidylethanolamine
ROG1	Protein with putative serine active lipase domain
SER3	3-phosphoglycerate dehydrogenase, catalyzes the first step in serine and glycine biosynthesis
THI3	Probable alpha-ketoisocaproate decarboxylase, may have a role in catabolism of amino acids to long-chain and complex alcohols
Nucleotide metabolism	
ATP1	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis
ATP15	Epsilon subunit of the F1 sector of mitochondrial F1F0 ATP synthase, which is a large, evolutionarily conserved enzyme complex required for ATP synthesis
COX5b	Subunit Vb of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain
FCY2	Purine-cytosine permease, mediates purine (adenine, guanine, and hypoxanthine) and cytosine accumulation
KTI11	Zn-ribbon protein that co-purifies with Dph1 and Dph2 in a complex required for synthesis of diphthamide on translation factor eEF2
NMA1	Nicotinic acid mononucleotide adenylyltransferase, involved in pathways of NAD biosynthesis and nicotinamide riboside salvage pathways
NPP1	Nucleotide pyrophosphatase/phosphodiesterase family member; mediates extracellular nucleotide phosphate hydrolysis along with Npp2p and Pho5p
QCR8	Subunit 8 of ubiquinol cytochrome-c reductase complex, which is a component of the mitochondrial inner membrane electron transport chain
RIB1	GTP cyclohydrolase II; catalyzes the first step of the riboflavin biosynthesis pathway
Lipid, fatty acid and isoprenoid biosynthesis	
ALE1	Broad-specificity lysophospholipid acyltransferase; may have role in fatty acid exchange at sn-2 position of mature glycerophospholipids
CHO1	Phosphatidylserine synthase, functions in phospholipid biosynthesis
CHO2	Phosphatidylethanolamine methyltransferase (PEMT), catalyzes the first step in the conversion of phosphatidylethanolamine to phosphatidylcholine during the methylation pathway of phosphatidylcholine biosynthesis
CST26	Protein required for incorporation of stearic acid into phosphatidylinositol
FAT1	Very long chain fatty acyl-CoA synthetase and long chain fatty acid transporter; activates imported fatty acids with a preference for very long lengths (C20-C26)
FPK1	Ser/Thr protein kinase that regulates the putative phospholipid translocases Lem3p-Dnf1p/Dnf2p
INP51	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase, plays a role in phosphatidylinositol 4,5-bisphosphate homeostasis and in endocytosis
IPK1	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase, nuclear protein required for synthesis of 1,2,3,4,5,6-hexakisphosphate (phytate)
IPT1	Inositolphosphotransferase, involved in synthesis of mannose-(inositol-P)2-ceramide (M(IP)2C), the most abundant sphingolipid
IRS4	EH domain-containing protein involved in regulating phosphatidylinositol 4,5-bisphosphate levels and autophagy

Table S1 – continued.

Gene/ORF*	Function**
LAS21	Integral plasma membrane protein involved in the synthesis of the glycosylphosphatidylinositol (GPI) core structure
LEM3	Membrane protein of the plasma membrane and ER, interacts specifically in vivo with the phospholipid translocase (flippase) Dnf1p
OPI1	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p function in negative regulation of phospholipid biosynthetic genes
OPI3	Phospholipid methyltransferase (methylene-fatty-acyl-phospholipid synthase), catalyzes the last two steps in phosphatidylcholine biosynthesis
OSH1	May be involved in ergosterol synthesis
PPT2	Phosphopantetheine:protein transferase (PPTase), activates mitochondrial acyl carrier protein (Acp1p) by phosphopantetheinylation
SAC1	Phosphatidylinositol phosphate (PtdInsP) phosphatase involved in hydrolysis of PtdIns[4]P
SCS2	Integral ER membrane protein that regulates phospholipid metabolism via an interaction with the FFAT motif of Opi1p
SCS7	Sphingolipid alpha-hydroxylase, functions in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids
SKN1	Protein involved in sphingolipid biosynthesis; type II membrane protein with similarity to Kre6p
SUR1	Probable catalytic subunit of a mannosylinositol phosphorylceramide (MIPC) synthase
SUR4	Elongase, involved in fatty acid and sphingolipid biosynthesis; synthesizes very long chain 20-26-carbon fatty acids from C18-CoA primers
TSC3	Protein that stimulates the activity of serine palmitoyltransferase (Lcb1p, Lcb2p) several-fold; involved in sphingolipid biosynthesis
VAC14	Protein involved in regulated synthesis of PtdIns(3,5)P(2)
YPR097W	Protein that contains a Phox homology (PX) domain and binds phosphoinositides
	CELL CYCLE AND DNA PROCESSING
DNA processing	
ADA2	Transcription coactivator, component of the ADA and SAGA transcriptional adaptor/HAT (histone acetyltransferase) complexes
ARP6	A component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
ARP8	Nuclear actin-related protein involved in chromatin remodeling
BUD21	Component of small ribosomal subunit (SSU) processosome that contains U3 snoRNA
BUD31	Component of the SF3b subcomplex of the U2 snRNP
CHD1	Nucleosome remodeling factor that functions in regulation of transcription elongation; component of both the SAGA and SLIK complexes
EAF6	Subunit of the NuA4 acetyltransferase complex that acetylates histone H4 and NuA3 acetyltransferase complex that acetylates histone H3
GCN2	Protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p) in response to starvation; contributes to DNA damage checkpoint control
GCN5	catalytic subunit of the ADA and SAGA histone acetyltransferase complexes
GRR1	F-box protein component of the SCF ubiquitin-ligase complex
HMO1	Chromatin associated high mobility group (HMG) family member involved in genome maintenance
HNT3	DNA 5' AMP hydrolase involved in DNA repair; member of the histidine triad (HIT) superfamily of nucleotide-binding proteins
HTZ1	Histone variant H2AZ, exchanged for histone H2A in nucleosomes by the SWR1 complex

Table S1 – continued.

Gene/ORF*	Function**
LDB7	Component of the RSC chromatin remodeling complex; interacts with Rsc3p, Rsc30p, Npl6p, and Htl1p to form a module important for a broad range of RSC functions
RAD55	Protein that stimulates strand exchange by stabilizing the binding of Rad51p to single-stranded DNA; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis
RAD57	Protein that stimulates strand exchange by stabilizing the binding of Rad51p to single-stranded DNA; involved in the recombinational repair of double-strand breaks in DNA during vegetative growth and meiosis
RNR3	The RNR complex catalyzes rate-limiting step in dNTP synthesis, regulated by DNA replication and DNA damage checkpoint pathways
RSC1	Component of the RSC chromatin remodeling complex; required for expression of mid-late sporulation-specific genes
RSC2	Component of the RSC chromatin remodeling complex; required for expression of mid-late sporulation-specific genes
RXT2	Subunit of the histone deacetylase Rpd3L complex; possibly involved in cell fusion and invasive growth
SAP30	Subunit of a histone deacetylase complex, along with Rpd3p and Sin3p, that is involved in silencing at telomeres, rDNA, and silent mating-type loci
SAS5	Subunit of the SAS complex (Sas2p, Sas4p, Sas5p), which acetylates free histones and nucleosomes and regulates transcriptional silencing
SGF29	Component of the HAT/Core module of the SAGA, SLIK, and ADA complexes; HAT/Core module also contains Gcn5p, Ngg1p, and Ada2p; binds methylated histone H3K4
SIN3	Component of the Sin3p-Rpd3p histone deacetylase complex, involved in transcriptional repression and activation of diverse processes
SNF2	Catalytic subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation
SET2	Histone methyltransferase with a role in transcriptional elongation, methylates a lysine residue of histone H3
SUS1	Component of both the SAGA histone acetylase and TREX-2 complexes
SWC3	Component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
SWC5	Component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
SWD3	Essential subunit of the COMPASS (Set1C) complex, which methylates histone H3 on lysine 4 and is required in transcriptional silencing near telomeres
SWH1	Protein similar to mammalian oxysterol-binding protein; contains ankyrin repeats
SWR1	Swi2/Snf2-related ATPase that is the structural component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
TOM1	E3 ubiquitin ligase of the hect-domain class; involved in degradation of excess histones
UBC4	Ubiquitin-conjugating enzyme (E2), mediates degradation of abnormal or excess proteins, including calmodulin and histone H3
ULS1	RING finger protein; member of the SWI/SNF family of DNA-dependent ATPases; plays a role in antagonizing silencing during mating-type switching
VPS71	Nucleosome-binding component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
VPS72	Htz1p-binding component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A
XRS2	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling
YAF9	Subunit of both the NuA4 histone H4 acetyltransferase complex and the SWR1 complex

Table S1 – continued.

Gene/ORF*	Function**
YNG2	Subunit of the NuA4 histone acetyltransferase complex that acetylates histone H4 and H2A
Cell cycle	
BEM2	Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis
BEM3	Rho GTPase activating protein (RhoGAP) involved in control of the cytoskeleton organization
BFR1	Component of mRNP complexes associated with polyribosomes; implicated in secretion and nuclear segregation
BIM1	Microtubule-binding protein that together with Kar9p makes up the cortical microtubule capture site and delays the exit from mitosis when the spindle is oriented abnormally
CDC10	Component of the septin ring that is required for cytokinesis
CDH1	Cell-cycle regulated activator of the anaphase-promoting complex/cyclosome (APC/C), which directs ubiquitination of cyclins resulting in mitotic exit
CIK1	Kinesin-associated protein required for both karyogamy and mitotic spindle organization
CKA1	Alpha catalytic subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation
CKA2	Alpha' catalytic subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation
CKB1	Beta regulatory subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation
CKB2	Beta' regulatory subunit of casein kinase 2 (CK2), a Ser/Thr protein kinase with roles in cell growth and proliferation
CNM67	Component of the spindle pole body outer plaque; required for spindle orientation and mitotic nuclear migration
EST3	Component of the telomerase holoenzyme, involved in telomere replication
HOF1	Bud neck-localized, SH3 domain-containing protein required for cytokinesis
HOP2	Meiosis-specific protein that localizes to chromosomes, preventing synapsis between nonhomologous chromosomes and ensuring synapsis between homologs
MCA1	Putative cysteine protease similar to mammalian caspases; may be involved in cell cycle progression
MCK1	Protein serine/threonine/tyrosine (dual-specificity) kinase involved in control of chromosome segregation and in regulating entry into meiosis
MRC1	S-phase checkpoint protein required for DNA replication
NEM1	Probable catalytic subunit of Nem1p-Spo7p phosphatase holoenzyme; regulates nuclear growth by controlling phospholipid biosynthesis
NIP100	Large subunit of the dynactin complex, which is involved in partitioning the mitotic spindle between mother and daughter cells
PHB1	Subunit of the prohibitin complex (Phb1p-Phb2p), involved in mitochondrial segregation
PUB1	Poly (A)+ RNA-binding protein, abundant mRNP-component protein that binds mRNA and is required for stability of many mRNAs
RBL2	Protein involved in microtubule morphogenesis, required for protection from excess free beta-tubulin
REC102	Protein involved in early stages of meiotic recombination; required for chromosome synapsis
RVS161	Amphiphysin-like lipid raft protein; interacts with Rvs167p and regulates polarization of the actin cytoskeleton, endocytosis, cell polarity, cell fusion and viability following starvation or osmotic stress

Table S1 – continued.

