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Glucose-induced MDR pump resynthesis in respiring yeast cells depends on nutrient level

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Abstract

Glucose addition to a stationary culture of wild-type *Saccharomyces cerevisiae* BY4742 cells with zero activity of MDR pumps resuspended in a fresh medium causes pump resynthesis (measured as pump-effected diS-C_3(3) efflux). In a stationary culture in its original growth medium, this glucose-induced pump resynthesis fails to occur due to depletion of essential nutrients or to extracellular metabolites produced by cells during growth. Direct pump inactivation by metabolites is excluded since exponential cells with high MDR pump activity cultured in a medium with high concentration of extracellular metabolites retain this activity for at least 2 h. The metabolites also do not affect pump synthesis on the level of gene expression as addition of concentrated growth medium or an amino acid mixture to stationary cells in spent growth medium restores glucose-induced pump synthesis. The block of MDR pump synthesis is therefore due to the lack of essential nutrients in spent medium.

Keywords: MDR pumps; Yeast; Fluorimetric assay; Growth medium composition

MDR transporters are important membrane proteins involved, among other things, in protecting cells against chemical stressors. In *Saccharomyces cerevisiae*, the best-known MDR transporters are the ATP-driven Pdr5p and Snq2p pumps, major components of the pleiotropic drug resistance (PDR) system [1–4]. Their operation can be readily monitored in situ, i.e., in intact cells and in real time, by the method based on monitoring, via changes in its spectroscopic properties, the uptake and efflux of the fluorescent potentiometric probe diS-C_3(3) [5] which is actively extruded from the cells by these pumps [6].

The activity of these pumps, especially in wild-type cells, has been shown to depend strongly on the metabolic state of the cells [6,7]; this finding is in accordance with the data obtained by other methods [8–10]. Wild-type cells in respiratory metabolic mode (post-diauxic glucose-grown cells or cells grown on glycerol or ethanol), which have virtually zero MDR pump activity, were found to regain this activity following an addition of glucose or fructose [6,7]. This pump reactivation, however, failed to occur in high-density cultures in original growth medium. A detailed study showed that this absence of glucose-induced pump reactivation in these cultures is not due to high cell concentration per se but to the composition of the spent culture medium [7]. Yet, even in those cultures in which the pump-restoring effect of sugar addition was missing, it could be re-established if the cells were resuspended in a fresh culture medium; this pump reactivation did not include the effect of freshly grown highly pump-endowed exponential cells [7].

The following questions remained to be answered: (1) Can interaction of Pdr5p and Snq2p pumps with extracellular metabolites present in post-diauxic and stationary yeast cultures lead to pump inhibition or can these metabolites inhibit pump synthesis? (2) What is the role of nutrient depletion in such spent media in the lack of sugar-induced resynthesis of PDR pumps?

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Materials and methods

Yeast strains. The *S. cerevisiae* strain BY 4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, and ura3Δ0) used in this study was from the EUROSCARF collection. *S. cerevisiae* wild-type strain IL 125-2B (MATα, his1) [11] and pump deficient mutant strain AD1-3 (Matα, PDR 1-3, ura3, his1, yor1Δ::hisG, snq2Δ::hisG, pdr5Δ::hisG) [12] were kindly provided by Prof. A. Goffeau and Prof. M. Ghislain, Faculté des sciences agronomiques, Université catholique de Louvain, Louvain-la-Neuve, Belgium.

Chemicals. DiS-$C_3(3)$ (3,3′-dipropylthiacarboxycyanine) was purchased from Molecular Probes, glucose, peptone, and amino acids from Sigma, yeast extract from Fluka, and citric acid and $Na_2HPO_4 \cdot 12H_2O$ (reagent grade) from Lachema.

Cell growth and staining conditions. Yeast was cultured in media containing 1% yeast extract, 1% bactopeptone, and 2% glucose. A preculture in YPD was performed for 15–20 h as described previously [13]. Cell culture densities were characterized by optical density at 578 nm of appropriately diluted cell suspension. The main culture was grown in the same fresh medium at 30 °C, starting with 2.5×10^6 cells/ml ($OD_{578} = 0.1$ on NovaSpec II absorption spectrometer), for different periods of time to reach the different growth phases.

