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Impact of the growth phase on the activity of multidrug resistance pumps and membrane potential of *S. cerevisiae*: effect of pump overproduction and carbon source

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Abstract

The potentiometric fluorescence probe diS-C_3(3) is expelled from *S. cerevisiae* by ABC pumps Pdr5 and Snq2 and can conveniently be used for studying their performance. The activity of these pumps in a strain with wild-type *PDR1* allele was shown to drop sharply on glucose depletion from the medium and then again at the end of the diauxic shift when the cells are adapted to growth on respiratory substrates. The presence of the *PDR1-3* allele causing pump overproduction prevented this second drop and the pump activity typical for diauxic cells was largely retained. Growth phase-dependent changes of membrane potential measured by the same probe in pump-free mutants included a $\Delta\psi$ drop in the late exponential and diauxic growth phase, indicating lowered activity of H^+-ATPase. Suppression of activity of both ABC pumps and H^+-ATPase obviously signifies cell transition to an energy-saving mode. Challenging respiration-adapted cells with glucose showed a novel feature of yeast ABC pumps—a strong dependence of pump activity on the type of the carbon source.

Keywords: Growth phases; *S. cerevisiae*; Membrane potential; Activity of MDR-pump; Fluorescence probe diS-C3(3)

1. Introduction

Multidrug resistance (MDR) transporters of the yeast *S. cerevisiae* are responsible for the resistance of the cells against a broad spectrum of structurally and functionally different xenobiotics [1].

The structure, substrate specificity and regulation of gene expression of the MDR pumps have been studied in considerable detail [2–5]. In contrast, practically no data exist on the impact of the yeast growth phase on the actual activity of the pumps. This has been mostly due to the lack of a reliable and sensitive method that could monitor changes in MDR pump activity accompanying changes in the physiological state of the cells. The methods commonly used for assessing MDR-related cell resistance to drugs and quantifying the substrate specificity of the pumps, the disc diffusion assay [6], and the plating test, rely on cell survival or death as the parameter reporting on pump action. Due to this fact, their applicability for assessing growth-phase-related changes in pump action is relatively low.

The fluorescence method described in our previous paper [7] can be used to measure changes in the activity of MDR pumps at any given time point and assign them to changes in the momentary physiological state of the cells. Importantly, vital functions of the cells are not altered by the dye since the measurements are performed with very low dye concentration (~10^{-8} M) and no major changes in gene expression are expected to occur during the measuring time (30 min).

This method was originally developed for real-time monitoring of yeast membrane potential by the cationic

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redistribution dye diS-C_3(3), whose intracellular/extracellular concentration ratio in equilibrium is directly proportional to the value of the membrane potential and can be estimated directly from simple fluorescence characteristics such as the position of the spectral maximum λ_{max} [8–10]. Staining curves, i.e. the time evolution of the λ_{max} after addition of the probe to the cell suspension, showed that the membrane potential can be determined not only from the equilibrium probe concentration, which is typically reached within 20–30 min, but also from the initial rate of the probe uptake [9]. This approach has been successfully used to monitor changes in yeast membrane potential induced by various stress factors [10–14].

The fluorescence response of diS-C_3(3) to membrane potential is critically affected by the action of MDR pumps because the charged diS-C_3(3) entering the cells of *S. cerevisiae* according to the membrane potential is expelled from cells possessing the ABC-type MDR transporters Pdr5, Snq2 and Yor1 [7]. This probe extrusion results in lowering of diS-C_3(3) concentration in cells with active MDR pumps relative to pump-free mutants. The pump action can be estimated by a simple subtraction of the staining curves for the two cell types [7].

Our study was performed to determine if and how the growth phase affects pump-effected probe extrusion from cells growing in YEPG medium. Another objective was to find out to what extent each of the three ABC-type MDR pumps (Pdr5p, Snq2p and Yor1p) is responsible for the extrusion of the probe from the cells, and how the presence of the *PDR1-3* allele leading to overproduction of MDR pumps affects the probe-extrusion efficiency.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae US 50–18C (*MATα*, *PDR1-3*, *ura3*, *his1*) and its mutants AD1-3 (*Matα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *snq2Δ::hisG*, *pdr5Δ::hisG*), AD1-8 (*Matα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *snq2Δ::hisG*, *pdr5Δ::hisG*, *pdr10Δ::hisG*, *pdr11Δ::hisG*, *ycf1Δ::hisG*, *pdr3Δ::hisG*, *pdr15Δ::hisG*;), AD12 (*Matα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *snq2Δ::hisG*), AD13 (*Matα*, *PDR1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *pdr5Δ::hisG*) and AD23 (*Matα*, *PDR1-3*, *ura3*, *his1*, *snq2Δ::hisG*, *pdr5Δ::hisG*) [15] and the wild-type strain IL 125-2B (*MATα*, *his1*) [16].