Gene/ORF*	Function**
SAC7	GTPase activating protein (GAP) for Rho1p, involved in signaling to the actin cytoskeleton
SIW14	Tyrosine phosphatase that plays a role in actin filament organization and endocytosis
SPC72	Component of the cytoplasmic Tub4p (gamma-tubulin) complex, binds spindle pole bodies and links them to microtubules; has roles in astral microtubule formation and stabilization
SPO7	Putative regulatory subunit of Nem1p-Spo7p phosphatase holoenzyme, regulates nuclear growth by controlling phospholipid biosynthesis, required for normal nuclear envelope morphology, premeiotic replication, and sporulation
SWM1	Subunit of the anaphase-promoting complex, which is an E3 ubiquitin ligase that regulates the metaphase-anaphase transition and exit from mitosis
YBR266C	Protein with a potential role in actin cytoskeleton organization
YTA7	Protein that localizes to chromatin and has a role in regulation of histone gene expression
	TRANSCRIPTION
RNA synthesis, processing and modification	
ARC1	Protein that binds tRNA and methionyl- and glutamyl-tRNA synthetases (Mes1p and Gus1p)
BDF1	Protein involved in transcription initiation at TATA-containing promoters; associates with the basal transcription factor TFIID
BMH1	Controls proteome at post-transcriptional level, binds proteins and DNA, involved in regulation of exocytosis, vesicle transport, Ras/MAPK signaling and rapamycin-sensitive signaling
BUD32	Protein kinase, component of the EKC/KEOPS complex with Kae1p, Cgi121p, Pcc1p, and Gon7p; EKC/KEOPS complex is required for t6A tRNA modification and may have roles in telomere maintenance and transcription
CBC2	Small subunit of the heterodimeric cap binding complex that also contains Sto1p, component of the spliceosomal commitment complex; interacts with Npl3p, possibly to package mRNA for export from the nucleus; contains an RNA-binding motif
CCR4	Component of the CCR4-NOT transcriptional complex, which is involved in regulation of gene expression
CSE2	Subunit of the RNA polymerase II mediator complex, required for regulation of RNA polymerase II activity
CSN12	Protein that forms a complex with Thp3p; may have a role in transcription elongation and/or mRNA splicing
CTK1	Catalytic (alpha) subunit of C-terminal domain kinase I (CTDK-I); phosphorylates both RNA pol II subunit Rpo21p to affect transcription and pre-mRNA 3' end processing, and ribosomal protein Rps2p to increase translational fidelity
CTK2	Beta subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates both RNA pol II subunit Rpo21p to affect transcription and pre-mRNA 3' end processing, and ribosomal protein Rps2p to increase translational fidelity
DST1	General transcription elongation factor TFIIS, enables RNA polymerase II to read through blocks to elongation
EAP1	eIF4E-associated protein, competes with eIF4G for binding to eIF4E; inhibits cap-dependent translation
FYV5	Protein involved in regulation of the mating pathway; binds with Matalpha2p to promoters of haploid-specific genes
IKI1	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA
IKI3	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA

Table S1 – continued.

Gene/ORF*	Function**
IOC2	Member of a complex (Isw1b) that exhibits nucleosome-stimulated ATPase activity and acts within coding regions to coordinate transcription elongation
LAG2	Protein that negatively regulates the SCF E3-ubiquitin ligase by interacting with and preventing neddyation of the cullin subunit, Cdc53p
LSM6	Lsm (Like Sm) protein; part of heteroheptameric complexes (Lsm2p-7p and either Lsm1p or 8p): cytoplasmic Lsm1p complex involved in mRNA decay; nuclear Lsm8p complex part of U6 snRNP and possibly involved in processing tRNA, snoRNA, and rRNA
MAL13	MAL-activator protein, part of complex locus MAL1
MED1	Subunit of the RNA polymerase II mediator complex; essential for transcriptional regulation
MKS1	Pleiotropic negative transcriptional regulator involved in Ras-CAMP and lysine biosynthetic pathways and nitrogen regulation
MSF1	Mitochondrial phenylalanyl-tRNA synthetase, active as a monomer, unlike the cytoplasmic subunit which is active as a dimer complexed to a beta subunit dimer
MSR1	Mitochondrial arginyl-tRNA synthetase
MTC5	Subunit of the SEA (Seh1-associated) complex, a coatomer-related complex that associates dynamically with the vacuole
NCS6	Protein required for thiolation of the uridine at the wobble position of Gln, Lys, and Glu tRNAs
NUT1	Component of the RNA polymerase II mediator complex, which is required for transcriptional activation and also has a role in basal transcription
PEX30	Peroxisomal integral membrane protein, involved in negative regulation of peroxisome number
PIB2	Protein binding phosphatidylinositol 3-phosphate, involved in telomere-proximal repression of gene expression
PRM4	Pheromone-regulated protein proposed to be involved in mating
PUS1	tRNA: pseudouridine synthase, introduces pseudouridines at positions 26-28, 34-36, 65, and 67 of tRNA; nuclear protein that appears to be involved in tRNA export
REG1	Regulatory subunit of type 1 protein phosphatase Glc7p, involved in negative regulation of glucose-repressible genes
RPA14	RNA polymerase I subunit A14
RPA34	RNA polymerase I subunit A34.5
RPB4	RNA polymerase II subunit B32; involved in recruitment of 3'-end processing factors to transcribing RNA polymerase II complex and in export of mRNA to cytoplasm under stress conditions
RRD2	Activator of the phosphotyrosyl phosphatase activity of PP2A, regulates G1 phase progression, the osmoresponse, microtubule dynamics
RTC1	Subunit of the SEA (Seh1-associated) complex, a coatomer-related complex that associates dynamically with the vacuole
RTS1	B-type regulatory subunit of protein phosphatase 2A (PP2A); Rts1p and Cdc55p are alternative regulatory subunits for PP2A
SAC3	Component of TREX-2 complex (Sac3p-Thp1p-Sus1p-Cdc31p) involved in transcription elongation and mRNA export from the nucleus
SEH1	Nuclear pore protein of the conserved Nup84p complex (Nup84p, Nup85p, Nup120p, Nup145p, and Seh1p); part of the SEA (Seh1-associated) complex, a coatamer-related complex that associates dynamically with the vacuole
SIN4	Subunit of the RNA polymerase II mediator complex; contributes to both postive and negative transcriptional regulation
SIS2	Negative regulatory subunit of protein phosphatase 1 Ppz1p and also a subunit of the phosphopantothenoylcysteine decarboxylase (PPCDC; Cab3p, Sis2p, Vhs3p) complex

Table S1 – continued.

Gene/ORF*	Function**
SLM5	Mitochondrial asparaginyl-tRNA synthetase
SRB8	Subunit of the RNA polymerase II mediator complex; essential for transcriptional regulation; involved in glucose repression
SSN2	Subunit of the RNA polymerase II mediator complex; required for stable association of Srb10p-Srb11p kinase; essential for transcriptional regulation
SSN8	Cyclin-like component of the RNA polymerase II holoenzyme, involved in phosphorylation of the RNA polymerase II C-terminal domain; involved in glucose repression and telomere maintenance
TCB1	Lipid-binding protein containing three calcium and lipid binding domains
TCB3	Lipid-binding protein, localized to the bud via specific mRNA transport
TFB5	Component of the RNA polymerase II general transcription and DNA repair factor TFIIH; involved in transcription initiation and in nucleotide-excision repair
THP1	Component of TREX-2 complex (Sac3p-Thp1p-Sus1p-Cdc31p) involved in transcription elongation and mRNA export from the nucleus
UBA4	Protein that activates Urm1p; also acts in thiolation of the wobble base of cytoplasmic tRNAs by adenylating and then thiolating Urm1p
URM1	Ubiquitin-like protein involved in thiolation of cytoplasmic tRNAs; receives sulfur from the E1-like enzyme Uba4p and transfers it to tRNA
WTM1	Transcriptional modulator involved in regulation of meiosis, silencing, and expression of RNR genes
Transcriptional factors	
AFT1	Transcription factor involved in iron utilization and homeostasis
CBF1	Binds the motif CACRTG present at several sites including MET gene promoters and centromere DNA element I (CDEI)
CRZ1	Transcription factor that activates transcription of genes involved in stress response
DAL81	Positive regulator of genes in multiple nitrogen degradation pathways
DIG1	MAP kinase-responsive inhibitor of the Ste12p transcription factor, involved in the regulation of mating-specific genes and the invasive growth pathway
GCN4	Transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation
GCR2	Transcriptional activator of genes involved in glycolysis
GLN3	Transcriptional activator of genes regulated by nitrogen catabolite repression (NCR)
MET31	Transcriptional regulation of the methionine biosynthetic genes
PHO2	Regulatory targets include genes involved in phosphate metabolism
RFX1	Major transcriptional repressor of DNA-damage-regulated genes, recruits repressors Tup1p and Cyc8p to their promoters
RIM101	Transcriptional repressor involved in response to pH and in cell wall construction
ROX1	Heme-dependent repressor of hypoxic genes; contains an HMG domain that is responsible for DNA bending activity
SFL1	Transcriptional repressor and activator; involved in repression of flocculation-related genes and activation of stress responsive genes
SKO1	Transcription factor of the ATF/CREB family; forms a complex with Tup1p and Cyc8p to both activate and repress transcription
STP3	Zinc-finger protein of unknown function, possibly involved in pre-tRNA splicing and in uptake of branched-chain amino acids
SWI4	A transcriptional activator that in concert with MBF (Mbp1-Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair
SWI5	Transcription factor that activates transcription of genes expressed at the M/G1 phase boundary and in G1 phase

Table S1 – continued.