Acquisition of spent medium. The spent medium was prepared by a sterile removal of BY4742 cells cultivated in YPD for a long time (90 h) to achieve high medium concentration of metabolites.

Concentrated YP medium and a mixture of amino acids. The composition and total amount of added concentrated nutrients were equivalent to the composition of the fresh standard YP medium (1 ml of concentrated medium containing 20% yeast extract and 20% bactopeptone was added to 20 ml of cell culture). The stock solution of amino acids contained histidine, methionine, lysine, leucine, and tryptophan (2 mg/1 ml of each); 1 ml of the mixture was to added to 20 ml of cell culture.

diS-$C_3(3)$ fluorescence assay. The cells were harvested, washed twice with double-distilled water, and resuspended in 10 mM citrate–phosphate (CP) buffer of pH 6.0 to $OD_{578} = 0.1$. DiS-$C_3(3)$ (10^{-4} M stock solution in ethanol) was added to 3 ml of yeast cell suspension to final dye concentrations of 2×10^{-7} M. The samples were kept at room temperature and occasionally gently stirred. Fluorescence emission spectra were measured on a FluoroMax 2 (Jobin-Yvon, SPEX) spectrofluorimeter equipped with a xenon lamp. Excitation wavelength was 531 nm, emission range 560–590 nm, duration of one spectral scan 20 s, and scattered light was eliminated by orange glass filter with a cut-off wavelength at 540 nm.

The *staining curves*, i.e., the dependence of the wavelength of emission maximum $\lambda_{max}(t)$ on the duration of staining, are characterized by the fit parameter λ^{eq}_{max} [7], which satisfactorily represents the state of equilibrium between passive probe uptake and its MDR-pump-mediated efflux.

Results and discussion

Effect of extracellular metabolites on the activity of MDR pumps

The diS-$C_3(3)$ fluorescence assay characterizes MDR pumps solely in terms of activity. Assuming an equal value of membrane potential in pump-competent and pump-free cells in the same growth phase [6,7], strong staining (i.e., high value of λ^{eq}_{max}) of pump-competent cells by the probe, equal to the staining of pump-free mutants, indicates a zero activity of the pumps while a decrease in staining (λ^{eq}_{max} drop) reflects increased pump activity.

The absence of λ^{eq}_{max} drop, i.e., of a pump-effected extrusion of diS-$C_3(3)$, after glucose addition to a *S. cerevisiae* culture grown to a high-density (or even when these cells are resuspended to a low density in the original medium [7]) can therefore result, e.g., from irreversible inactivation of existing pump molecules by extracellular metabolites present in the medium.

As shown previously [5], the inhibition of MDR pumps Pdr5p and Snq2p is readily visualized by the diS-$C_3(3)$ fluorescence assay. The inhibition causes a fast and marked change in cell staining—due to the blockage of pump-effected probe efflux, the originally low staining of pump-competent cells changes to intensive staining and a consequent red shift of λ^{eq}_{max} to a value given solely by membrane potential.

To assess possible direct pump inactivation by components of the "spent" medium, we used exponential BY4742 cells (10-h cultivation, OD = 2.9) with a high pump activity (first point in the curve in Fig. 1). These cells were resuspended to the original OD into the same volume of the spent medium (see Materials and methods) without and with 1% glucose. After resuspending, the cells were cultivated for another 10 h and OD changes due to cell growth and changes in their diS-$C_3(3)$ staining (λ^{eq}_{max}) were determined throughout this interval (Fig. 1).

It was important to know if the transfer of exponential cells into the spent medium and their further cultivation does not cause a drop in membrane potential, which would lead to passive probe outflow and drop in cell staining that could erroneously be interpreted as preservation of pump activity. Like in the preceding studies, we used for the purpose the strain *S. cerevisiae* AD1-3 whose staining by diS-$C_3(3)$ is given solely by membrane potential due to the deletion of genes coding for Pdr5p and Snq2p pumps [6,7] (Fig. 1).

