

RESEARCH ARTICLE

Robust, pleiotropic drug resistance 5 (Pdr5)-mediated multidrug resistance is vigorously maintained in *Saccharomyces cerevisiae* cells during glucose and nitrogen limitation

Hadiar Rahman, Joshua Carneglia[#], Molly Lausten[#], Michael Robertello[#], John Choy and John Golin^{*}

Department of Biology, The Catholic University of America, Washington, DC 20064, USA

^{*}Corresponding author: Department of Biology, The Catholic University of America, McCort-Ward Building, room 103, 620 Michigan Avenue NE, Washington, DC 20064. Tel: 202-319-5722; E-mail: golin@cua.edu

[#]These authors contributed equally to this work. ML was a summer intern from Haverford College.

One sentence summary: Multidrug resistance is maintained in yeast cells even when glucose and nitrogen supplies are low.

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ABSTRACT

Saccharomyces cerevisiae has sophisticated nutrient-sensing programs for responding to harsh environments containing limited nutrients. As a result, yeast cells can live in diverse environments, including animals, as a commensal or a pathogen. Because they live in mixed populations with other organisms that excrete toxic chemicals, it is of interest to know whether yeast cells maintain functional multidrug resistance mechanisms during nutrient stress. We measured the activity of Pdr5, the major *Saccharomyces* drug efflux pump under conditions of limiting nutrients. We demonstrate that the steady-state level of this transporter remains unchanged during growth in low concentrations of glucose and nitrogen even though two-dimensional gel electrophoresis revealed a decrease in the level of many proteins. We also evaluated rhodamine 6G transport and resistance to three xenobiotic agents in rich (synthetic dextrose) and starvation medium. We demonstrate that Pdr5 function is vigorously maintained under both sets of conditions.

Keywords: multidrug resistance; glucose and nitrogen starvation; Pdr5; ABC transporter

INTRODUCTION

Saccharomyces cerevisiae is a strong model organism for the study of basic cellular functions, including the response to environmental stress and the development of multiple drug resistance (Conrad et al. 2014; Gonzalez and Hall 2017). With respect to the latter, identification of the yeast multidrug transporter Pdr5 (Leppert et al. 1990; Balzi et al. 1994) established this efflux pump as the founding member of a unique subfamily of ATP-binding cassette (ABC) transporters often overexpressed in pathogenic

fungi (Vanden et al. 1998; Konotoylannis and Lewis 2002; Lage 2003). For instance, Pdr5 and the Cdr1 transporter of *Candida albicans* show >70% amino acid identity.

Despite intensive study, the natural niche of baker's yeast remains puzzling (Goodard and Greig 2015). In fact, these cells are found in a broad range of environments, including grain, fruit, tree bark, insects, plants and even animals. At the same time, yeast cells are always a minority organism in a population. As a result, it is not surprising that yeast has developed multiple

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intricate mechanisms for dealing with the presence of harsh environments (Conrad *et al.* 2014; Gonzalez and Hall 2017). During starvation, selective transcription and translation of genes are required for the stress response (Zid and O'shea 2014) and a general reduction in translation (Pavanapuresan *et al.* 2014). Furthermore, membrane proteins that are normally recycled by endosomal trafficking are often shipped to the vacuole for degradation (Lang *et al.* 2014). The genetic characteristics of yeast strains vary; in some, starvation can induce a novel filamentation program (Gimeno *et al.* 1992; Cullen and Sprague 2000).

The effect of glucose and nitrogen limitation on the function of multiple resistance transporters has never been evaluated in yeast, even though the presence of xenobiotic compounds under such conditions would be expected (Goodard and Greig 2015). Therefore, we looked at the effect of limiting nitrogen and glucose on Pdr5-mediated drug resistance in *Saccharomyces* and observed that robust, Pdr5 transport is maintained.

MATERIALS AND METHODS

Yeast growth and strains

All the cultures used in this study were grown at 30°C in a shaking incubator. We used YPD, SD and the same culture medium (SLALD; synthetic low dextrose (0.05%), low (50 μ M) ammonium sulfate) described by Johnson *et al.* (2014) for the induction of the filamentation response. Save for their nitrogen and glucose concentrations, SD and SLALD media are equivalent (both are supplemented with 1% uracil and histidine). We used two isogenic yeast strains in this study. The wild-type (WT) strain JG2001 (Golin *et al.* 2007) overexpresses the major Pdr5 multidrug transporter and lacks the other yeast ABC transporters that mediate drug resistance. The Pdr5-mediated ATPase of the G312A mutant strain is catalytically dead and has no transport capability (Furman *et al.* 2013).