Gene/ORF*	Function**
UME6	Key transcriptional regulator of early meiotic genes, binds URS1 upstream regulatory sequence, couples metabolic responses to nutritional cues with initiation and progression of meiosis
ZAP1	Transcription factor, binds to zinc-responsive promoters to induce transcription of certain genes in presence of zinc, represses other genes in low zinc
	PROTEIN SYNTHESIS
Ribosome biogenesis	
MRP1	Mitochondrial ribosomal protein of the small subunit; MRP1 exhibits genetic interactions with PET122 and PET123
MRPL51	Mitochondrial ribosomal protein of the large subunit
MRPS35	Mitochondrial ribosomal protein of the small subunit
NOP6	rRNA-binding protein required for 40S ribosomal subunit biogenesis
NSR1	Nucleolar protein that binds nuclear localization sequences, required for pre-rRNA processing and ribosome biogenesis
REH1	Cytoplasmic 60S subunit biogenesis factor, associates with pre-60S particles
RPL13B	Protein component of the large (60S) ribosomal subunit
RPL1B	N-terminally acetylated protein component of the large (60S) ribosomal subunit
RPL21A	Protein component of the large (60S) ribosomal subunit
RPL31B	Protein component of the large (60S) ribosomal subunit
RPL38	Protein component of the large (60S) ribosomal subunit
RPL41B	Ribosomal protein L47 of the large (60S) ribosomal subunit
RPL42B	Protein component of the large (60S) ribosomal subunit
RPL8A	Ribosomal protein L4 of the large (60S) ribosomal subunit
RPL9B	Protein component of the large (60S) ribosomal subunit
RPS0A	Protein component of the small (40S) ribosomal subunit
RPS0B	Protein component of the small (40S) ribosomal subunit
RPS10B	Protein component of the small (40S) ribosomal subunit
RPS11B	Protein component of the small (40S) ribosomal subunit
RPS14A	Ribosomal protein 59 of the small subunit, required for ribosome assembly and 20S pre-rRNA processing
RPS14B	Ribosomal protein 59 of the small subunit, required for ribosome assembly and 20S pre-rRNA processing
RPS16A	Protein component of the small (40S) ribosomal subunit
RPS16B	Protein component of the small (40S) ribosomal subunit
RPS17A	Ribosomal protein 51 (rp51) of the small (40s) subunit
RPS17B	Ribosomal protein 51 (rp51) of the small (40s) subunit
RPS18A	Protein component of the small (40S) ribosomal subunit
RPS18B	Protein component of the small (40S) ribosomal subunit
RPS19A	Protein component of the small (40S) ribosomal subunit, required for assembly and maturation of pre-40 S particles
RPS1A	Ribosomal protein 10 (rp10) of the small (40S) subunit
RPS1B	Ribosomal protein 10 (rp10) of the small (40S) subunit
RPS21A	Protein component of the small (40S) ribosomal subunit
RPS21B	Protein component of the small (40S) ribosomal subunit

Table S1 – continued.

Gene/ORF*	Function**
RPS22B	Protein component of the small (40S) ribosomal subunit
RPS24B	Protein component of the small (40S) ribosomal subunit
RPS26B	Protein component of the small (40S) ribosomal subunit
RPS27A	Protein component of the small (40S) ribosomal subunit
RPS27B	Protein component of the small (40S) ribosomal subunit
RPS28A	Protein component of the small (40S) ribosomal subunit
RPS28B	Protein component of the small (40S) ribosomal subunit
RPS29A	Protein component of the small (40S) ribosomal subunit
RPS29B	Protein component of the small (40S) ribosomal subunit
RPS30A	Protein component of the small (40S) ribosomal subunit
RPS30B	Protein component of the small (40S) ribosomal subunit
RPS4B	Protein component of the small (40S) ribosomal subunit
RPS6B	Protein component of the small (40S) ribosomal subunit
RPS7B	Protein component of the small (40S) ribosomal subunit
RPS9B	Protein component of the small (40S) ribosomal subunit
SQS1	Acts with Prp43p to stimulate 18s rRNA maturation by Nob1p; component of pre-ribosomal particles
Translation	
ASC1	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p; ortholog of RACK1 that inhibits translation; core component of the small (40S) ribosomal subunit
CAM1	Nuclear protein required for transcription of MXR1; binds the MXR1 promoter in the presence of other nuclear factors
CBS1	Mitochondrial translational activator of the COB mRNA; membrane protein that interacts with translating ribosomes
DOM34	Protein that facilitates ribosomal subunit dissociation when translation is stalled
EFG1	Essential protein required for maturation of 18S rRNA
FYV7	Essential protein required for maturation of 18S rRNA
HBS1	GTPase with similarity to translation release factors; together with binding partner Dom34p, facilitates ribosomal subunit dissociation and peptidyl-tRNA release when translation is stalled
HCR1	Protein involved in translation initiation as a substoichiometric component (eIF3j) of translation initiation factor 3 (eIF3) and required for processing of 20S pre-rRNA
HEK2	RNA binding protein involved in the asymmetric localization of ASH1 mRNA
MRN1	RNA-binding protein proposed to be involved in translational regulation
NEW1	ATP binding cassette protein that cosediments with polysomes and is required for biogenesis of the small ribosomal subunit
PAT1	Topoisomerase II-associated deadenylation-dependent mRNA-decapping factor; also required for faithful chromosome transmission, maintenance of rDNA locus stability, and protection of mRNA 3'-UTRs from trimming
PET494	Mitochondrial translational activator specific for the COX3 mRNA
RPP2B	Ribosomal protein P2 beta, involved in the interaction between translational elongation factors and the ribosome
RPS23A	Ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit, required for translational accuracy
RPS23B	Ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit, required for translational accuracy

Table S1 – continued.

Gene/ORF*	Function**
RTS1	B-type regulatory subunit of protein phosphatase 2A (PP2A); PP2A-Rts1p protects cohesin when recruited by Sgo1p to the pericentromere
SBP1	Putative RNA binding protein; involved in translational repression and associated with small nucleolar RNAs snR10 and snR11
SLX9	Protein required for pre-rRNA processing; associated with the 90S pre-ribosome and 43S small ribosomal subunit precursor
SCP160	Essential RNA-binding G protein effector of mating response pathway, interacts in mRNA-dependent manner with translating ribosomes
SCH9	AGC family protein kinase; phosphorylated by Tor1p and required for TORC1-mediated regulation of ribosome biogenesis, translation initiation, and entry into G0 phase
SRO9	Cytoplasmic RNA-binding protein that associates with translating ribosomes
TMA19	Protein that associates with ribosomes; homolog of translationally controlled tumor protein
TPD3	Regulatory subunit A of the heterotrimeric protein phosphatase 2A (PP2A), which also contains regulatory subunit Cdc55p and either catalytic subunit Pph21p or Pph22p
TRM1	tRNA methyltransferase; two forms of the protein are made by alternative translation starts
TRM9	tRNA methyltransferase, catalyzes esterification of modified uridine nucleotides in tRNA(Arg3) and tRNA(Glu), likely as part of a complex with Trm112p
TSR2	Protein with a potential role in pre-rRNA processing
TSR3	Protein required for correct processing of the 20S pre-rRNA at site D to generate mature 18S rRNA
	CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES
Transport routes	
AGE1	ADP-ribosylation factor (ARF) GTPase activating protein (GAP) effector, involved in the secretory and endocytic pathways
APL4	Gamma-adaptin, large subunit of the clathrin-associated protein (AP-1) complex; involved in vesicle mediated transport
APS1	Small subunit of the clathrin-associated adaptor complex AP-1, which is involved in protein sorting at the trans-Golgi network
APS2	Small subunit of the clathrin-associated adaptor complex AP-2, which is involved in protein sorting at the plasma membrane
ART5	Protein proposed to regulate the endocytosis of plasma membrane proteins
ATG11	Adapter protein for pexophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway
ATG15	Lipase required for intravacuolar lysis of autophagic bodies and Cvt bodies
AVL9	Conserved protein involved in exocytic transport from the Golgi
BST1	GPI inositol deacylase of the ER that negatively regulates COPII vesicle formation, prevents production of vesicles with defective subunits
CHS5	Component of the exomer complex and is involved in export of selected proteins from the Golgi to the plasma membrane
COG6	Component of the conserved oligomeric Golgi complex (Cog1p through Cog8p), a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments
ENT3	Protein containing an N-terminal epsin-like domain involved in clathrin recruitment and traffic between the Golgi and endosomes
GCS1	ADP-ribosylation factor GTPase activating protein (ARF GAP), involved in ER-Golgi transport
GEA2	Guanine nucleotide exchange factor for ADP ribosylation factors (ARFs), involved in vesicular transport between the Golgi and ER, Golgi organization, and actin cytoskeleton organization

Table S1 – continued.