The data in Fig. 1 show that, for a minimum of 2 h of cultivation in the spent medium, strain BY4742 retains the low level of staining; strain AD1-3 displays only a slight decrease in cell staining during the cultivation, which indicates only a marginal change in membrane potential due to starvation. Throughout the cultivation interval, the optical density of both strains in the absence of glucose did not change at all, with 1% glucose as carbon source it increased by about 10%.

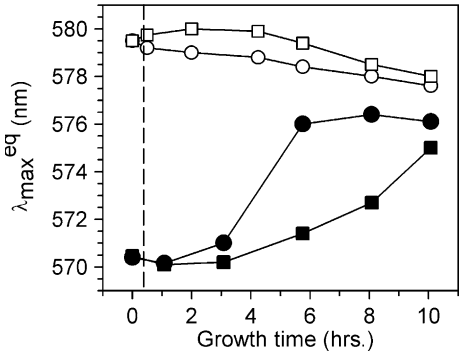

Fig. 1. Time course of the staining level (λ^{eq}_{max}) during the cultivation of exponential BY4742 (full symbols) and AD1-3 (empty symbols) cells in YPD after resuspension in original spent "stationary" medium (●) (OD_{BY} *3 → 3.09*, OD_{AD} *1.1 → 1.2*) and in spent "stationary" medium with added glucose (1%) (■) (OD_{BY} *3 → 3.4*, OD_{AD} *1.1 → 1.45*). Dashed line indicates the transfer of cells into spent medium.

In view of the minute change in membrane potential during the cultivation of AD1-3 cells in growth-depleted medium, i.e., lack of any dramatic drop in $\Delta\Psi$ caused by starvation, and the unchanged culture density, i.e., negligible growth of new pump-competent cells, the low staining of BY4742 cells persisting for 2 h can be ascribed to an active removal of the probe from the cells. Hence, the pump activity of exponential BY4742 cells resuspended in spent medium is retained. This excludes a direct inhibitory effect of extracellular metabolites present in the spent medium on pump activity.

The increase in the staining of BY4742 cells after 3 h of cultivation indicating inhibition of the transport activity of the pumps is apparently associated with depletion of cellular ATP level since the presence of glucose in the medium delays this drop in staining by about 2 h although it brings about only negligible cell growth (approximately 10% increase in optical density).

Although the extracellular metabolites in spent medium clearly do not directly inhibit the activity of MDR pumps, we cannot exclude their inhibitory effect on the level of protein synthesis because the persisting pump activity may be due either to the originally present pumps or, alternatively, to newly synthesized ones while the original pumps are being degraded.

Effect of extracellular metabolites on the synthesis of MDR pumps

Previous study [7] showed that the transfer and resuspension to lower culture density of long-cultured cells into a fresh complex YPD medium bring about an increase in the activity of Pdr5p and Snq2p pumps caused by their *de novo* synthesis. To assess the possible effect of extracellular metabolites in spent medium on the expression of appropriate genes and protein synthesis, we monitored changes in the diS-C_3(3) staining of BY4742 cells (analogous data for IL125-2B are not shown) cultivated for a long period in YPD medium, which was subsequently supplied with concentrated YP medium and with glucose to reach nutrient and carbon source concentrations equal to those in the fresh medium, or with a mixture of amino acids (Fig. 2). Controls were cells whose culture medium was supplied with glucose only, with concentrated YP medium alone, or with a mixture of amino acids alone, and also cells that were transferred into a fresh medium to a density equal to that of the stationary culture. The last of these controls was used to see whether resuspension of cells in a fresh medium to a high-density brings about a similar drop in staining observed previously [7] on resuspending them to a lower density.