2.2. Cell growth and staining conditions

Yeast was pre-cultured in YEPG medium (1% yeast extract, 1% bactopeptone, 2% glucose) at 30 °C for 24 h. A volume of 150 μl inoculum was added to 20 ml fresh YEPG medium and the main culture was grown for different periods of time to reach the different growth phases. The cells were harvested, washed twice with double-distilled water and resuspended in citrate phosphate (CP) buffer of pH 6.0 to OD_{578}=0.1.

2.3. Probe uptake into cells

Cells (3 ml in fluorimetric cuvettes) were labeled by diS-C_3(3) (10^{-5} M stock solution in ethanol) under gentle stirring at room temperature to a final dye concentration of 10^{-8} M.

2.4. Fluorescence measurements

Fluorescence spectra were measured on FluoroMax 2 (Jobin-Yvon, SPEX) spectrofluorimeter equipped with a xenon lamp. Excitation wavelength was 531 nm, fluorescence range 560–590 nm, duration of one spectral scan 20 s, scattered light was eliminated by orange glass filter with a cutoff wavelength at 540 nm.

2.5. Determination of glucose concentration in growth medium

Glucose concentration was determined by the Gluco-PHAN assay strips (Pliva-Lachema) containing glucose oxidase, peroxidase and tetramethylbenzidine.

2.6. Chemicals

DiS-C_3(3) (3,3′ -dipropylthiacarboxycyanine) was purchased from Molecular Probes, glucose and yeast extract from Sigma, citric acid and $Na_2HPO_4 \cdot 12\ H_2O$ (reagent grade) from Lachema, Czech Republic, and bactopeptone from Difco.

3. Results and discussion

3.1. Effect of MDR pumps on the growth of S. cerevisiae

The strains had the following characteristics:

Strain	Genotype	Consequences
US50-18C	*MATα*, *PDR 1-3*, *ura3*, *his1*	overproduction of Pdr5, Snq2 and Yor1 pumps
AD12	*Matα*, *PDR 1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *snq2Δ::hisG*	overproduction of Pdr5p
AD13	*Matα*, *PDR 1-3*, *ura3*, *his1*, *yor1Δ::hisG*, *pdr5Δ::hisG*	overproduction of Snq2p
AD23	*Matα*, *PDR 1-3*, *ura3*, *his1*, *snq2Δ::hisG*, *pdr5Δ::hisG*	overproduction of Yor1p

Strain	Genotype	Consequences
AD1-3	*Matα*, *PDR 1-3*, *ura3*, *his1*, *yor1Δ*::*hisG*, *snq2Δ*::*hisG*, *pdr5Δ*::*hisG*	deletant lacking Pdr5, Snq2 and Yor1 pumps
AD1-8	*Matα*, *PDR 1-3*, *ura3*, *his1*, *yor1Δ*::*hisG*, *snq2Δ*::*hisG*, *pdr5Δ*::*hisG*, *pdr10Δ*::*hisG*, *pdr11Δ*::*hisG*, *ycf1Δ*::*hisG*, *pdr3Δ*::*hisG*, *pdr15Δ*::*hisG*	deletant lacking Pdr5, Snq2 and Yor1 and other pumps
IL125-2B	*MATα*, *his1*	wild-type strain with normal production of the three proteins

When grown under identical conditions, individual strains reach different optical densities and need different times to complete the transition to the diauxic growth phase [17,18] (Fig. 1A). Strains AD1-8 and AD1-3, as well as AD23 with functional Yor1p, reach the diauxic phase (dashed lines in Fig. 1A) later than strains US50-18C, AD12, AD13 and IL125-2B having functional genes for Pdr5p and/or Snq2p. We suppose that the faster transition to the diauxic phase in cells expressing Pdr5p and/or Snq2p is caused by a faster depletion of glucose from the growth medium; this may be associated with a higher consumption of ATP by these pumps.