Gene/ORF*	Function**
GEF1	Voltage-gated chloride channel localized to the golgi, the endosomal system, and plasma membrane, and involved in cation homeostasis
GGA2	Protein that interacts with and regulates Arf1p and Arf2p in a GTP-dependent manner to facilitate traffic through the late Golgi
HSE1	Subunit of the endosomal Vps27p-Hse1p complex required for sorting of ubiquitinated membrane proteins into intralumenal vesicles prior to vacuolar degradation, as well as for recycling of Golgi proteins and formation of lumenal membranes
KES1	Member of the oxysterol binding protein family, which includes seven yeast homologs; involved in negative regulation of Sec14p-dependent Golgi complex secretory functions
LDB19	Protein involved in regulating the endocytosis of plasma membrane proteins by recruiting the ubiquitin ligase Rsp5p to its target
LST4	Protein possibly involved in a post-Golgi secretory pathway
LST7	Protein possibly involved in a post-Golgi secretory pathway
MDM10	Subunit of both the ERMES complex that links the ER to mitochondria, and of the mitochondrial sorting and assembly machinery (SAM complex) that functions in import and assembly of outer membrane beta-barrel proteins
MDM12	Mitochondrial outer membrane protein, required for transmission of mitochondria to daughter cells; component of the ERMES complex that links the ER to mitochondria; may influence import and assembly of outer membrane beta-barrel proteins
MDM34	Mitochondrial component of the ERMES complex that links the ER to mitochondria and may promote inter-organellar calcium and phospholipid exchange as well as coordinating mitochondrial DNA replication and growth
MEH1	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex, which is required for proper sorting of amino acid permease Gap1p
MON1	Protein required for fusion of cvt-vesicles and autophagosomes with the vacuole
NHX1	Na+/H+ and K+/H+ exchanger; required for osmotolerance to acute hypertonic shock and for vacuolar fusion
PEP1	Type I transmembrane sorting receptor for multiple vacuolar hydrolases; cycles between the late-Golgi and prevacuolar endosome-like compartments
PEP4	Vacuolar aspartyl protease (proteinase A), required for the posttranslational precursor maturation of vacuolar proteinases
PEP8	Vacuolar protein sorting protein that forms part of the multimeric membrane-associated retromer complex along with Vps35p, Vps29p, Vps17p, and Vps5p; essential for endosome-to-Golgi retrograde protein transport
SBH1	Beta subunit of the Sec61p ER translocation complex (Sec61p-Sss1p-Sbh1p); involved in protein translocation into the endoplasmic reticulum
SBH2	Ssh1p-Sss1p-Sbh2p complex component, involved in protein translocation into the endoplasmic reticulum
SEC66	Subunit of Sec63 complex (Sec63p, Sec62p, Sec66p and Sec72p); with Sec61 complex, Kar2p/BiP and Lhs1p forms a channel competent for SRP-dependent and post-translational SRP-independent protein targeting and import into the ER
SEC72	Subunit of Sec63 complex (Sec63p, Sec62p, Sec66p and Sec72p); with Sec61 complex, Kar2p/BiP and Lhs1p forms a channel competent for SRP-dependent and post-translational SRP-independent protein targeting and import into the ER
SLM4	Component of the EGO complex, which is involved in the regulation of microautophagy, and of the GSE complex; required for proper sorting of amino acid permease Gap1p
SNF7	Component of the ESCRT-III involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway
SNF8	Component of the ESCRT-II complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome
SRN2	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome

Table S1 – continued.

Gene/ORF*	Function**
SRO7	Effector of Rab GTPase Sec4p, forms a complex with Sec4p and t-SNARE Sec9p; involved in exocytosis and docking and fusion of post-Golgi vesicles with plasma membrane
SSH1	Subunit of the Ssh1 translocon complex
STP22	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome
SYN8	Endosomal SNARE related to mammalian syntaxin 8
TLG2	Syntaxin-like t-SNARE that forms a complex with Tlg1p and Vti1p and mediates fusion of endosome-derived vesicles with the late Golgi
TRK1	Component of the Trk1p-Trk2p potassium transport system
VAM10	Protein involved in vacuole morphogenesis; acts at an early step of homotypic vacuole fusion that is required for vacuole tethering
VAM6	Vacuolar protein that plays a critical role in the tethering steps of vacuolar membrane fusion by facilitating guanine nucleotide exchange on small guanosine triphosphatase Ypt7p
VAM7	Vacuolar SNARE protein that functions with Vam3p in vacuolar protein trafficking
VPS1	Dynamin-like GTPase required for vacuolar sorting
VPS21	Rab family GTPase required for endocytic transport and for sorting of vacuolar hydrolases
VPS25	Component of the ESCRT-II complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome
VPS28	Component of the ESCRT-I complex (Stp22p, Srn2p, Vps28p, and Mvb12p), which is involved in ubiquitin-dependent sorting of proteins into the endosome«
VPS29	Endosomal protein that is a subunit of the membrane-associated retromer complex essential for endosome-to-Golgi retrograde transport
VPS36	Component of the ESCRT-II complex
VPS38	Part of a Vps34p phosphatidylinositol 3-kinase complex that functions in carboxypeptidase Y (CPY) sorting
VPS41	Subunit of the HOPS complex; essential for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport
VPS5	Nexin-1 homolog required for localizing membrane proteins from a prevacuolar/late endosomal compartment back to the late Golgi apparatus
VPS51	Component of the GARP (Golgi-associated retrograde protein) complex, which is required for the recycling of proteins from endosomes to the late Golgi
VPS52	Component of the GARP (Golgi-associated retrograde protein) complex, which is required for the recycling of proteins from endosomes to the late Golgi
VPS53	Component of the GARP (Golgi-associated retrograde protein) complex, which is required for the recycling of proteins from endosomes to the late Golgi
VPS54	Component of the GARP (Golgi-associated retrograde protein) complex, which is required for the recycling of proteins from endosomes to the late Golgi
VPS74	Protein required for Golgi localization of glycosyltransferases; binding to PtdIns4P required for Golgi targeting and function
YBL104C	Subunit of the SEA (Seh1-associated) complex, a coatomer-related complex that associates dynamically with the vacuole
YMR1	Phosphatidylinositol 3-phosphate (PI3P) phosphatase, involved in various protein sorting pathways, including CVT targeting and endosome to vacuole transport
YPT11	Rab family GTPase that interacts with the C-terminal tail domain of Myo2p; mediates distribution of mitochondria and endoplasmic reticuli to daughter cells
YPT7	GTP-binding protein of the rab family; required for homotypic fusion event in vacuole inheritance

Table S1 – continued.

Gene/ORF*	Function**
Transported compounds	
AGP1	Low-affinity amino acid permease with broad substrate range, involved in uptake of asparagine, glutamine, and other amino acids
APQ12	Protein required for nuclear envelope morphology, nuclear pore complex localization, mRNA export from the nucleus
CTR1	High-affinity copper transporter of the plasma membrane, mediates nearly all copper uptake under low copper conditions
DHH1	Cytoplasmic DExD/H-box helicase, may have a role in mRNA export and translation
DRS2	Aminophospholipid translocase (flippase) that maintains membrane lipid asymmetry in post-Golgi secretory vesicles; contributes to clathrin-coated vesicle formation and endocytosis
ERP4	Protein with similarity to Emp24p and Erv25p, member of the p24 family involved in ER to Golgi transport
FET3	Ferro-O2-oxidoreductase required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity
FTR1	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3p
GTR1	Cytoplasmic GTP binding protein and negative regulator of the Ran/Tc4 GTPase cycle; component of GSE complex, which is required for sorting of Gap1p; involved in phosphate transport and telomeric silencing
GTR2	Putative GTP binding protein that negatively regulates Ran/Tc4 GTPase cycle; activates transcription; subunit of EGO and GSE complexes; required for sorting of Gap1p
GUP1	Plasma membrane protein involved in remodeling GPI anchors; proposed to be involved in glycerol transport
IZH1	Membrane protein involved in zinc ion homeostasis
LTV1	Component of the GSE complex, which is required for proper sorting of amino acid permease Gap1p; required for ribosomal small subunit export from nucleus
MCH5	Plasma membrane riboflavin transporter; facilitates the uptake of vitamin B2; required for FAD-dependent processes
NUP84	Subunit of the nuclear pore complex (NPC); forms a subcomplex with Nup85p, Nup120p, Nup145p-C, Sec13p, and Seh1p that plays a role in nuclear mRNA export, and NPC biogenesis
OPT2	Oligopeptide transporter; also plays a role in formation of mature vacuoles
PDR5	Plasma membrane ATP-binding cassette (ABC) transporter, multidrug transporter actively regulated by Pdr1p
PHO86	ER resident protein required for ER exit of the high-affinity phosphate transporter Pho84p, specifically required for packaging of Pho84p into COPII vesicles
SEM1	Component of the lid subcomplex of the regulatory subunit of the 26S proteasome; involved in mRNA export mediated by the TREX-2 complex (Sac3p-Thp1p)
SNX3	Sorting nexin required to maintain late-Golgi resident enzymes in their proper location by recycling molecules from the prevacuolar compartment
SNX41	Sorting nexin, involved in the retrieval of late-Golgi SNAREs from the post-Golgi endosome to the trans-Golgi network
TOM5	Component of the TOM (translocase of outer membrane) complex responsible for recognition and initial import of all mitochondrially directed proteins
YOR1	Plasma membrane ATP-binding cassette (ABC) transporter, multidrug transporter mediates export of many different organic anions including oligomycin
YSY6	Protein whose expression suppresses a secretory pathway mutation in E. coli; has similarity to the mammalian RAMP4 protein involved in secretion

Table S1 – continued.

