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Saccharomyces cerevisiae Rds2 transcription factor involvement in cell wall composition and architecture

Inmaculada Moreno, Nunzia Tutrone, Rafael Sentandreu, Eulogio Valentín*

GMCA Research Group, Department of Microbiology and Ecology, Faculty of Pharmacy, University of Valencia, Spain

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Summary. Although the cell wall is very important in yeasts, relatively little is known about the relationship between its structure and function. In *Saccharomyces cerevisiae*, a family of 55 transcription factor proteins unique to fungi, so-called zinc cluster proteins, has been described. Of these, Rds2 has been identified as an activator/inhibitor of gluconeogenesis. However, previous studies have pointed out additional roles for this protein, specifically, in the modulation of cell-wall architecture and drug sensitivity. In this work, evidence regarding the role of Rds2 as a regulator of cell-wall architecture and composition is presented based on phenotypical analysis of the cell walls prepared from a *S. cerevisiae* Rds2 mutant strain. Analyses of the sensitivity of this *rds2Δ* mutant to different drugs and to osmotic stress showed that Rds2 is indeed involved in the drug-sensitivity response and plays a role in determining osmotic sensitivity. [**Int Microbiol** 2008; 11(1): 57-63]

Key words: *Saccharomyces cerevisiae* · transcription factors · gene *RDS2* · cell wall

Introduction

The fungal cell wall is an essential extracellular network made up of polysaccharides and proteins that contribute to protecting the cell internally from turgor pressure and externally from harmful physical, chemical, and biological agents. It also plays a role in the selective uptake and excretion of various compounds and in communication with the intra- and extracellular environments [for recent reviews, see 21,25]. In *Saccharomyces cerevisiae*, the cell wall is mainly composed of glucans (1,3-β- and 1,6-β-glucan), mannoproteins, and chitin [39]. The β-glucans are the main components, accounting for 50–60% (by weight) of the cell wall in *S. cerevisiae* and other yeasts.

***Corresponding author:** E. Valentín
Departamento de Microbiología y Ecología
Facultad de Farmacia, Universidad de Valencia
Av. Vicente Andrés Estellés, s/n
46100 Burjassot, Valencia, Spain
Tel. +34-963543614. Fax +34-963544543
E-mail: eulogio.valentin@uv.es

Chitin, a linear polymer of 1,4-β-linked *N*-acetylglucosamine units, is a relatively minor (1–10%), albeit important, constituent. Together, β-glucans and chitin polymers confer rigidity and contribute the morphology of the fungal cell wall [31]. Mannoproteins, which represent 30–40% (by weight) of the total cell wall, determine its surface properties and are present in at least three forms: (i) proteins that are not covalently linked to the cell wall and can be extracted from the glucan mesh simply by boiling in SDS, (ii) proteins that are attached to other cell wall proteins through disulfide bridges and thus can be extracted with reducing agents, and (iii) proteins that are covalently linked to the insoluble glucan network of the wall. In this last group, two further types of proteins have been described: (a) GPI (glycosylphosphatidylinositol) cell-wall proteins, which are linked to β-1,3-glucan, via β-1,6-glucan, through a GPI moiety [12,22], and can be selectively removed from the cell wall by treatment with β-1,6-endoglucanase or hydrofluoric acid [20], and (b) ASL (alkali sensitive linked) cell-wall proteins, which are directly linked to β-1,3-glucan through an alkali-sensitive linkage [6,7].

Changes and adaptations of the cell wall in response to alterations in the intra- and extracellular environments are modulated, in part, by transcription factors. A family of transcription factors found exclusively in fungi contains a zinc cluster motif that includes the consensus sequence $CysX_2CysX_6CysX_{5–16}CysX_2CysX_{6–8}$. In the *S. cerevisiae* genome, there are 55 genes encoding putative zinc cluster proteins [for a complete list, see 2,3]. Some of these proteins have been shown to be involved in cellular processes within the cell, including regulation of primary and secondary metabolism, drug resistance, and cell-wall architecture [36]. However, the functions of many of these putative zinc cluster proteins are still unknown. In a previous study, several zinc cluster transcription factors thought to be involved in modulating the architecture of the *S. cerevisiae* cell wall were identified [2]. Among them, Rds2 has also been described as a regulator of drug sensitivity [1] and gluconeogenesis [35], suggesting that this protein assumes different functions, i.e., as an activator or repressor, depending on the needs of the cell.