To measure the growth rate of the WT and G312A mutant strains in SD or SLALD medium, overnight cultures were grown to stationary phase in YPD medium. SD or SLALD broth was inoculated with $\sim 8 \times 10^5$ cells/ml. Growth was followed over time using spectroscopy.

Assessment of budding indices

To determine the bud indices, cells grown in SD or SLALD medium for 5.5, 8 and 17.5 h were fixed in 3.7% formaldehyde. Before counting, we centrifuged cells for 1.5 m at 15 000 rpm and discarded the supernatant. The cell pellets were resuspended in 100 μ l of sterile MQ water. Following this, we placed 3 μ l of cells onto a microscopic slide with a cover slip. Cells on the slides were observed under a Nikon Alphaphot Ys microscope at $\times 40$ magnification and photographed. Then cells were counted and classified using the Microsoft Paint image application.

Chemicals

Chemicals were added to media post sterilization. Cerulenin, clotrimazole, rhodame 6G (R6G) and tributyltin chloride were purchased from Sigma-Aldrich. They were diluted in dimethyl sulfoxide (DMSO) without additional purification.

Preparation of whole-cell lysates and western blotting

We prepared whole-cell lysates with a microbead beater (BioSpec Products) suspended in ice water from 50 ml

logarithmic-phase cultures in 50 mM Tris (pH 7.5), 2.5 mM EDTA buffer (2:1 ratio of buffer volume to pellet weight) containing a protease cocktail (Downes *et al.* 2013). We performed five 1-m cycles of lysis with a 1-m cooling period in between. Following this, the lysates were centrifuged at 15 000 rpm (4°C) to pellet the unbroken cells and the glass beads. The supernatants were frozen at -80°C . The protein concentration of the lysates was determined with a bicinchoninic acid kit (Thermo Fisher) using bovine serum albumin as the protein standard. Prior to gel electrophoresis, samples were solubilized in 5X SDS-PAGE. We ran equivalent samples from the same extract on separate 7% tris-acetate gels. We performed western blots as recently described (Downes *et al.* 2013) with anti-Pdr5 (Santa Cruz Biotechnology) and anti-GAPDH antibodies (Protein tech). The Pdr5 primary antibody produced in goat was diluted 1:1000 and incubated at 4°C overnight. We used Pdr5 secondary antibody anti-goat produced in mouse in 1:5000 dilution and incubated the filter at room temperature for 2 h. For a loading control, we used GAPDH primary antibody diluted 1:10 000 and incubated 4°C for 2 h. The GAPDH secondary antibody was diluted 1:7000 and the filter incubated at room temperature for 1.5 h. The chemiluminescent signal was developed by the addition of enhanced chemiluminescence reagent for 1 m before viewing the signal on a BioRad ChemiDoc MP Imaging System.

To compare the levels of Pdr5 in the various lysates, the ratio of Pdr5 to GAPDH signals was calculated using Image J software using a scan with a resolution of 600 dpi (rsbweb.nih.gov/ij/).

Two-dimensional gel electrophoresis of whole cell lysates

Two-dimensional (2D) electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (O'Farrell, 1975; Burgess-Cassler *et al.* 1989) by Kendrick Labs, Inc. (Madison, WI). Isoelectric focusing was carried out in a glass tube of inner diameter 3.3 mm using 2.0% pH 3–10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 20 000 V-h. One microgram of an isoelectric focusing internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33 000 and pI 5.2; an arrow on the stained gels marks its position. After equilibration for 10 m in buffer 'O' (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (1.0 mm thick). SDS slab gel electrophoresis was carried out for about 5 h at 25 mA/gel. The following proteins (MilliporeSigma) were used as molecular weight standards: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), carbonic anhydrase (29 000) and lysozyme (14 000). These standards appear as bands at the basic edge of the 10% acrylamide slab gels. The gels were stained with Coomassie Brilliant Blue R-250 and dried between sheets of cellophane paper with the acid edge to the left.