As expected (see [7]), no changes in staining occurred on adding only glucose, only concentrated YP medium without glucose, or only the amino acid mixture. In contrast, shortly after transferring the cells into a fresh medium with glucose, the cells exhibited an increase in staining followed by a conspicuous λ^{eq}_{max} drop indicating a rise in the activity of the pumps; this also occurred after addition of concentrated YP medium and glucose. Interestingly, when the cells were supplied with the mixture of amino acids plus glucose, the transient increase in staining was not observed. The 30-min interval at which the lack of increase in staining on addition of amino acids plus glucose was observed is hardly sufficient for pump synthesis. The effect is therefore very likely to be due to a lower value of membrane potential caused by, e.g., H^+-ATPase activation with simultaneous uptake of the amino acids by proton symport.

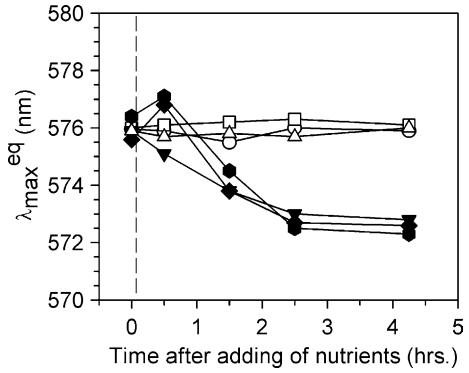

Fig. 2. Time course of the staining level (λ^{eq}_{max}) after adding nutrients to BY4742 cells subjected to a long-term cultivation in YPD. Addition of glucose (empty circles), concentrated YP medium (empty squares), glucose with concentrated YP medium (full diamonds), mixture of amino acids (empty triangles), glucose with a mixture of amino acids (full inverted triangles), and resuspension in a fresh medium with glucose (full hexagons). Dashed line indicates nutrient and/or glucose addition into spent medium.

This assumption was confirmed in an experiment with the pump-free strain AD1-3, in which the staining reflects solely membrane potential. As seen in Fig. 3, addition of glucose plus concentrated YP medium to the cells, or their resuspension in a fresh YPD medium, caused an increase in staining while addition of amino acids plus glucose did not bring about this increase in staining.

It should be noted that the optical density of cultures of strains BY4742 and AD1-3 does not change in any of these cases (Figs. 2 and 3), except when both glucose and

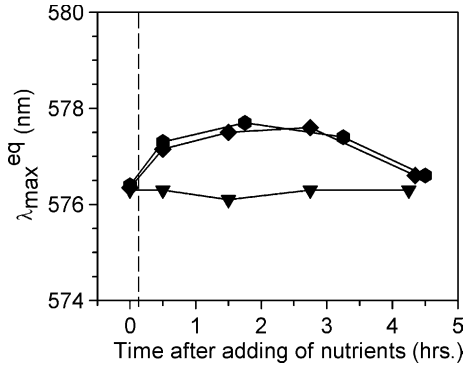

Fig. 3. Time course of the staining level (λ^{eq}_{max}) after adding nutrients to AD1-3 cells subjected to a long-term cultivation in YPD. Addition of glucose with concentrated YP medium (full diamonds), glucose with a mixture of amino acids (full inverted triangles), and resuspension in a fresh medium with glucose (full hexagons). Dashed line indicates nutrient and/or glucose addition into spent medium.

nutrients are added; then it mildly increases by less than 10% and the number of cells in the culture thus remains practically constant. Since the drop in the staining of BY4742 cells in spent medium supplied with concentrated nutrients (either YP medium or an amino acid mixture) is analogous to that observed in cells transferred into fresh medium containing no growth-accumulated metabolites, which was shown to be associated with the resynthesis of the pumps [7], we can conclude that even in this case it can be attributed to the resynthesis of pump molecules in existing cells. In other words—the presence of extracellular metabolites has no inhibitory effect on the synthesis of MDR pumps, whether on the level of expression of appropriate genes or on the level of direct inhibition, and the lack of pump resynthesis by glucose is the result of depletion of nutrients, in particular amino acids, from the culture medium. The negative impact of nitrogen starvation on pump synthesis has been noted by Mamnun et al. [10].

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