Gene/ORF*	Function**
	PROTEIN FATE
Protein folding, modification, degradation	
ALG12	Alpha-1,6-mannosyltransferase localized to the ER; responsible for the addition of the alpha-1,6 mannose to dolichol-linked Man7GlcNAc2, acts in the dolichol pathway for N-glycosylation
ALG9	Mannosyltransferase, involved in N-linked glycosylation; catalyzes the transfer of mannose from Dol-P-Man to lipid-linked oligosaccharides
BER1	Protein involved in microtubule-related processes, N-acetylation
CAX4	Dolichyl pyrophosphate (Dol-P-P) phosphatase with a luminally oriented active site in the ER, cleaves the anhydride linkage in Dol-P-P, required for Dol-P-P-linked oligosaccharide intermediate synthesis and protein N-glycosylation
DER1	Endoplasmic reticulum membrane protein, required for ER-associated protein degradation of misfolded or unassembled proteins
DUG2	Probable di- and tri-peptidase; forms a complex with Dug1p and Dug3p to degrade glutathione (GSH) and other peptides containing a gamma-glu-X bond
EOS1	Protein involved in N-glycosylation
ERI1	Endoplasmic reticulum membrane protein that binds to and inhibits GTP-bound Ras2p at the ER
ERJ5	Type I membrane protein with a J domain is required to preserve the folding capacity of the endoplasmic reticulum
FMP30	Mitochondrial inner membrane protein proposed to be involved in N-acylethanolamine metabolism
HSL7	Protein arginine N-methyltransferase that exhibits septin and Hsl1p-dependent bud neck localization and periodic Hsl1p-dependent phosphorylation
LSM1	Lsm (Like Sm) protein; forms heteroheptameric complex (with Lsm2p, Lsm3p, Lsm4p, Lsm5p, Lsm6p, and Lsm7p) involved in degradation of cytoplasmic mRNAs
MNN1	Alpha-1,3-mannosyltransferase, required for addition of alpha1,3-mannose linkages to N-linked and O-linked oligosaccharides
OCT1	Mitochondrial intermediate peptidase, cleaves destabilizing N-terminal residues of a subset of proteins upon import, after their cleavage by mitochondrial processing peptidase (Mas1p-Mas2p)
OST4	Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes protein asparagine-linked glycosylation
OST6	Subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins
PTC6	Mitochondrial type 2C protein phosphatase (PP2C) involved in mitophagy
SAP155	Protein that forms a complex with the Sit4p protein phosphatase and is required for its function
SPC2	Subunit of signal peptidase complex (Spc1p, Spc2p, Spc3p, Sec11p), which catalyzes cleavage of N-terminal signal sequences of proteins targeted to the secretory pathway
SSE1	ATPase that is a component of the heat shock protein Hsp90 chaperone complex; binds unfolded proteins
SSQ1	Mitochondrial hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins at a step after cluster synthesis
TUL1	Golgi-localized RING-finger ubiquitin ligase (E3), involved in ubiquitinating and sorting membrane proteins that contain polar transmembrane domains to multivesicular bodies for delivery to the vacuole for quality control purposes
UMP1	Short-lived chaperone required for correct maturation of the 20S proteasome; degraded by proteasome upon completion of its assembly

Table S1 – continued.

Gene/ORF*	Function**
VID22	Glycosylated integral membrane protein, which plays a role in fructose-1,6-bisphosphatase (FBPase) degradation; involved in FBPase transport from the cytosol to Vid (vacuole import and degradation) vesicles
VIP1	Inositol hexakisphosphate (IP6) and inositol heptakisphosphate (IP7) kinase; involved in cortical actin cytoskeleton function, and invasive pseudohyphal growth analogous to S. pombe asp1
	BIOGENESIS OF CELLULAR COMPONENTS
Cell wall	
BCK2	Protein rich in serine and threonine residues involved in protein kinase C signaling pathway, which controls cell integrity
BGL2	Endo-beta-1,3-glucanase, major protein of the cell wall, involved in cell wall maintenance
BNI4	Targeting subunit for Glc7p protein phosphatase, required for localization of chitin synthase III to the bud neck via interaction with the chitin synthase III regulatory subunit Skt5p
CCW12	Cell wall mannoprotein with a role in maintenance of newly synthesized areas of cell wall
CWH43	Putative sensor/transporter protein involved in cell wall biogenesis
FKS1	Catalytic subunit of 1,3-beta-D-glucan synthase, functionally redundant with alternate catalytic subunit Gsc2p; involved in cell wall synthesis and maintenance; localizes to sites of cell wall remodeling
GAS1	Beta-1,3-glucanosyltransferase, required for cell wall assembly and also has a role in transcriptional silencing
GAS2	1,3-beta-glucanosyltransferase, involved with Gas4p in spore wall assembly
KRE1	Cell wall glycoprotein involved in beta-glucan assembly
MKC7	GPI-anchored aspartyl protease, member of the yapsin family of proteases involved in cell wall growth and maintenance
MUC1	GPI-anchored cell surface glycoprotein (flocculin) required for pseudohyphal formation, invasive growth, flocculation, and biofilms
PUN1	Plasma membrane protein with a role in cell wall integrity; transcription induced upon cell wall damage and metal ion stress
ROT2	Glucosidase II catalytic subunit required for normal cell wall synthesis
SMI1	Protein involved in the regulation of cell wall synthesis; proposed to be involved in coordinating cell cycle progression with cell wall integrity
	CELL RESCUE, DEFENSE AND VIRULENCE
Stress response	
CSF1	Protein required for fermentation at low temperature
DFG16	Probable multiple transmembrane protein, involved in diploid invasive and pseudohyphal growth upon nitrogen starvation; required for accumulation of processed Rim101p
HAL5	Putative protein kinase; overexpression increases sodium and lithium tolerance, whereas gene disruption increases cation and low pH sensitivity and impairs potassium uptake, suggesting a role in regulation of Trk1p and/or Trk2p transporters
HOG1	Mitogen-activated protein kinase involved in osmoregulation
NBP2	Protein involved in the HOG (high osmolarity glycerol) pathway, negatively regulates Hog1p by recruitment of phosphatase Ptc1p the Pbs2p-Hog1p complex
PBS2	MAP kinase kinase of the HOG signaling pathway; activated under severe osmotic stress
PTC1	Type 2C protein phosphatase (PP2C); dephosphorylates Hog1p, inactivating osmosensing MAPK cascade

Table S1 – continued.

Gene/ORF*	Function**
RHR2	Constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis, induced in response to both anaerobic and, along with the Hor2p/Gpp2p isoform, osmotic stress
RIM13	Calpain-like cysteine protease involved in proteolytic activation of Rim101p in response to alkaline pH
RIM20	Protein involved in microtubule morphogenesis, required for protection from excess free beta-tubulin
RIM21	Component of the RIM101 pathway, has a role in cell wall construction and alkaline pH response
RIM8	Protein involved in proteolytic activation of Rim101p in response to alkaline pH; interacts with ESCRT-1 subunits Stp22p and Vps28p
RIM9	Protein involved in the proteolytic activation of Rim101p in response to alkaline pH
SCH9	Protein kinase involved in transcriptional activation of osmostress-responsive genes; regulates G1 progression, cAPK activity, nitrogen activation of the FGM pathway
SSK2	MAP kinase kinase kinase of the HOG1 mitogen-activated signaling pathway; interacts with Ssk1p, leading to autophosphorylation and activation of Ssk2p which phosphorylates Pbs2p
STE11	Signal transducing MEK kinase involved in pheromone response and pseudohyphal/invasive growth pathways where it phosphorylates Ste7p, and the high osmolarity response pathway, via phosphorylation of Pbs2p
SVF1	Protein with a potential role in cell survival pathways, required for the diauxic growth shift
YGL046W	Protein involved in proteolytic activation of Rim101p in response to alkaline pH; interacts with ESCRT-1 subunits Stp22p and Vps28p
YVH1	Protein phosphatase involved in vegetative growth at low temperatures, sporulation, and glycogen accumulation
Disease, virulence and defense	
ETT1	Nuclear protein that inhibits replication of Brome mosaic virus in *S. cerevisiae*
MAK3	Catalytic subunit of N-terminal acetyltransferase of the NatC type; required for replication of dsRNA virus
OCA4	Cytoplasmic protein required for replication of Brome mosaic virus in *S. cerevisiae*
OCA5	Cytoplasmic protein required for replication of Brome mosaic virus in *S. cerevisiae*
OCA6	Cytoplasmic protein required for replication of Brome mosaic virus in *S. cerevisiae*
	UNKNOWN/DUBIOUS
ACF4	Protein of unknown function, possible role in actin cytoskeleton organization
AIM25	Putative protein of unknown function
AIM26	Putative protein of unknown function; null mutation confers sensitivity to tunicamycin and DTT
API2	Dubious open reading frame, unlikely to encode a protein;
DPH6	Putative protein of unknown function
ECM33	GPI-anchored protein of unknown function, has a possible role in apical bud growth
FYV12	Protein of unknown function, required for survival upon exposure to K1 killer toxin
FYV6	Protein of unknown function, required for survival upon exposure to K1 killer toxin; proposed to regulate double-strand break repair via non-homologous end-joining
GDS1	Protein of unknown function, required for growth on glycerol as a carbon source
ILM1	Protein of unknown function; may be involved in mitochondrial DNA maintenance; required for slowed DNA synthesis-induced filamentous growth
IME4	Probable mRNA N6-adenosine methyltransferase required for entry into meiosis

Table S1 – continued.