Computerized comparisons of 2D gels

Duplicate Coomassie blue-stained gels were obtained from each sample. The gels were scanned with a quantitative densitometer (GE Imagescanner III, GE Healthcare, Piscataway, NJ). The scanner was checked for linearity prior to scanning with a NIST-calibrated step tablet (GE Healthcare, Piscataway, NJ). The image was analyzed using Progenesis Same Spots software (version 4.5, 2011, TotalLab, UK) and Progenesis PG240 software (version 2006, TotalLab, UK). The general method of computerized analysis for the gel included spot finding, background subtraction (average

on boundary), quantification in conjunction with detailed manual checking. Spot % is equal to spot integrated density above background (volume) expressed as a percentage of total density above background of all spots measured. Difference is defined as fold-change of spot percentages. For example, if corresponding protein spots from different samples (e.g. SD versus SLALD) have the same spot %, the difference field will show 1.0; if the spot % from the mutant is twice as large as wild type, the difference field will display 2.0 indicating 2-fold upregulation. If the spot % from the mutant has a value half as large, the difference field will display -2.0 indicating a 2-fold downregulation. The montage panels cannot be individually contrasted and sometimes appear overly dark. However, image contrasting at any level does not affect the spot percentage data or any calculations.

Assay of R6G transport

We used a previously described assay (Mehla et al. 2014) to compare the ability of our strains to transport 10 μ M rhodamine 6G (R6G) in SD, SLALD and YPD media against a concentration gradient at 30°C for up to 17.5 h. Overnight cultures of either WT or G312A mutant cells were inoculated into either SD or SLALD medium. The low initial inocula ensured that the cells were in the logarithmic phase of growth throughout the experiment. Ninety milliseconds prior to the end of the incubation period, R6G was added to a final concentration of 10 μ M. After incubation, 3×10^6 cells were pelleted, the supernatant was removed, and the pellet was resuspended in 500 μ l of medium. Cells were placed on ice in 12 \times 75 mm Falcon polystyrene round-bottom tubes suitable for fluorescence activated cell sorting. The cells were analyzed with a FACSsort using an excitation wavelength of 529 nm and an emission wavelength of 553 nm. We analyzed the data with a CellQuest program. We expressed retained fluorescence in arbitrary units (a.u.).

Assays of drug resistance

To assess relative resistance to Pdr5 transport substrates quantitatively, yeast strains were inoculated in 2 ml YPD broth and grown at 30°C with shaking overnight. The absorption was measured using a spectrophotometer at ABS_{600} and the value was used to calculate the cell concentration. Cells (0.5×10^5) were added to 2 ml of SD or SLALD medium following the addition of a fixed concentration of the drug to be tested. Tubes were incubated for 48 h at 30°C with shaking. The % inhibition was calculated by determining the ratio of the absorption at 600 nm of culture grown at a particular drug concentration to the absorption of same strain grown in medium without drug. GraphPad Prism 6 software was used to plot the data points and generate the graph. The IC_{50} values were determined using a non-linear transformation (log [drug], variable slope, four parameters). The error bars represent the standard error of the mean.

RESULTS

Cell division in SLALD medium occurs more slowly than in SD medium

We examined the growth of the WT and phenotypically null G312A strains in SD and SLALD medium for up to 18 h in a series ($n = 3$) of independent experiments (Fig. 1A). The growth curves of the WT and G312A mutant strain were statistically indistinguishable in both media. With each strain, growth in SD medium was about 1.5 \times faster and the final cell concentration at 18 h was higher (1.5×10^7 cells/ml) than in SLALD medium

(0.8×10^7 cells/ml). Therefore, the lower concentration of nutrients was clearly growth limiting. These data also demonstrate that Pdr5 is not required for growth in either medium.

We also evaluated the budding indices of WT cells grown in the two media (Fig. 1B) at 5.5, 8.0 and 17.5 h (Fig. 1B). These results are remarkably consistent with the growth curve data. For instance, at 5.5 h when the SD and SLALD curves were only starting to diverge, the proportion of unbudded cells in SD was 45.5% (65/146) and in SLALD it is 55.5% (76/137). Although statistically significant ($P = 0.01$), the difference was modest. At 8 h, however, the proportion of unbudded cells was 16.9% (27/160) and 40.8% (141/346) in SD and SLALD media, respectively. In SLALD medium, the proportion of unbudded cells never dropped below 40%.