Studies on the homologous transcription factor Cwt1 in the pathogenic fungus *Candida albicans* revealed an important role for this protein in several cellular processes, such as correct regulation of cell-wall architecture, sensitivity to antifungal agents, and the metabolism of various sugars [17,27,28]. These results are consistent with what is currently known about Rds2, and suggest that these two transcriptional factors not only have homologous structures, but also similar functions. Moreover, it has been shown that the particular activity expressed by Cwt1 depends on the amount of protein within the cell and on the position of the cell in the cell cycle [28, and Moreno et al., unpublished results]. In the present study, the composition of the cell wall in *S. cerevisiae* cells lacking Rds2 was examined in exponentially growing and stationary-phase cells to better understand the role of this transcriptional regulator in the construction of the yeast cell wall.

Materials and methods

Strains and growth conditions. The *S. cerevisiae* strains used in this study were FY73 (*MATα, his3-Δ200, ura3-52*), FZH (*MATα, rds2*::*HIS3, ura3-52*) (both from [2]) and FZR (*MATα, rds2*::*HIS3, RDS2*::*URA3*). *S. cerevisiae* cells were routinely grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or SD (0.7% yeast nitrogen base without amino acids, 2% glucose) medium supplemented with the appropriate nutrients in the amounts specified by Sherman [33]. *Escherichia coli* DH5α [F, ϕ80, *lac*4M15, *rec*A1, *end*A1, *gyr*A96, *thi*$^-$1, (r^-_k, m^-_k), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F) U169] was used routinely for plasmid propagation, as described by Hanahan [16]. This strain was grown in LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl) supplemented with 50 μg ampicillin/ml as required.

S. cerevisiae cells were transformed using the lithium/acetate method according to Gietz and Woods [15].

DNA preparation. Genomic DNA was prepared from *S. cerevisiae* as described by Fujimura and Sakuma [13]. Standard DNA manipulation techniques were carried out using standard protocols [32].

Plasmid construction. The reintegration plasmid containing the complete *RDS2* gene was constructed by PCR amplification of the ORF using genomic DNA as template. A 1.3-kb fragment was obtained using the sense primer RDS2-1 (CACACCCGGGGATGTCAGCAAACAGTGGTGTAAAAC) and the antisense primer RDS2-2 (CACAGTCGACTGACAAAGGGATGAAATTCCCGACG) containing, respectively, an engineered *Sma*I and *Sal*I restriction site (underlined). To reintegrate *RDS2* into the genome of the null mutant strain, FZH, the 1.3-kb DNA fragment containing the *RDS2* gene was inserted into plasmid PWS93 [34] in frame with the HA epitope present in the plasmid. The resulting plasmid, pWS-RDS2, was used to transform the mutant strain (FZH), yielding the over-expressing strain FZR.

Phenotypic analysis of *RDS2* mutants. Strain sensitivity to NaCl, LiCl, calcofluor white, hygromycin B, quinidine, bleomycin, amphoterycin B, and ketoconazole was tested by streaking cells on plates containing different concentrations of these compounds, following the method described by Van der Vaart et al. [40]. Aliquots (3 μl) of serial 1:10 dilutions of cells grown overnight (OD_{600} = 1) were deposited on YPD plates containing a range of concentrations of NaCl (0–2 M), LiCl (0–300 mM), calcofluor white (0–70 μg/ml), hygromycin B (0–150 μg/ml), quinidine (0–1.5 mg/ml), bleomycin (0–25 μg/ml), amphoterycin B (0–7 μg/ml), and ketoconazole (0–6 μg/ml), grown at 28ºC and monitored after 3 days. Sensitivity to zymolyase was also tested following the method described by Van der Vaart et al. [40]. Exponentially growing cells were adjusted to an OD_{600} of 0.5 in 10 mM Tris-HCl, pH 7.5, containing 100 μg zymolyase 20T/ml of cells.The decreases in the optical density were monitored over a 90-min period.