Gene/ORF*	Function**
IRC13	Dubious open reading frame unlikely to encode a protein
JJJ3	Protein of unknown function, contains a J-domain, which is a region with homology to the E. coli DnaJ protein
LCL1	Putative protein of unknown function; deletion mutant is fluconazole resistant and has long chronological lifespan
LDB16	Protein of unknown function; null mutants have decreased net negative cell surface charge; GFP-fusion protein expression is induced in response to the DNA-damaging agent MMS
LNP1	Putative protein of unknown function; GFP-fusion protein is induced in response to the DNA-damaging agent MMS
MNL2	Putative protein of unknown function
MSC1	Protein of unknown function; mutant is defective in directing meiotic recombination events to homologous chromatids
MTC1	Protein of unknown function that may interact with ribosomes;GFP-fusion protein localizes to the cytoplasm and to COPI-coated vesicles (early Golgi)
NST1	Protein of unknown function, mediates sensitivity to salt stress
PIH1	Protein of unresolved function; may function in protein folding and/or rRNA processing, interacts with a chaperone (Hsp82p), two chromatin remodeling factors (Rvb1p, Rvb2p) and two rRNA processing factors (Rrp43p, Nop58p)
RGI2	Protein of unknown function involved in energy metabolism under respiratory conditions
RRT12	Probable subtilisin-family protease with a role in formation of the dityrosine layer of spore walls
RRT14	Putative protein of unknown function; predicted to be involved in ribosome biogenesis
RRT2	Putative protein of unknown function; non-essential gene identified in a screen for mutants with increased levels of rDNA transcription
RTC2	Protein of unknown function; mutant produces large lipid droplets, is resistant to fluconazole, has decreased levels of rDNA transcription, growth defects on minimal media, and suppresses cdc13-1
RTP1	Putative protein of unknown function
SND1	Putative protein of unknown function; may interact with ribosomes
TMA10	Protein of unknown function that associates with ribosomes
TMA20	Protein of unknown function that associates with ribosomes and has a putative RNA binding domain
TMA22	Protein of unknown function; associates with ribosomes and has a putative RNA binding domain
VHS2	Cytoplasmic protein of unknown function; suggesting a role in G1/S phase progression;
VPS69	Dubious open reading frame, unlikely to encode a protein
WWM1	WW domain containing protein of unknown function; binds to Mca1p, a caspase-related protease that regulates H2O2-induced apoptosis; overexpression causes G1 phase growth arrest and clonal death that is suppressed by overexpression of MCA1
YAR044W	Unknown
YBL071C-B	Putative protein of unknown function
YBR174C	Dubious open reading frame unlikely to encode a protein
YCL001W-B	Putative protein of unknown function; YCL001W-B gene has similarity to DOM34
YCR062W	Protein of unknown function; induced by treatment with 8-methoxypsoralen and UVA irradiation
YCR075W-A	Putative protein of unknown function; identified by homology to Ashbya gossypii
YCR085W	Dubious open reading frame unlikely to encode a protein

Table S1 – continued.

Gene/ORF*	Function**
YCR087W	Dubious open reading frame unlikely to encode a protein
YCR095W-A	Putative protein of unknown function
YDL057W	Putative protein of unknown function
YDR455C	Dubious open reading frame unlikely to encode a protein
YDR491C	Dubious open reading frame unlikely to encode a functional protein
YDR524C-B	Putative protein of unknown function
YDR524W-A	Putative protein of unknown function; deletion strains are moderately sensitive to the radiomimetic drug bleomycin
YER119C-A	Dubious open reading frame; deletion mutation blocks replication of Brome mosaic virus in S. cerevisiae, but this is likely due to effects on the overlapping gene SCS2
YGL036W	Putative protein of unknown function
YGL041C-B	Putative protein of unknown function; identified by fungal homology and RT-PCR
YGL042C	Dubious open reading frame, not conserved in closely related Saccharomyces species; deletion mutation blocks replication of Brome mosaic virus in S. cerevisiae, but this is likely due to effects on the overlapping gene DST1
YGL188C-A	Putative protein of unknown function
YGL218W	Dubious open reading frame, unlikely to encode a protein
YGR011W	Dubious open reading frame unlikely to encode a functional protein
YGR022C	Dubious open reading frame unlikely to encode a protein
YGR025W	Dubious open reading frame unlikely to encode a functional protein
YGR122W	Probable ortholog of A. nidulans PalC, which is involved in pH regulation and binds to the ESCRT-III complex
YGR201C	Putative protein of unknown function
YHR131C	Putative protein of unknown function; overexpression causes cell cycle delay or arrest; contains a PH domain and binds phosphatidylinositols and other lipids in a large-scale study
YIR042C	Putative protein of unknown function
YJL075C	Dubious open reading frame, unlikely to encode a protein
YJL169W	Dubious open reading frame unlikely to encode a protein
YKL096C-B	Putative protein of unknown function
YKR023W	Putative protein of unknown function
YKR073C	Dubious open reading frame unlikely to encode a protein
YLF023W	Unknown
YLL007C	Putative protein of unknown function
YLR111W	Dubious open reading frame unlikely to encode a protein
YLR169W	Dubious open reading frame unlikely to encode a functional protein
YLR171W	Dubious open reading frame unlikely to encode a functional protein
YLR264C-A	Putative protein of unknown function
YLR326W	Putative protein of unknown function, predicted to be palmitoylated
YLR358C	Dubious open reading frame unlikely to encode a protein
YLR366W	Dubious open reading frame unlikely to encode a protein
YLR374C	Dubious open reading frame unlikely to encode a protein
YLR413W	Putative protein of unknown function
YLR434C	Unknown

Table S1 – continued.

Gene/ORF*	Function**
YMR001C-A	Putative protein of unknown function
YMR031W-A	Dubious open reading frame unlikely to encode a protein; null mutant displays shortened telomeres
YMR057C	Dubious open reading frame unlikely to encode a protein
YMR075C-A	Dubious open reading frame unlikely to encode a protein,
YMR242W-A	Putative protein of unknown function
YMR326C	Dubious open reading frame unlikely to encode a protein
YNL120C	Dubious open reading frame unlikely to encode a protein; deletion enhances replication of Brome mosaic virus in S. cerevisiae
YNL198C	Dubious open reading frame unlikely to encode a protein
YNR005C	Dubious open reading frame unlikely to encode a functional protein
YNR042W	Dubious open reading frame unlikely to encode a protein
YOL073C	Putative protein of unknown function
YOR008C-A	Putative protein of unknown function, includes a potential transmembrane domain; deletion results in slightly lengthened telomeres
YOR055W	Dubious open reading frame unlikely to encode a functional protein
YOR199W	Dubious open reading frame unlikely to encode a protein
YOR223W	Protein of unknown function found in the ER and vacuole lumen
YOR228C	Protein of unknown function, localized to the mitochondrial outer membrane
YPL150W	Putative protein kinase of unknown cellular role; binds phosphatidylinositols and cardiolipin in a large-scale study
YPL199C	Putative protein of unknown function, predicted to be palmitoylated
YPL205C	Hypothetical protein; deletion of locus affects telomere length
YPR053C	Dubious open reading frame unlikely to encode a protein
YPR092W	Dubious open reading frame unlikely to encode a functional protein
YPR109W	Predicted membrane protein; dipoid deletion strain has high budding index
YPR114W	Putative protein of unknown function
YPR148C	Protein of unknown function that may interact with ribosomes

*Genes marked in **bold** represent the genes whose deletions resulted in hypersensitive to chitosan.

**Biological function is based on the information available in *Saccharomyces* Genome Database (SGD) project (www.yeastgenome.org).

ATTACHMENTS Supplemental Tables

Table S 2- Genes whose deletion confers resistance to chitosan.

Gene/ORF*	Function**
	METABOLISM
ADK1	Adenylate kinase, required for purine metabolism
ARD1	Subunit of N-terminal acetyltransferase NatA (Nat1p, Ard1p, Nat5p); acetylates many proteins and thus affects telomeric silencing, cell cycle, heat-shock resistance, mating, and sporulation
ERG3	C-5 sterol desaturase, catalyzes the introduction of a C-5(6) double bond into episterol, a precursor in ergosterol biosynthesis
ERG4	C-24(28) sterol reductase, catalyzes the final step in ergosterol biosynthesis
ERG6	Delta(24)-sterol C-methyltransferase, converts zymosterol to fecosterol in the ergosterol biosynthetic pathway by methylating position C-24
GSH1	Gamma glutamylcysteine synthetase catalyzes the first step in glutathione (GSH) biosynthesis
HOC1	Alpha-1,6-mannosyltransferase involved in cell wall mannan biosynthesis
IDH2	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle
LCB4	Sphingoid long-chain base kinase, responsible for synthesis of long-chain base phosphates
RAM1	Beta subunit of the CAAX farnesyltransferase (FTase) that prenylates the a-factor mating pheromone and Ras proteins
TCO89	Subunit of TORC1 (Tor1p or Tor2p-Kog1p-Lst8p-Tco89p), a complex that regulates growth in response to nutrient availability
	CELL CYCLE AND DNA PROCESSING
ALF1	Alpha-tubulin folding protein, required for the folding of alpha-tubulin and may play an additional role in microtubule maintenance
ARC18	Subunit of the ARP2/3 complex, which is required for the motility and integrity of cortical actin patches
BEM4	Protein involved in establishment of cell polarity and bud emergence;; involved in maintenance of proper telomere length
CAC2	Component of the chromatin assembly complex (with Rlf2p and Msi1p) that assembles newly synthesized histones onto recently replicated DNA
CTF18	Subunit of a complex with Ctf8p that shares some subunits with Replication Factor C and is required for sister chromatid cohesion
DEF1	RNAPII degradation factor, forms a complex with Rad26p in chromatin, enables ubiquitination and proteolysis of RNAPII present in an elongation complex
DOC1	Processivity factor required for the ubiquitination activity of the anaphase promoting complex (APC)
MLH1	Protein required for mismatch repair in mitosis and meiosis as well as crossing over during meiosis
NPL6	Component of the RSC chromatin remodeling complex; involved in nuclear protein import and maintenance of proper telomere length
PMS1	ATP-binding protein required for mismatch repair in mitosis and meiosis
PPS1	Protein phosphatase with specificity for serine, threonine, and tyrosine residues; has a role in the DNA synthesis phase of the cell cycle
RAD27	5' to 3' exonuclease, 5' flap endonuclease, required for Okazaki fragment processing and maturation as well as for long-patch base-excision repair
RAI1	Nuclear protein with decapping endonuclease activity targeted toward mRNAs with unmethylated 7-methylguanosine cap structures
RFM1	DNA-binding protein required for vegetative repression of middle sporulation genes; involved in telomere maintenance
RIM1	Single-stranded DNA-binding protein essential for mitochondrial genome maintenance; involved in mitochondrial DNA replication

Table S2 – continued.