Similar levels of Pdr5 are found in whole cell extracts made from cells grown in SD and SLALD medium

We monitored the steady-state level of Pdr5 over a 17.5-h period in extracts made from cells grown in SD or SLALD medium (Fig. 2). Because YPD medium is also used frequently in studies with Pdr5, we also evaluated the steady-state level of the transporter in this medium. The Pdr5 antibody is highly specific. There is no signal in the *pdr5* Δ control (Fig. 2A), although the levels of the GAPDH loading control are the same in both strains. Although there is large reprogramming of protein synthesis during nutrient starvation, the level of Pdr5 was the same in extracts made from SD or SLALD grown cells at 5.5 and 8 h (Fig. 2B). A modest difference in the ratios was seen at 17.5 h when the SLALD and SD extracts were compared. In this sample, the Pdr5 level in the SLALD extract was 80% of that observed in the SD counterpart. The GAPDH level in SLALD was 87% that found with the SD sample. In a second set of 17.5 h lysates used to perform 2-D gel electrophoresis (see below), the reduction in SLALD was even smaller. Thus, relative to the 17.5 h SD lysate, the levels of Pdr5 and GAPDH in the SLALD extract were 93% and 92%.

Our attempts to make purified plasma membranes from SD-grown cells (rather than YPD-grown cultures) were repeatedly unsuccessful so a quantitative assessment of Pdr5 localization under these conditions was not possible. Data, below, however clearly demonstrated robust Pdr5 function suggesting that PM level of the transporter was not greatly altered when grown under nutrient stress. In any case, the results from the western blotting suggested that steady-state levels of Pdr5 were unaltered even though the levels of nitrogen and glucose were considerably reduced.

There are significant changes in the steady-state levels of many other proteins after cells are cultured in SLALD medium

To determine whether significant numbers of proteins were reduced when cells were cultured under nutrient limitation, we analyzed the steady-state level of proteins in lysates obtained from cells grown in either SD or SLALD medium by 2D gel electrophoresis (Fig. 3A). To evaluate reproducibility, a pair of gels from each lysate was compared. A qualitative comparison of the two gels suggests that many proteins show reduced levels in the extract made from SLALD-grown cells.

Quantitative analysis of 1489 distinct protein spots was performed. Potential differences between the same proteins from the two lysates were statistically analyzed using a t-test. A t-test value of $P = 0.05$ was obtained for 459 proteins. This