Cell-wall purification. Purified cell walls were obtained as described previously for *S. cerevisiae* [29,38] except that intact cells were broken with glass beads (1.5 g/mg dry cells) by shaking 1 min in a vortex mixer at room temperature eight times, with 1-min intervals during which the samples were cooled on ice. This method resulted in disruption of the entire cell population, as monitored by phase-contrast microscopy. The purification procedure was continued by repeated washing (1200 ×*g*, 5 min) of the cell-wall pellet in 1 mM cold phenylmethylsulfonyl fluoride. The pellet was collected and operationally defined as containing cell walls, while the supernatant consisted of the cytosol plus membrane fractions.

Cell wall chemical analysis. Protein was extracted with 1 M NaOH for 30 min in a boiling water bath and measured using the Bradford method [5] with BSA as the standard. Neutral sugars were measured as described by Dubois et al. [10], with glucose as the standard. Chitin was measured as described by Kapteyn et al. [19], with *N*-acetylglucosamine (GlcNAc) as the standard. β-Glucans were measured as described by Dijkgraaf et al. [9]. Mannan content was determined by precipitating the reducing sugars with Fehling solution [18]. All assays were done in triplicate from at least two wall samples; the data did not differ by more than 10%.

SDS-PAGE and Western blot analysis. Proteins were separated basically as described by Laemmli [24] on SDS-10% (w/v) polyacrylamide gels. For Western blot analysis, proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters (Hybond-C Extra, General Electric, Little Chalfont, England) according to the method of Towbin et al. [37]. Filters were probed with mouse antibody against the HA epitope (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:10,000, followed by goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). Antiserum binding was visualized using the ECL (enhanced chemiluminescence) fluorescent labeling kit (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Luminescence

was recorded by exposing the filter to a radioautographic X-Omat film (Kodak, Rochester, NY, USA).

Results and Discussion

Effect of Rds2 depletion on cell sensitivities to calcofluor white, Congo red, and zymolyase. Sensitivity to compounds that interfere with the cell-wall architecture, such as calcofluor white [11,30], Congo red [23], and β-1,3-glucanases [40], have been used to determine changes in the composition of the yeast cell wall. Here, droplets of serial dilutions prepared from cultures of the FY73 parental and FZH null mutant (*rds2Δ*) strains were plated on YPD plates containing different amounts of calcofluor white and Congo red. The results showed that the FZH mutant strain was clearly hypersensitive to the cell-wall-perturbing agent calcofluor white (Fig. 1A). This result was in agreement with previous findings [2], in which a role for Rds2 in cell-wall architecture was proposed. By contrast, the two strains did not differ in their sensitivities to Congo red (data not shown), suggesting that β-glucan microfibrils are not altered in the cell wall of the FZH mutant. To better characterize the effect of *RDS2* deletion on cell-wall construction, the sensitivity of the mutant to zymolyase was quantified in exponentially growing and stationary-phase cells. The absence of Rds2 caused a slight resistance of the mutant to zymolyase in exponentially growing cells (Fig. 1B), whereas no differences between the *rds2Δ* mutant and the parental strain were observed when the cells reached stationary phase (Fig. 1B). This minor decrease in zymolyase sensitivity was consistent with changes in the structure of the glucan network, i.e., an increment in the thickness of the mannoprotein outer layer of the cell wall, as suggested by Van der Vaart et al. [40], or a difference in the relative contents of cell-wall polymers. Similar observations were made in the *C. albicans* mutant *cwt1/cwt1*, in which the amounts of the cell-wall components are also altered [27].