We used the following procedure to make all growth curves comparable. (1) Critical points—inoculation, beginning of the exponential phase, onset of the late exponential phase, end of this phase/transition to the diauxic lag, end of the diauxic lag/start of the post-diauxic phase—were determined for AD1-3 as a reference strain. The moment of inoculation (0 h) was denoted phase point 1, all other points were denoted (time (h)+1). (2) In all other strains, the distances between these critical points were made equidistant with those of the AD1-3 strain irrespective of their actual duration. This gave normalized growth curves, which were comparable for all strains and in which the cultivation time was replaced by phase points. This procedure provided a common platform on which the growth of all strains could be compared (Fig. 1B).

3.2. Growth phase-dependent probe export by the pumps in US50-18C cells

To compare the probe extrusion from the cells in individual growth phases, we measured staining curves of all the above yeast strains at a number of points during these growth phases.

As shown previously, the concentration of the probe in cells of the pump-free AD1-3 and AD1-8 strains is not affected by the pump activity, and the staining curves therefore reflect solely their membrane potential [7]. These strains can therefore be used as negative controls for assessing the action of MDR pumps.

The staining curves of these pump-free strains were found to change markedly depending on the growth phase (Fig. 2A). At phase point 16.5 (beginning of the late exponential phase) the staining is considerably slower and the final λ_{max} value is lower than at points 11 and 29 (middle of the exponential phase and advanced post-diauxic phase). We chose the λ_{max} value attained after 21.5 min of staining as the parameter which characterizes the level of cell staining in all growth phases (dashed line in Fig. 2A), and denoted it $\lambda_{max/20}$. The values of this parameter given in Fig. 2B show that late exponential and diauxic cells stain less than exponential and post-diauxic cells. Thus, the lower staining of these cells indicates their lower membrane potential [7,10]. In yeasts, membrane potential is generated predominantly by the activity of the plasma membrane H^+-ATPase. Our results therefore suggest that the activity of the H^+-ATPase in these two phases is lowered—see Nso et al. [19].

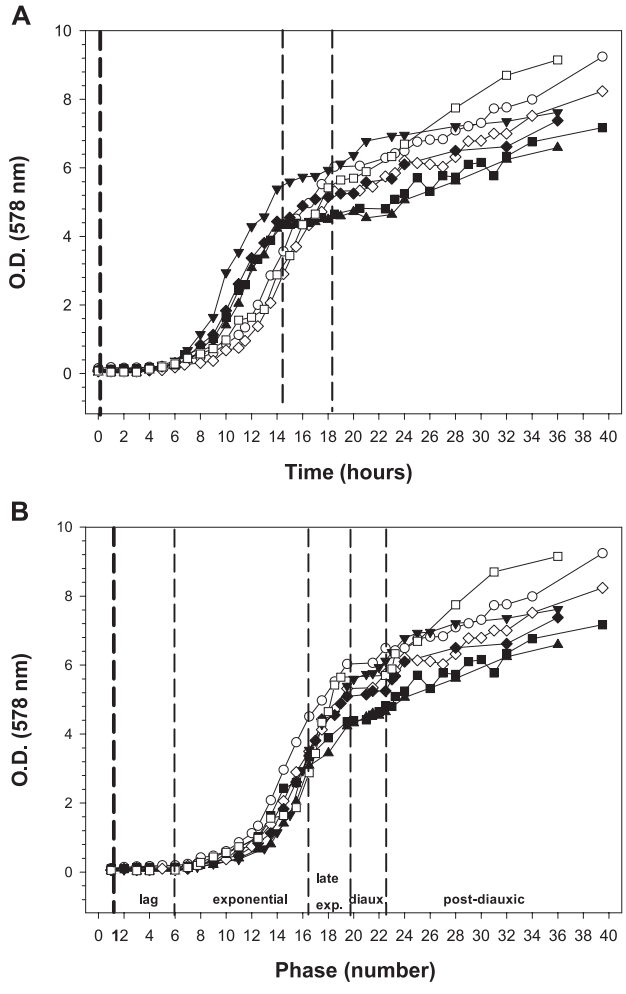

Fig. 1. Effect of pump equipment of the strains on cell growth. Growth curves (A) and normalized growth curves (B) of *S. cerevisiae* AD1-3 (○), AD1-8 (◇), US50-18C (■), IL125-2B (▼), AD12 (▲), AD13 (◆) and AD23 (□). Thick dashed line in A—growth time zero; in B—phase 1 of the normalized growth curves.