Gene/ORF*	Function**
SGF73	SAGA complex subunit; has a role in anchoring the deubiquitination module into SAGA and SLIK complexes
SIF2	WD40 repeat-containing subunit of the Set3C histone deacetylase complex, which represses early/middle sporulation genes
SIR2	Conserved NAD+ dependent histone deacetylase of the Sirtuin family involved in regulation of lifespan; negatively regulates initiation of DNA replication
SIR3	Silencing protein that interacts with Sir2p and Sir4p, and histone H3 and H4 tails, to establish a transcriptionally silent chromatin state
SIR4	Silent information regulator that, together with SIR2 and SIR3, is involved in assembly of silent chromatin domains at telomeres and the silent mating-type loci
SLX5	Subunit of the Slx5-Slx8 SUMO-targeted ubiquitin ligase (STUbL) complex, stimulated by SUMO-modified substrates; contains a RING domain and two SIMs (SUMO-interacting motifs); forms SUMO-dependent nuclear foci, including DNA repair centers
STE20	Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family
SPT10	Putative histone acetylase with a role in transcriptional silencing; sequence-specific activator of histone genes
XRN1	Evolutionarily-conserved 5'-3' exonuclease component of cytoplasmic processing (P) bodies involved in mRNA decay; plays a role in microtubule-mediated processes, filamentous growth, ribosomal RNA maturation, and telomere maintenance
YFR024C	Protein containing a C-terminal SH3 domain; involved in actin patch assembly and actin polymerization
YKE2	Subunit of the heterohexameric Gim/prefoldin protein complex involved in the folding of alpha-tubulin, beta-tubulin, and actin
	TRANSCRIPTION
Rna synthesis and processing	
ASK10	Component of RNA polymerase II holoenzyme, phosphorylated in response to oxidative stress
BUD13	Subunit of the RES complex, which is required for nuclear pre-mRNA retention and splicing; involved in bud-site selection
CDC73	Component of the Paf1p complex; binds to and modulates the activity of RNA polymerases I and II
GAL11	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme
IST3	Component of the U2 snRNP, required for the first catalytic step of splicing and for spliceosomal assembly
IWR1	RNA polymerase II transport factor, conserved from yeast to humans; involved in both basal and regulated transcription from RNA polymerase II (RNAP II) promoters, but not itself a transcription factor
*LEA1**	Component of U2 snRNP; disruption causes reduced U2 snRNP levels
LRP1	Nuclear exosome-associated nucleic acid binding protein; involved in RNA processing, surveillance, degradation, tethering, and export
NAB6	Putative RNA-binding protein that associates with mRNAs encoding cell wall proteins in high-throughput studies
NAM7	ATP-dependent RNA helicase of the SFI superfamily involved in nonsense mediated mRNA decay
NMD2	Protein involved in the nonsense-mediated mRNA decay (NMD) pathway; involved in telomere maintenance
NOT3	Subunit of the CCR4-NOT complex, which is a global transcriptional regulator with roles in transcription initiation and elongation and in mRNA degradation
NPR2	Subunit of the conserved Npr2/3 complex that mediates downregulation of TORC1 activity upon amino acid limitation; subunit of SEA (Seh1-associated) complex

Table S2 – continued.

Gene/ORF*	Function**
NPR3	Subunit of the conserved Npr2/3 complex that mediates downregulation of TORC1 activity upon amino acid limitation; subunit of SEA (Seh1-associated) complex
PGD1	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme
RIC1	Protein involved in retrograde transport to the cis-Golgi network; involved in transcription of rRNA and ribosomal protein genes
RPI1	Putative transcriptional regulator
*RTT103**	Protein that interacts with exonuclease Rat1p and Rai1p and plays a role in transcription termination by RNA polymerase II
*SNT309**	Member of the NineTeen Complex (NTC) that contains Prp19p and stabilizes U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs
SNU66	Component of the U4/U6.U5 snRNP complex involved in pre-mRNA splicing via spliceosome; also required for pre-5S rRNA processing
SPT21	Protein with a role in transcriptional silencing; required for normal transcription at several loci including HTA2-HTB2 and HHF2-HHT2
SPT4	Protein involved in the regulating Pol I and Pol II transcription, pre-mRNA processing, kinetochore function, and gene silencing
TOP1	Topoisomerase I, nuclear enzyme that relieves torsional strain in DNA by cleaving and re-sealing the phosphodiester backbone
TRF5	Non-canonical poly(A) polymerase, involved in nuclear RNA degradation as a component of the TRAMP complex; catalyzes polyadenylation of hypomodified tRNAs, and snoRNA and rRNA precursors
UPF3	Component of the nonsense-mediated mRNA decay (NMD) pathway; involved in decay of mRNA containing nonsense codons; involved in telomere maintenance
Transcriptional factors	
IXR1	Protein that binds DNA containing intrastrand cross-links formed by cisplatin, contains two HMG (high mobility group box) domains
STP1	Transcription factor, undergoes proteolytic processing by SPS (Ssy1p-Ptr3p-Ssy5p)-sensor component Ssy5p in response to extracellular amino acids
SUM1	Transcriptional repressor required for mitotic repression of middle sporulation-specific genes; also acts as general replication initiation factor
YAP1	Transcription factor required for oxidative stress tolerance; activated by H2O2 through the multistep formation of disulfide bonds and transit from the cytoplasm to the nucleus
	PROTEIN SYNTHESIS
*FES1**	Hsp70 (Ssa1p) nucleotide exchange factor
MSD1	Mitochondrial aspartyl-tRNA synthetase, required for acylation of aspartyl-Trna
MSY1	Mitochondrial tyrosyl-tRNA synthetase
NAT1	Subunit of the N-terminal acetyltransferase NatA (Nat1p, Ard1p, Nat5p)
PPQ1	Putative protein serine/threonine phosphatase
RPL12B	Protein component of the large (60S) ribosomal subunit
RPL16B	N-terminally acetylated protein component of the large (60S) ribosomal subunit, binds to 5.8 S rRNA
RPL19B	Protein component of the large (60S) ribosomal subunit
RPL34A	Protein component of the large (60S) ribosomal subunit
RPL8B	Ribosomal protein L4 of the large (60S) ribosomal subunit
RPP1A	Ribosomal stalk protein P1 alpha, involved in the interaction between translational elongation factors and the ribosome
RPS8A	Protein component of the small (40S) ribosomal subunit
RSM22	Mitochondrial ribosomal protein of the small subunit; also predicted to be an S-adenosylmethionine-dependent methyltransferase

Table S2 – continued.

Gene/ORF*	Function**
SWS2	Putative mitochondrial ribosomal protein of the small subunit; participates in controlling sporulation efficiency
TEF4	Gamma subunit of translational elongation factor eEF1B, stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes
	PROTEIN FATE (folding, modification, destination)
ATG10	Conserved E2-like conjugating enzyme that mediates formation of the Atg12p-Atg5p conjugate, which is a critical step in autophagy
DGK1	Diacylglycerol kinase, localized to the endoplasmic reticulum (ER); contains a CTP transferase domain
ERD1	Predicted membrane protein required for the retention of lumenal endoplasmic reticulum proteins
GIM5	Subunit of the heterohexameric cochaperone prefoldin complex which binds specifically to cytosolic chaperonin and transfers target proteins to it
KEX2	Subtilisin-like protease (proprotein convertase), a calcium-dependent serine protease involved in the activation of proproteins of the secretory pathway
KTR6	Probable mannosylphosphate transferase involved in the synthesis of core oligosaccharides in protein glycosylation pathway
MDM20	Non-catalytic subunit of the NatB N-terminal acetyltransferase, which catalyzes N-acetylation of proteins with specific N-terminal sequences
MNN11	Subunit of a Golgi mannosyltransferase complex that also contains Anp1p, Mnn9p, Mnn10p, and Hoc1p, and mediates elongation of the polysaccharide mannan backbone
MNN4	Putative positive regulator of mannosylphosphate transferase (Mnn6p), involved in mannosylphosphorylation of N-linked oligosaccharides
MUB1	MYND domain-containing protein required for ubiquitination and turnover of Rpn4p
OST3	Gamma subunit of the oligosaccharyltransferase complex of the ER lumen, which catalyzes asparagine-linked glycosylation of newly synthesized proteins
RCE1	Type II CAAX prenyl protease involved in the proteolysis and maturation of Ras and the a-factor mating pheromone
RPL40B	Fusion protein, that is cleaved to yield ubiquitin and a ribosomal protein of the large (60S) ribosomal subunit
RPN10	Non-ATPase base subunit of the 19S regulatory particle (RP) of the 26S proteasome; N-terminus plays a role in maintaining the structural integrity of the RP
SEY1	GTPase with a role in ER morphology; interacts physically and genetically with Yop1p and Rtn1p
SCT1	Glycerol 3-phosphate/dihydroxyacetone phosphate dual substrate-specific sn-1 acyltransferase of the glycerolipid biosynthesis pathway
TRE1	Plasma membrane protein that binds to Bsd2p and regulates ubiquitylation and vacuolar degradation of the metal transporter Smf1p
UBP15	Ubiquitin-specific protease involved in protein deubiquitination; catalytic activity regulated by an N-terminal TRAF-like domain and and C-terminal sequences
UBP6	Ubiquitin-specific protease situated in the base subcomplex of the 26S proteasome, releases free ubiquitin from branched polyubiquitin chains
XDJ1	Putative chaperone, homolog of E. coli DnaJ, closely related to Ydj1p
YLR194C	Structural constituent of the cell wall attached to the plasma membrane by a GPI-anchor
YND1	Apyrase with wide substrate specificity, helps prevent inhibition of glycosylation by hydrolyzing nucleoside tri- and diphosphates that inhibit glycotransferases
	CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES
Tranport routes	
APL2	Beta-adaptin, large subunit of the clathrin-associated protein (AP-1) complex; binds clathrin; involved in clathrin-dependent Golgi protein sorting

Table S2 – continued.