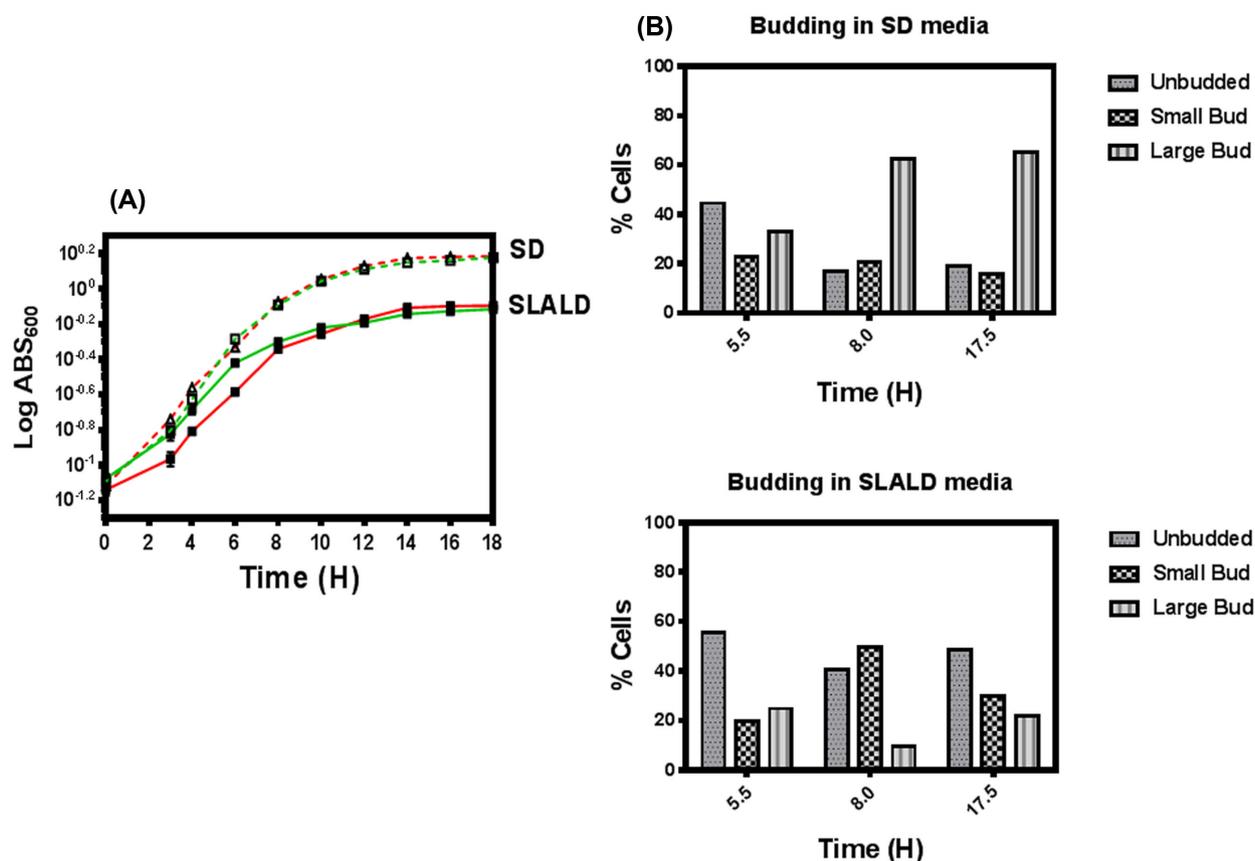


Figure 1. Growth of the WT strain JG2001 and the G312A mutant was slower in SLALD medium than in SD medium. (A) The strains were cultured overnight in YPD both prior to inoculation in either SD (broken line) or SLALD media (solid line). In this figure, the WT strain growth curve lines are colored green, and the G312A mutant lines red. Growth was determined from the spectroscopic absorption at 600 nm. The lines were constructed from the mean values ($n = 3$). (B) Budding indices were determined in SD and SLALD medium for the WT strain as described in Materials and Methods section. Between 100 and 200 cells were scored for each determination shown.

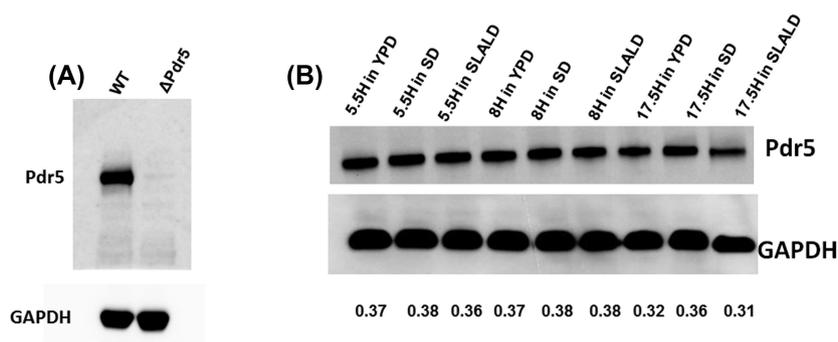


Figure 2. Steady-state levels of Pdr5 were unaltered in SLALD medium. YPD overnight cultures of the WT strain were used to inoculate 50 ml SD, SLALD and YPD cultures. Cell lysates were prepared and western blotting of 40 μ g of solubilized protein was carried out as described in the Materials and Methods section. The blots of Pdr5 and GAPDH protein which serves as a loading control are from the same lysates. (A) Lysates of WT and a Δ Pdr5 strains were prepared from SD medium and blotted as described in Materials and Methods section. (B) Lysates of the WT strain were prepared from 5.5, 8 and 17.5 h cultures and blotted as described in Materials and Methods section. The numbers in each lane represent the ratio of Pdr5/GAPDH as determined using the Image J software.

usually meant a difference of at least $2\times$ in spot density. Differences less than this were generally not statistically significant, but probably excluded many proteins from the analysis. Nevertheless, we observed that 186 proteins (0.405) showed a significant reduction in SLALD medium; the levels of 273 (0.599) were increased.