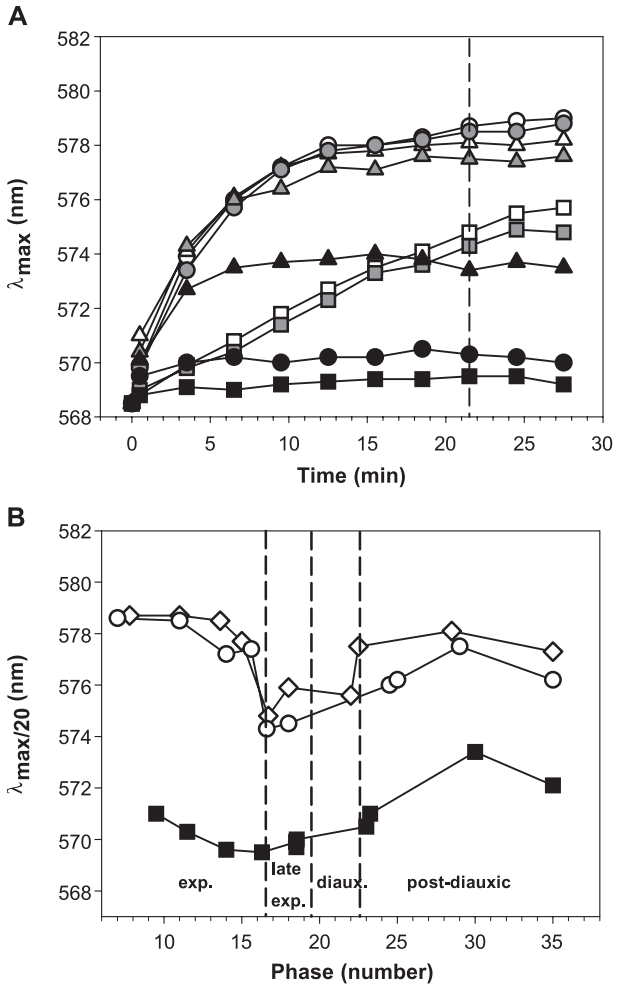

Fig. 2. Effect of growth phase on diS-C_3(3) staining of pump-free and pump-overproducing strains. (A) DiS-C_3(3) staining curves of AD1-3 (grey symbols), AD1-8 (empty symbols) and US50-18C (black symbols) cells at three different phase points: point 11 (○), point 16.5 (□) and point 29 (△). Dashed line indicates $\lambda_{max/20}$—the parameter used for characterizing the cell staining level. (B) Dependence of cell staining levels ($\lambda_{max/20}$ values) on the growth phase for AD1-3 (○), AD1-8 (◇) and US50-18C (■) cells.

US50-18C cells (Fig. 2A) overproducing the Pdr5, Snq2 and Yor1 pumps, in which the staining reflects not only the membrane potential but also the pumping activity [7], also stain differently in different growth phases. Exponential and late exponential cells are not stained at all. In contrast, the probe uptake by post-diauxic cells during the first minutes of staining is comparable to that in pump-free mutants in the same growth phase; after this period, the staining curve levels off far below the curves for the pump-free cells. The comparable initial uptake rate indicates that the membrane potential of both pump-free and pump-overproducing cells is about the same, i.e. its magnitude is not affected by the presence of the pumps. This can be presumed to hold in all other growth phases as well. Then any differences in staining between pump-free and pump-competent cells must be due to the action of the pumps. The parameter $\Delta\lambda_{max/20}$, obtained by a simple subtraction of the $\lambda_{max/20}$ value at given growth phase points of the US50-18C from the corresponding values for AD1-3 cells in Fig. 2B, yields information on the activity of pumps in US50-18C cells in all phases of growth (Fig. 3).

Fig. 3 shows that the pumping activity is high throughout the exponential phase until the transition to the late exponential phase, when the pumping activity drops to a lower level. This then remains practically constant during all subsequent growth phases.

3.3. Which of the Pdr5, Snq2, and Yor1 pumps can be studied with diS-C_3(3)?

To determine whether all three ABC pumps (Pdr5p, Snq2p and Yor1p) are responsible for probe extrusion, we measured the staining curves of AD12 (having only Pdr5p), AD13 (having only Snq2p) and AD23 (having only Yor1p) strains in individual growth phases and determined the levels of staining ($\lambda_{max/20}$ values).

As shown in Fig. 4A, the probe concentration (staining level) in pre-diauxic and diauxic AD12 cells is very similar to that found in the US50-18C strain (dotted curves taken from Fig. 2B). The same holds for AD13 cells, which, however, already start staining better during the transition to the diauxic growth phase. A significantly different staining can be observed with strain AD23, whose $\lambda_{max/20}$ profile copies the staining of the pump-free mutants.