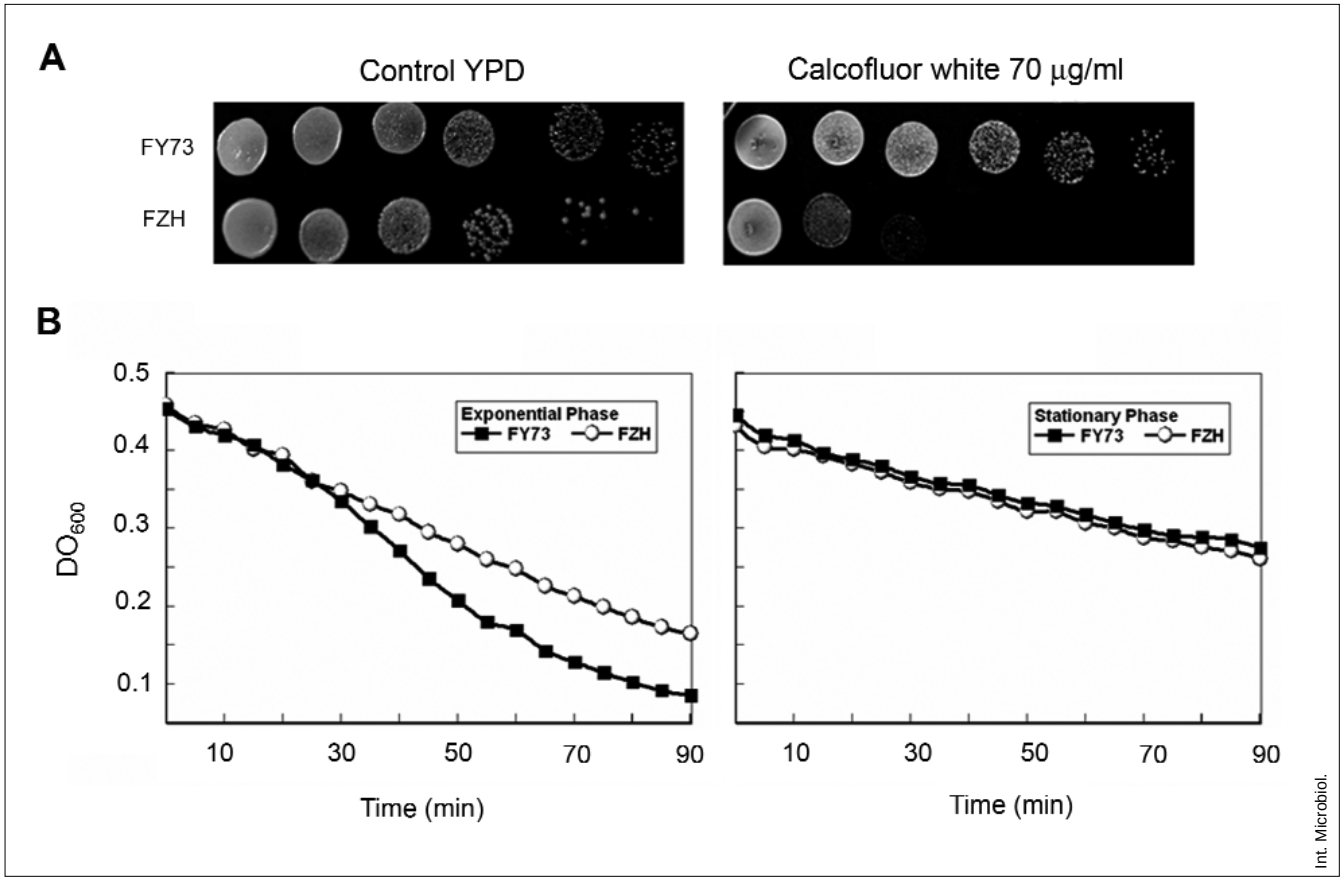

Fig. 1. (**A**) Calcofluor white sensitivities of the *Saccharomyces cerevisiae* wild-type strain FY73 and the deletion strain FZH. Cells were grown in YPD medium and aliquots of serial 1:10 dilution series were spotted on YPD plates with or without calcofluor white. (**B**) Zymolyase sensitivities of the two strains during the exponential and stationary phases of growth. Cells were incubated in 100 µg zymolyase 20T/ml and decreases in OD_{600} were monitored.