We assessed the level of Pdr5 in the extracts used for 2D electrophoresis by western blotting (Fig. 3B). The Pdr5 and GAPDH

levels in SLALD medium were 92% and 93% those observed in SD medium and therefore essentially unchanged.

Robust Pdr5-mediated R6G transport takes place in SLALD medium

We measured Pdr5-specific transport against a concentration gradient of 10 μ M R6G in the WT and catalytically dead G312A

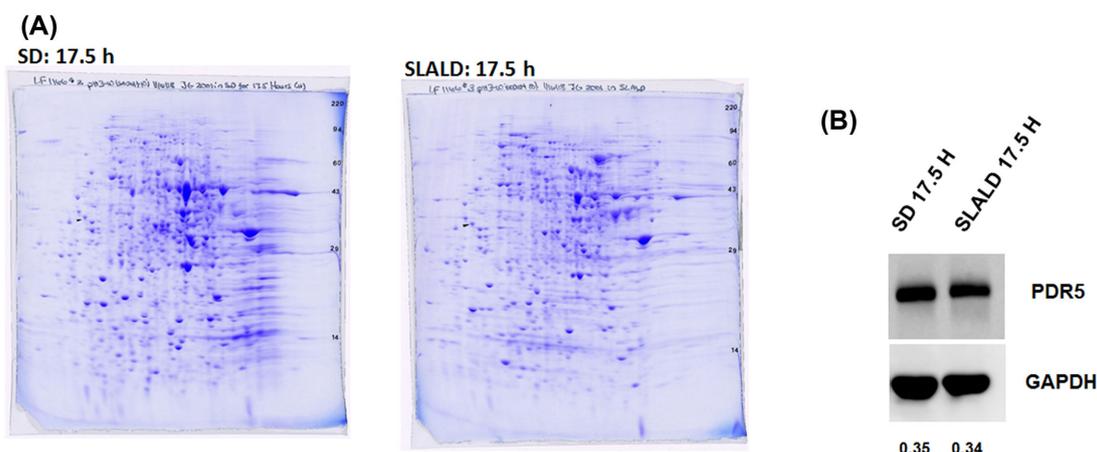


Figure 3. Analysis of proteins by two-dimensional gel electrophoresis indicated altered levels of many proteins after growth in SLALD medium. (A) Whole-cell lysates were prepared from WT cells grown for 17.5 h in either SD or SLALD medium. The protein concentrations were determined and 2 mg of proteins were solubilized in 5X SDS-PAGE before shipment on dry ice to Kendrick Laboratories for analysis as described in the Materials and Methods section. Statistical comparison was performed on duplicate sets of gels. One example from SD and SLALD medium are shown. (B) A western blot of Pdr5 and GAPDH was performed with 40 μ g of solubilized lysate protein as described in the Materials and Methods section from the same material used to perform two-dimensional gel electrophoresis.

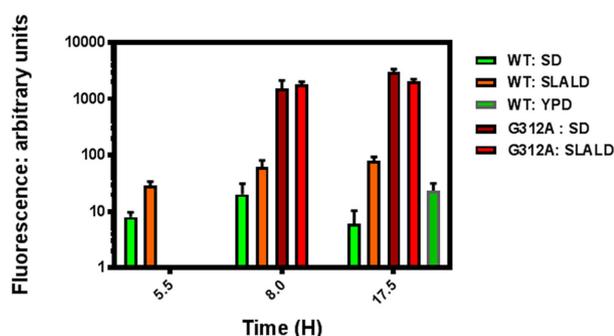


Figure 4. Vigorous R6G transport was maintained in SLALD medium. R6G efflux assays were performed with cells from both strain grown in SD or SLALD medium containing 10 μ M R6G as described in the Materials and Methods section ($n = 3$). Error bars represent the standard error of the mean.

strains. The WT strain exhibited robust transport in SD medium (Fig. 4), with a retained mean of only 6.1 ± 2.4 a.u. at 17.5 h fluorescence. In contrast, the G312A mutant accumulated nearly 400 \times as much R6G (3000 ± 200 a.u.) as the WT. In SLALD medium, the WT strain accumulated 81 ± 7.4 a.u. The results with SD and SLALD were also compared to the transport activity observed in 17.5 h YPD WT cultures which had a retained fluorescence of 24 ± 4.5 a.u. Unfortunately, most (90%) of the G312A cells died in YPD even at this relatively low concentration of R6G. Thus, a valid comparison between the two strains could not be made in this medium.