Gene/ORF*	Function**
APL5	Delta adaptin-like subunit of the clathrin associated protein complex (AP-3); functions in transport of alkaline phosphatase to the vacuole via the alternate pathway
*APL6**	Beta3-like subunit of the yeast AP-3 complex; functions in transport of alkaline phosphatase to the vacuole via the alternate pathway
*APM3**	Mu3-like subunit of the AP-3; functions in transport of alkaline phosphatase to the vacuole via the alternate pathway
*APS3**	Small subunit of the clathrin-associated adaptor complex AP-3, which is involved in vacuolar protein sorting
*CDC50**	Endosomal protein that interacts with phospholipid flippase Drs2p
EDE1	Key endocytic protein involved in a network of interactions with other endocytic proteins, binds membranes in a ubiquitin-dependent manner
EMC1	Member of a transmembrane complex required for efficient folding of proteins in the ER
EMC4	Member of a transmembrane complex required for efficient folding of proteins in the ER
EMC5	Member of a transmembrane complex required for efficient folding of proteins in the ER
EMC6	Member of a transmembrane complex required for efficient folding of proteins in the ER
EMP24	Component of the p24 complex; binds to GPI anchor proteins and mediates their efficient transport from the ER to the Golgi
END3	EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis
*ERV14**	Protein localized to COPII-coated vesicles, involved in vesicle formation and incorporation of specific secretory cargo
*FPS1**	Plasma membrane channel, member of major intrinsic protein (MIP) family; involved in efflux of glycerol and in uptake of acetic acid and the trivalent metalloids arsenite and antimonite
GET1	Subunit of the GET complex; involved in insertion of proteins into the ER membrane
GET2	Subunit of the GET complex; involved in insertion of proteins into the ER membrane
*GET3**	Guanine nucleotide exchange factor for Gpa1p; subunit of the GET complex, which is involved in Golgi to ER trafficking and insertion of proteins into the ER membrane
GET4	Protein with a role in insertion of tail-anchored proteins into the ER membrane
*GLO3**	ADP-ribosylation factor GTPase activating protein (ARF GAP), involved in ER-Golgi transport
GOS1	v-SNARE protein involved in Golgi transport, homolog of the mammalian protein GOS-28/GS28
GRH1	Acetylated, cis-golgi localized protein involved in ER to Golgi transport; forms a complex with the coiled-coil protein Bug1p
GSG1	Subunit of TRAPPIII (transport protein particle), a multimeric guanine nucleotide-exchange factor for Ypt1p, required for membrane expansion during autophagy and the CVT pathway
GYP6	GTPase-activating protein (GAP) for the yeast Rab family member, Ypt6p; involved in vesicle mediated protein transport
ICE2	Integral ER membrane protein with type-III transmembrane domains; mutations cause defects in cortical ER morphology in both the mother and daughter cells
INP53	Polyphosphatidylinositol phosphatase, dephosphorylates multiple phosphatidylinositols; involved in trans Golgi network-to-early endosome pathway
KRE11	Subunit of TRAPPII, a multimeric guanine nucleotide-exchange factor for Ypt1p; involved in intra-Golgi traffic and the retrograde pathway from the endosome to Golgi
PEX13	Integral peroxisomal membrane protein required for translocation of peroxisomal matrix proteins
PEX15	Phosphorylated tail-anchored type II integral peroxisomal membrane protein required for peroxisome biogenesis
PEX19	Chaperone and import receptor for newly-synthesized class I peroxisomal membrane proteins (PMPs)

Table S2 – continued.

Gene/ORF*	Function**
PEX22	Putative peroxisomal membrane protein required for import of peroxisomal proteins
PEX4	Peroxisomal ubiquitin conjugating enzyme required for peroxisomal matrix protein import and peroxisome biogenesis
*RCY1**	F-box protein involved in recycling plasma membrane proteins internalized by endocytosis
RER1	Protein involved in retention of membrane proteins, including Sec12p, in the ER; localized to Golgi; functions as a retrieval receptor in returning membrane proteins to the ER
SAC6	Fimbrin, actin-bundling protein; cooperates with Scp1p in the organization and maintenance of the actin cytoskeleton
SEC28	Epsilon-COP subunit of the coatomer; regulates retrograde Golgi-to-ER protein traffic
SFT2	Non-essential tetra-spanning membrane protein found mostly in the late Golgi; may be part of the transport machinery
TED1	Conserved phosphoesterase domain-containing protein that acts together with Emp24p/Erv25p in cargo exit from the ER
VRP1	Proline-rich actin-associated protein involved in cytoskeletal organization and cytokinesis
YPT6	Rab family GTPase, Ras-like GTP binding protein involved in the secretory pathway, required for fusion of endosome-derived vesicles with the late Golgi
Tranport compounds	
PET8	S-adenosylmethionine transporter of the mitochondrial inner membrane, member of the mitochondrial carrier family
INH1	Protein that inhibits ATP hydrolysis by the F1F0-ATP synthase
COX17	Copper metallochaperone that transfers copper to Sco1p and Cox11p for eventual delivery to cytochrome c oxidase
ACB1	Acyl-CoA-binding protein, transports newly synthesized acyl-CoA esters from fatty acid synthetase (Fas1p-Fas2p) to acyl-CoA-consuming processes
AGP2	High affinity polyamine permease; plasma membrane carnitine transporter, also functions as a low-affinity amino acid permease
AVT5	Putative transporter, member of a family of seven S. cerevisiae genes (AVT1-7) related to vesicular GABA-glycine transporters
ITR1	Myo-inositol transporter with strong similarity to the minor myo-inositol transporter Itr2p, member of the sugar transporter superfamily
TIM18	Component of the mitochondrial TIM22 complex involved in insertion of polytopic proteins into the inner membrane
	INTERACTION WITH THE CELLULAR ENVIRONMENT
Ionic homeostasis	
*PKR1**	V-ATPase assembly factor, functions with other V-ATPase assembly factors in the ER to efficiently assemble the V-ATPase membrane sector (V0)
*PMR1**	High affinity Ca2+/Mn2+ P-type ATPase required for Ca2+ and Mn2+ transport into Golgi
*RAV1**	Subunit of the RAVE complex which promotes assembly of the V-ATPase holoenzyme; required for transport between the early and late endosome/PVC and for localization of TGN membrane proteins
*RAV2**	Subunit of RAVE complex, that associates with the V1 domain of the vacuolar membrane (H+)-ATPase (V-ATPase) and promotes assembly and reassembly of the holoenzyme
VMA11	Vacuolar ATPase V0 domain subunit c', involved in proton transport activity
VMA16	Subunit c'' of the vacuolar ATPase, which functions in acidification of the vacuole
VMA21	Integral membrane protein that is required for vacuolar H+-ATPase (V-ATPase) functions and in the assembly of the V-ATPase.
VMA8	Subunit D of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase); plays a role in the coupling of proton transport and ATP hydrolysis
VPH1	Subunit a of vacuolar-ATPase V0 domain, one of two isoforms (Vph1p and Stv1p)

Table S2 – continued.

Gene/ORF*	Function**
YHR039C-B	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase); involved in vacuolar acidification
	UNKNOWN/DUBIOUS
CNL1	Protein of unknown function; likely member of BLOC complex involved in endosomal cargo sorting
DUF1	Putative protein of unknown function
*EMP65**	Putative protein of unknown function; genetic interactions suggest a role in folding of ER membrane proteins
FYV10	Protein of unknown function, involved in proteasome-dependent catabolite inactivation of FBPase
FYV8	Protein of unknown function
GEP5	Protein of unknown function, required for mitochondrial genome maintenance
*HUR1**	Protein of unknown function
LGE1	Protein of unknown function; may be involved DNA synthesis and reduced efficiency of meiotic nuclear division
OXR1	Protein of unknown function required for normal levels of resistance to oxidative damage
RTC3	Protein of unknown function involved in RNA metabolism
SLP1	Integral membrane protein of unknown function; member of the SUN-like family of proteins; genetic interactions suggest a role in folding of ER membrane proteins
VPS63	Dubious open reading frame, unlikely to encode a protein
YBL012C	Dubious open reading frame unlikely to encode a protein
YBR062C	Protein of unknown function that interacts with Msb2p; may play a role in activation of the filamentous growth pathway.
YBR085C-A	Putative protein of unknown function
YDL041W	Dubious open reading frame unlikely to encode a protein
YDL118W	Non-essential protein of unconfirmed function; mutants are defective in telomere maintenance, and are synthetically sick or lethal with alpha-synuclein
YDR203W	Dubious open reading frame unlikely to encode a functional protein
YDR290W	Dubious open reading frame unlikely to encode a protein
YER084W	Dubious open reading frame unlikely to encode a protein
YGL007C-A	Putative protein of unknown function; deletion exhibits slow-growth phenotype; computationally predicted to have a role in cell budding
YGL007W	Dubious ORF located in the upstream region of PMA1
YGL072C	Dubious open reading frame unlikely to encode a protein
YGL199C	Dubious open reading frame unlikely to encode a protein
YGR064W	Dubious open reading frame unlikely to encode a protein
YGR176W	Dubious open reading frame unlikely to encode a functional protein
YHL005C	Dubious open reading frame unlikely to encode a protein
YHR078W	High osmolarity-regulated gene of unknown function
YJR087W	Dubious open reading frame, unlikely to encode a protein
YKL031W	Dubious open reading frame, unlikely to encode a protein
YKL199C	Unknown
YLR184W	Dubious ORF unlikely to encode a functional protein
*YLR338W**	Dubious open reading frame unlikely to encode a protein
YML007C-A	Putative protein of unknown function

Table S2 – continued.

Gene/ORF*	Function**
*YML095C-A**	Unknown
YML102C-A	Unknown
YML117W-A	Unknown
*YMR010W**	Putative protein of unknown function
YMR086C-A	Dubious open reading frame unlikely to encode a functional protein
YNL043C	Dubious open reading frame unlikely to encode a protein
YNL089C	Dubious open reading frame unlikely to encode a functional protein
YNL319W	Dubious open reading frame unlikely to encode a protein
YOL050C	Dubious open reading frame unlikely to encode a protein; deletion confers sensitivity to 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide (GSAO)
YOR135C	Dubious open reading frame unlikely to encode a protein
YOR309C	Dubious open reading frame unlikely to encode a protein

*Genes marked with asterisk represent the genes whose deletions resulted in resistance to chitosan.
**Biological function is based on the information available in Saccharomyces Genome Database (SGD) project (www.yeastgenome.org).