Table 1. Chemical composition of the cell walls in theRsd2 mutant FZH as a function of growth phase[a]

Component	Content (%)	
	Exponential phase	Stationary phase
Total glucan	100	174
β-1,3-glucan	102	177
β-1,6-glucan	91	147
Mannan	168	240
Chitin	70	108
Protein	104	78

[a]Cell walls isolated from exponentially growing and stationary-phase cells of *Saccharomyces cerevisiae* strain FZH were chemically analyzed as described in Materials and methods. The results are the percentages of each polymer with respect to the parental strain (FY73), defined as 100% in each case. The values of the different polymers are the average of three independent experiments.

Chemical composition of the cell wall. To determine whether the absence of Rds2 affected the amounts and proportions of the different cell-wall polymers, the chemical compositions of the cell walls from the parental (FY73) and mutant (FZH) strains were compared. Analysis of cell walls isolated from exponentially growing cells showed that the content of cell-wall polymers differed between the two strains (Table 1). However, unlike in the parental strain, there were no significant differences in the amounts of the two types of β-glucans in the cell wall of the mutant strain, consistent with the phenotype obtained in the Congo red sensitivity test. The most important differences were a higher mannan content (1.8-fold) and a lower amount of chitin (0.7-fold) in the cell walls of the *rds2Δ* null mutant compared to the parental strain. The increased amount of mannan in the outermost surface of the mutant cell walls may have hampered the accessibility of zymolyase to its β-1,3-glucan target, accounting for the slight resistance of the *rds2* mutant to zymolyase digestion. Similarly, a decrease in the chitin content of the cell wall of the mutant strain likely explains its hypersensitivity to calcofluor white, which is known to interfere with correct formation of the chitin lattice of growing cells [11].

Analysis of the cell walls from stationary-phase cells showed that the content of β-1,3-glucan, β-1,6-glucan, and mannan was higher in the null mutant than in the parental strain by, respectively, 1.7-, 1.5-, and 2.4-fold (Table 1). Although the amount of mannan in the mutant cell wall was even higher in stationary phase than in exponential phase, the zymolyase sensitivity of the two strains was very similar. This may have been due to a general increase in the amounts of glucans and mannan, such that the proportion of these components within the cell wall was sufficient to resist zymolase digestion. Taken together, these results suggested that, as in *C. albicans* [27], the absence of Rds2 in *S. cerevisiae* leads to changes in the architecture of the yeast cell wall, since depletion of the protein altered the amounts of the different cell-wall polymers. In addition, these results clearly showed that the composition of the cell wall of the *rds2* mutant changes depending on the position of the cell in the cell cycle.

Effect of Rds2 depletion on sensitivity to drugs. It was previously reported that Rds2 regulates drug sensitivity. To determine the sensitivities of the parental and mutant strains to antifungal agents, droplets of serial dilutions from overnight cultures of each of the strains were plated on YPD containing various amounts of amphotericin B or ketoconazole. The *rds2Δ* null mutant was very susceptible to the antifungal agent ketoconazole (Fig. 2), which is used extensively in the treatment of fungal infections [1], whereas its sensitivity to the antifungal agent amphotericin B was reduced (Fig. 2). These two drugs have different mechanisms of action. Ketoconazole interferes with ergosterol synthesis by inhibiting an enzyme of the cytochrome P450 complex that converts lanosterol into ergosterol [41]. Amphotericin B binds hydrophobically to ergosterol in the plasma membrane, thereby altering membrane permeability with subsequent leakage of cytoplasmic components [14]. The different responses of the *rds2Δ* null mutant to these antifungal agents indicated that Rds2 regulates drug sensitivity either specifically or as part of an overall alteration in the permeability of the cell surface. The susceptibilities of *S. cerevisiae* wild-type and mutant cells to different drugs were also tested by a spot assay. Accordingly, serial dilution droplets of each strain were plated on YPD plates containing various amounts of hygromycin B, quinidine, or bleomycin. The *rds2Δ* mutant was clearly hypersensitive to hygromycin B (Fig. 2), a phenotype commonly seen in mutants with altered glycosylation [4,8], and to the anticancer drug bleomycin (Fig. 2); however, the *rds2Δ* mutant was slightly more resistant than the parental strain FY73 to the antimalarial drug quinidine (Fig. 2).