A modest loss of drug resistance in SLALD medium is not attributable to decreased Pdr5 activity

We also looked at the relative multidrug resistance of the WT and G312A mutant strains to Pdr5-specific transport substrates clotrimazole and cerulenin, which are antifungal agents, and tributyltin chloride, which is a significant environmental pollutant (Fig. 5). The three compounds are structurally distinct and define at least two distinct Pdr5 transport sites (Golin et al. 2003; Mehla et al. 2014). We evaluated clotrimazole resistance quantitatively in three media following incubation for 48 h at 30 $^{\circ}$ C (6): YPD, SD and SLALD media (Fig. 5A). The growth curves, fitted with GraphPad Prism software, had R-squared values ranging

from 0.74 (WT strain in SD medium plus tributyltin chloride) to 0.99 with a median value of 0.89. To a large extent, these results mirrored the transport studies. For instance, when clotrimazole was the test compound, the IC₅₀ of the WT strain in SD medium was ~ 62.5 μ M, or about three times as high as the concentration observed in SLALD (~ 22 μ M; Fig. 4A). Significantly, the G312A strain, which had no ATPase activity and thus no Pdr5-mediated transport capability, also showed about three times the resistance in SD (~ 0.50 μ M) as in SLALD (~ 0.15 μ M). This means that the relatively modest decrease in resistance and transport that the WT exhibited in the former medium must be attributable to a non-Pdr5-mediated mechanism, because both the WT and G312A strains showed a similar loss in resistance in the starvation cultures. In each case, the WT strain had an IC₅₀ that was >100 times the G312A mutant. Furthermore, strains grown in nutrient-rich YPD were more sensitive to clotrimazole than were strains grown in SLALD medium. The WT strain had an IC₅₀ of about 10 μ M in YPD. Thus, the resistance of the WT strain was six times greater in SD than in YPD.

We also observed robust resistance in the WT strain when we tested the two other transport substrates. With cerulenin (Fig. 5B), a much more polar substrate than clotrimazole, we found very little difference in the IC₅₀ values of the WT or G312A mutant strains between SD and SLALD. The results with tributyltin chloride (Fig. 5C) mirrored those with clotrimazole. Both strains had IC₅₀ values in SLALD medium that were three times lower than in SD medium.

DISCUSSION

It is well established that PDR5 is upregulated in response to damaged mitochondrial DNA, which leads to unbalanced lipid and amino acid biosynthesis (Moye-Rowley 2005). The results reported here present functional evidence that *S. cerevisiae* also maintains a vigorous multidrug efflux system during the stress of glucose and nitrogen limitation. Thus, when drug resistance to three Pdr5 transport substrates was evaluated in the WT and G312A mutant strains, the same differential in IC₅₀ values was seen in SD and SLALD media.

Pdr5 is the founding member of a large subfamily of ABC transporters that appear only in fungi and slime molds. These transporters have striking asymmetry including catalytic