To assess the growth phase-dependence of the pump activity of AD12, AD13 and AD23 strains overproducing only a single pump, we subtracted the $\lambda_{max/20}$ values for individual strains from corresponding $\lambda_{max/20}$ values of the pump-free strain AD1-3 used as negative control. The values of the resulting parameter $\Delta\lambda_{max/20}$, which reports on the activity of individual pumps in Fig. 4B, indicate that exponential AD12 cells maintain a high activity of the Pdr5

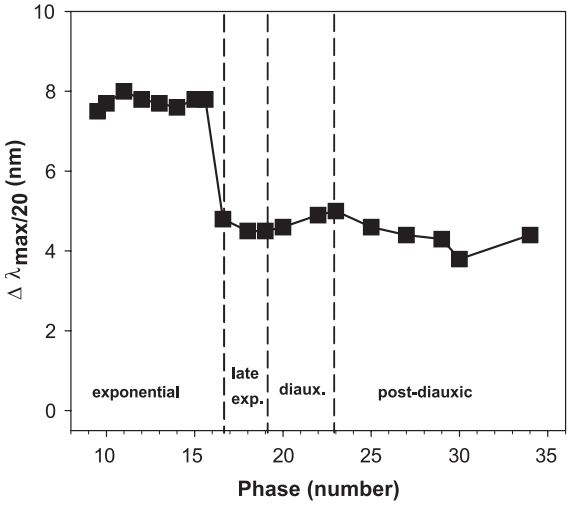

Fig. 3. Effect of growth phase on pump activity of pump-overproducing US50-18C cells. The measure of pump activity, $\Delta\lambda_{max/20}$, was obtained by subtracting the $\lambda_{max/20}$ value for US50-18C from the corresponding $\lambda_{max/20}$ value of the better-staining pump-free strain AD1-3.

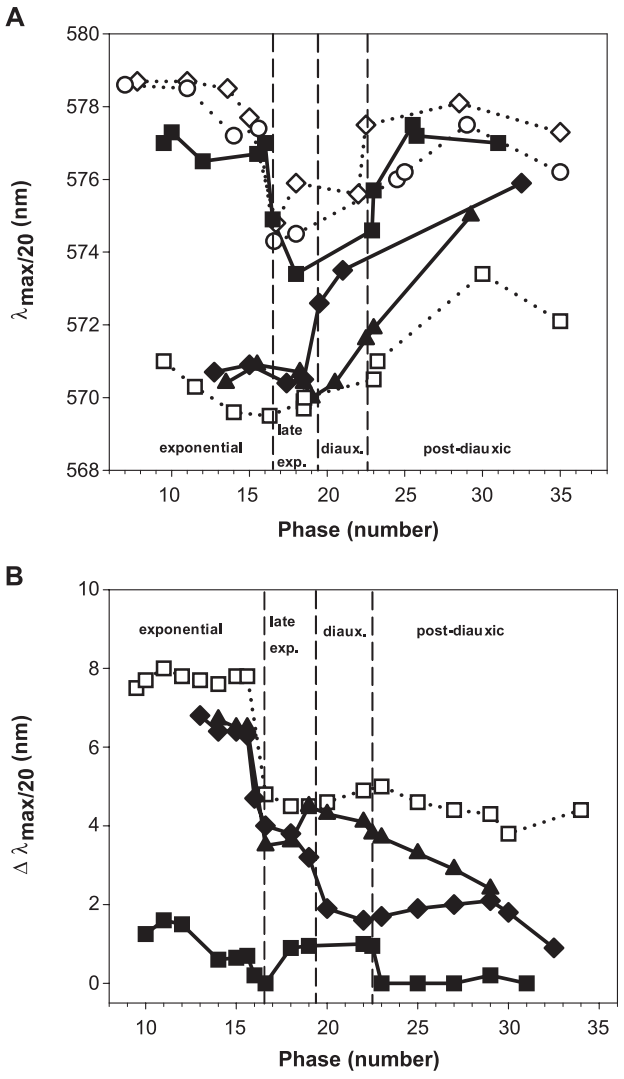

Fig. 4. The growth phase-dependence of the staining level (A) and pump activity (B) of AD12, AD13 and AD23 strains overproducing only a single pump. (A) The level of staining ($\lambda_{max/20}$ value) of AD12 (▲), AD13 (♦) and AD23 (■) cells; values for reference strains AD1-3 (○, dotted), AD1-8 (◇, dotted), US50-18C (□, dotted) taken from Fig. 2B. (B) Activity of individual pumps ($\Delta\lambda_{max/20}$ values) for the strains AD12 (Pdr5; ▲), AD13 (Snq2; ♦) and AD23 (Yor1; ■). The $\Delta\lambda_{max/20}$ values were obtained by subtracting the $\lambda_{max/20}$ values for individual strains from corresponding $\lambda_{max/20}$ values of the pump-free strain AD1-3 used as negative control. The $\Delta\lambda_{max/20}$ curve for US50-18C (□, dotted) from Fig. 3 is shown for comparison.