It has been reported that *S. cerevisiae* mutants defective in *N*-glycosylation often display hypersensitivity to hygromycin B [8]. However, in the *rds2Δ* mutant, and unlike the glycosylation mutants, the cell-wall content of mannan is higher than in the parental strain, although hypersensitivity to hygromycin B is still observed. Thus, while the results suggested a relationship between cell-wall glycoproteins and the sensitivity of *S. cerevisiae* to hygromycin B, the molecular mechanisms by which the mannan content modifies sensitiv-

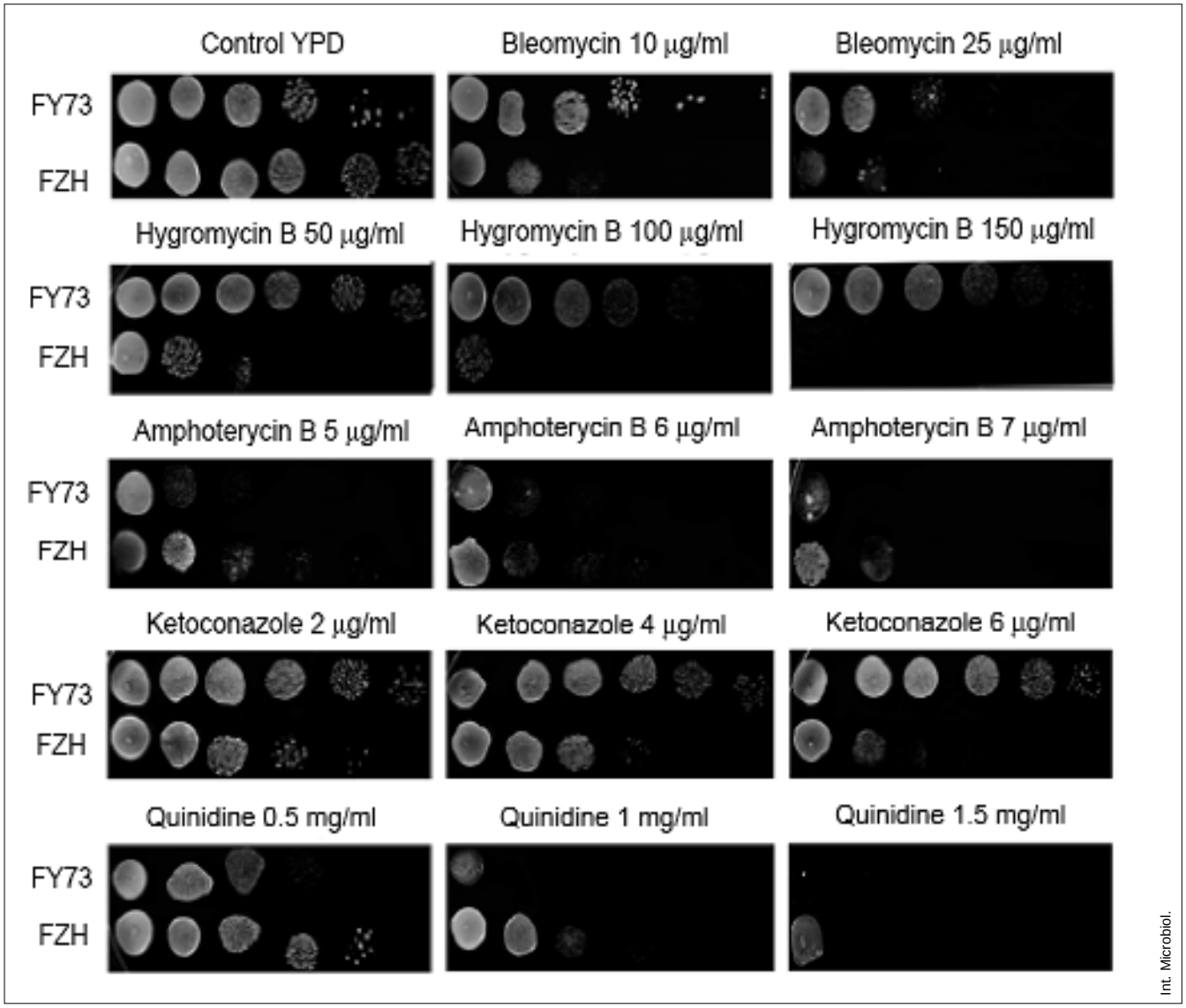

Fig. 2. Antibiotic sensitivities of the *Saccharomyces cerevisiae* wild-type (FY73) and the deletion strain (FZH). Cells were grown in YPD medium and aliquots of serial 1:10 dilution series were spotted on YPD plates with or without bleomycin, hygromycin, amphoterycin B, ketoconazole, and quinidine.