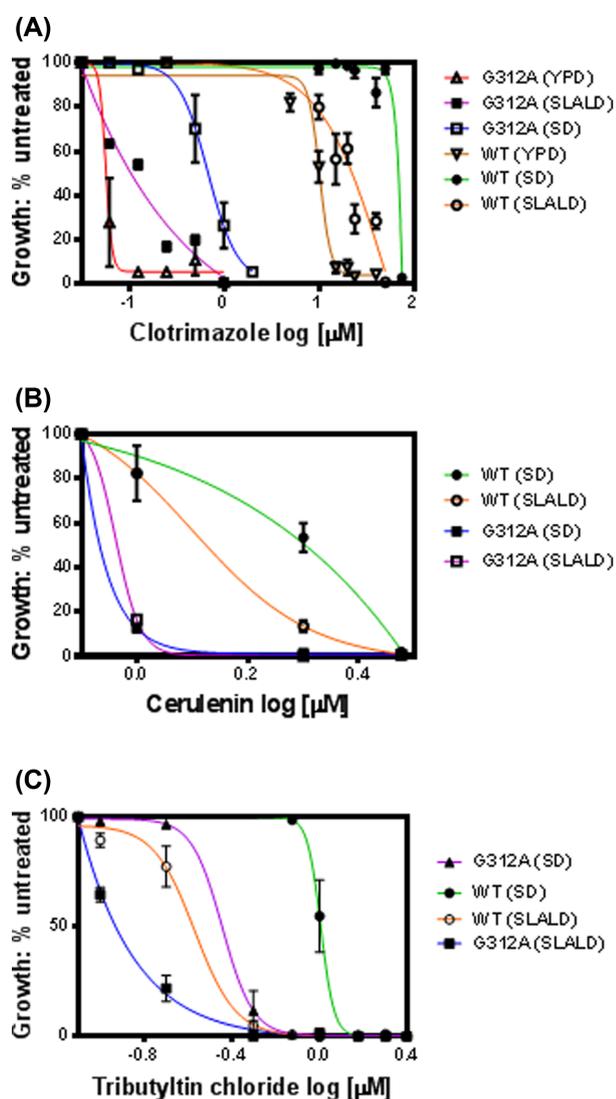


Figure 5. Pdr5-mediated resistance to transport substrates is maintained under conditions of reduced nitrogen and glucose. Growth in the presence of transport substrates was measured in liquid culture as described in the Materials and Methods section ($n = 4$) and the data were analyzed using GraphPad Prism software. Error bars represent the standard error of the mean. (A) Response of the WT strain and the G312A mutant strain to clotrimazole in YPD, SD and SLALD medium after 48 h incubation at 30° C. The resistance of both strains to cerulenin (B) and tributyltin chloride (C) was evaluated in SD and SLALD medium.

and non-catalytic ATP-binding sites (reviewed in Golin and Ambudkar 2015). Pdr5 has high amino acid identity with the *C. albicans* multidrug efflux pumps Cdr1 and Cdr2. A study by Rodaki *et al.* (2009) that looked at the transcriptional response of *C. albicans* to glucose is particularly instructive. This organism is highly sensitive to a range of glucose levels found in the human blood stream (0.01%–1.0%), but in a manner, that is the reverse of *S. cerevisiae*. *Candida albicans* stress genes were upregulated in response to increased glucose levels. These include the genes encoding the Cdr1, Cdr2 and Snq2 efflux pumps. When the authors did a comparative study of the analogous homologs in *S. cerevisiae*, they were already active at the lowest glucose concentration and there was very little change in their gene transcription (PDR5 was slightly upregulated at higher glucose concentrations). The *S. cerevisiae* transcriptional data

are thus in excellent agreement with our functional studies of Pdr5 and suggest that, in contrast to *C. albicans*, the broader environmental range of baker's yeast might require a broader window of drug pump activity. In contrast, when yeast cells are switched to medium that has a non-fermentable carbon source PDR5 transcription drops considerably (Mamnun, Schuller and Kuchler 2004). These conditions are different from the ones used in this study where small amounts of glucose remain. However, even under conditions where a non-fermentable carbon source is present, addition of Pdr5 transport substrates quickly restores mRNA production. In the present study, the resistance to clotrimazole and the transport of R6G was highly dependent on culture medium. For instance, the IC_{50} for clotrimazole with the WT strain ranged from 10 μM in YPD to 62 μM in SD medium. This observation underscores the need for quantitative assessment of drug resistance in multiple media especially in an organism that occupies a broad ecological niche. Once this is done, it might be better to think of IC_{50} values in terms of a range of concentrations rather than as a single value. Often, analysis is limited to qualitative spot tests on YPD plates with a single drug concentration.

The large difference in IC_{50} values observed with clotrimazole in YPD and SD broth cultures could be due to differential activity of genes in different media. Alternatively, this variation might reflect differential passive uptake or internal sequestration of some substrates. This may be a function of compound chemistry.

In summary, although two-dimensional gel electrophoresis demonstrated that the steady-state levels of many proteins are reduced during nitrogen and glucose limitation, the level of the Pdr5 efflux pump remained unchanged and continues to function vigorously. Thus, preservation of functional efflux activity may contribute to the survival of *S. cerevisiae* to the diverse environments they inhabit. It will be interesting if this is also the case for other organisms with diverse niches.

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Conflict of interest. None declared.

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