ity to drugs such as hygromycin B remains to be established. An altered response was also observed in *rds2Δ* cells growing in the presence of bleomycin or quinidine, which are not antifungal compounds, suggesting that the effects of these drugs on the mutant are not specific; instead, they may have been due to changes in the permeability of the cell wall. Alternatively, Rds2 may regulate plasma membrane proteins that are directly or indirectly involved in detoxification systems, such as multidrug transporters.

Effect of Rds2 depletion on osmotic stress tolerance. A potential role for Rds2 in the cellular response to osmotic stress was also evaluated. Serial dilutions droplets from cultures of both parental and *rds2Δ* strains were plated on YPD plates containing different amounts of NaCl or LiCl. The *rds2Δ* null mutant cells displayed a clear osmoresistant phenotype (Fig. 3) to salt stress, in contrast to the parental strain, which was almost unable to grow on YPD-NaCl or YPD-LiCl plates (Fig. 3). These results suggest that Rds2 is indeed involved in the adaptation of *S. cerevisiae* to a hyperosmolar (hyperionic) environment. It is known that the yeast cell wall plays an important role in cellular osmoregulation [for a review, 26] through the cell-integrity pathway. Defects in both the cell wall and the plasma membrane could lead to altered permeability to saline compounds.

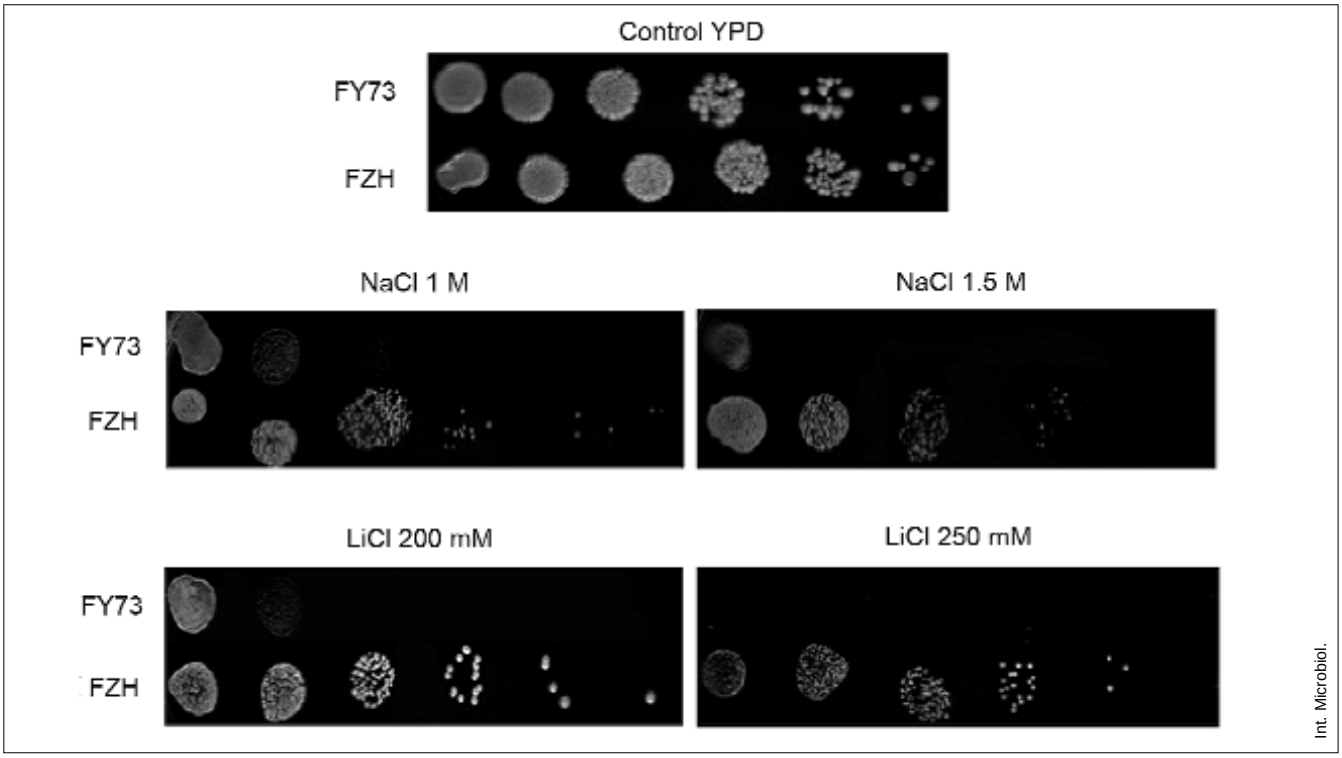

Fig. 3. Sensitivity to osmotic stress of the *Saccharomyces cerevisiae* wild-type (FY73) and the deletion strain (FZH). Cells were grown in YPD medium and aliquots of serial 1:10 dilution series were spotted on YPD plates with or without NaCl and LiCl.

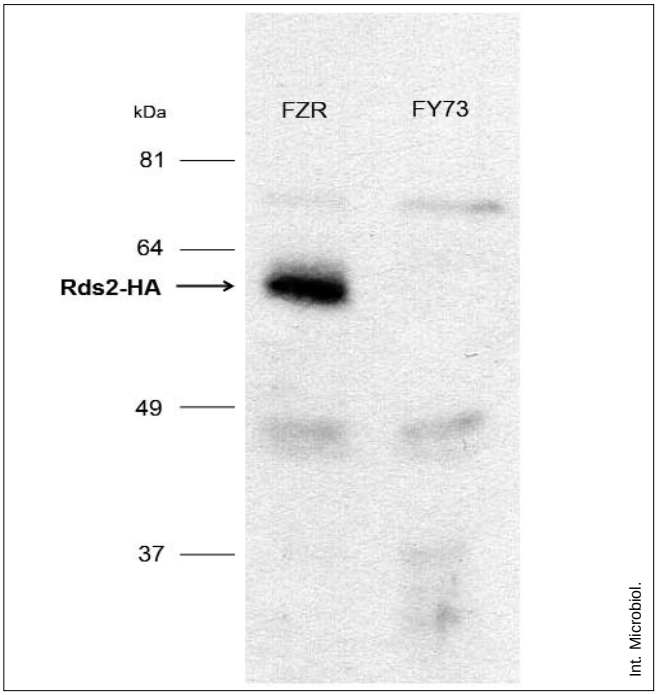

Fig. 4. Western blot analysis of cytosolic extracts obtained from the *Saccharomyces cerevisiae* wild-type strain (FY73) and the *RDS2*-overexpressing strain (FZR), harboring Rds2 tagged with the HA epitope. The membranes were incubated with a polyclonal anti-HA antibody.

Overexpression of *RDS2*. *RDS2* was overexpressed by subcloning a PCR-generated fragment containing the *RDS2* ORF into a pWS93 plasmid, thereby placing the gene under the control of the *ADH1* promoter. *RDS2* expression was confirmed by Western blot analysis of a cytoplasmic extract obtained from the *rds2Δ* null mutant transformed with the recombinant plasmid (Fig. 4). Overexpression of *RDS2* did not induce changes in the morphology or growth rate of the cells, but did increase the sensitivity of the overexpressing strain to calcofluor white, hygromycin, and bleomycin, such that the behaviors of the null mutant strain and the overexpressing strain in the presence of these drugs were very similar (data not shown). Moreover, the cell-wall composition of the two strains did not differ. These results suggest that any deviation from a certain amount of the transcription factor Rds2 increases cell sensitivity to calcofluor white, which interferes with the assembly of the cell wall, and to other drugs (data not shown). In short, the behavior of the overexpressing strain FZR was very similar to that of the *rds2Δ* null mutant.

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