pump. On transition to the late exponential phase, the activity of this pump drops to a lower level, which remains fairly constant until the transition to the post-diauxic phase and then it slowly decreases. The activity of the Snq2 pump in exponential and late exponential AD13 cells equals that of the Pdr5p in AD12 cells but it drops again during the transition to the diauxic phase and the resulting low activity then remains constant. The activity of the Yor1 pump in the AD23 cells is very low throughout, and completely vanishes in the post-diauxic phase. The contribution of this pump to the extrusion of our probe from the cells is therefore negligible.

These results indicate that of the three pumps overproduced in US50-18C cells, only Pdr5p and Snq2p are responsible for probe extrusion and our method thus cannot be used for monitoring the activity of Yor1p.

3.4. Effect of the PDR1-3 allele on the activity of pumps in individual growth phases

To assess the effect of pump overproduction caused by the *PDR1-3* allele on probe export, we compared the level of staining of strain US50-18C with that of strain IL125-2B having the wild-type *PDR1* allele, and strain AD1-3 used as a negative pump-free control. The staining (given by the $\lambda_{max/20}$ values) of pre-diauxic and diauxic cells of US50-18C and IL125-2B was about the same (Fig. 5A), a difference appearing only upon transition to the post-diauxic phase of growth: while US50-18C cells displayed only a mild increase in staining, in IL125-2B cells the staining increased sharply to the level of the pump-free AD1-3 cells. Hence, as seen from the $\Delta\lambda_{max/20}$ values describing the pump activity (Fig. 5B), cells with the *PDR1-3* allele, when entering the post-diauxic phase, exhibit only a slight drop in efflux pump activity, whereas in cells with the wild-type *PDR1* allele, the pumps become either inactivated or their genes are no longer expressed because the pump activity nearly disappears.

3.5. Response of pump activity in post-diauxic cells to glucose challenge

The obvious reason for the dramatic drop in the activity of the two pumps in wild-type strain is the preceding exhaustion of glucose from the medium. A question therefore arises how these cells, which are at the end of the diauxic shift and should be adapted to growth on respiratory substrates, respond to re-addition of glucose. As seen in Fig. 6, addition of glucose (final concentration 1%) to cells of the pump-deleted strain AD1-3 at the point of transition from the diauxic to the post-diauxic phase (phase point 22.5) had practically no effect on cell staining during subsequent growth.

In contrast, addition of glucose to cells of the wild-type strain IL125-2B in the same phase brought about a considerable and relatively rapid decrease in cell staining. A similar drop in staining was also found in glucose-supplied pump-overproducing strain US50-18C (Fig. 6A). In both strains, glucose exhaustion brought the level of cell staining back to that of control glucose-free sample; the different rapidity of glucose exhaustion is caused by different cell concentrations (OD). $\Delta\lambda_{max/20}$ values in Fig. 6B clearly demonstrate that, in both pump-competent strains, addition of glucose to cells in the diauxic/post-diauxic transition leads to a rapid increase of activity of the two ABC pumps, which persists until the cells change their metabolic mode to respiration.

Depletion of a substrate from the growth medium is known to trigger a series of metabolic and gene expression changes in yeast cells. With fermentable substrates such as glucose, these changes include derepression of genes supporting respiratory metabolism and activation of regulatory networks that cause the expression of genes participating in cell defenses against oxidative and other stresses [17,20,21], a sharp drop in the level of transcription [22,23] and activation of proteolytic systems degrading a number of proteins [21]. All these processes appear to be parts of a "saving program" preparing the cell for a transition to energy shortage conditions. The energy-saving measures the cell takes are likely to involve downregulation (repression or degradation) of high-energy-consuming systems, e.g. active membrane transports such as H^+-ATPase [19]. To our knowledge, no data exist on possible diauxic-shift-associated activation of the PDR network regulating yeast ABC pumps; in fact, the energy-saving programs are likely to also entail a downregulation of this network and its component pumps, which are certain to pose high demands on cellular energy resources. This pump shut-down clearly occurs in

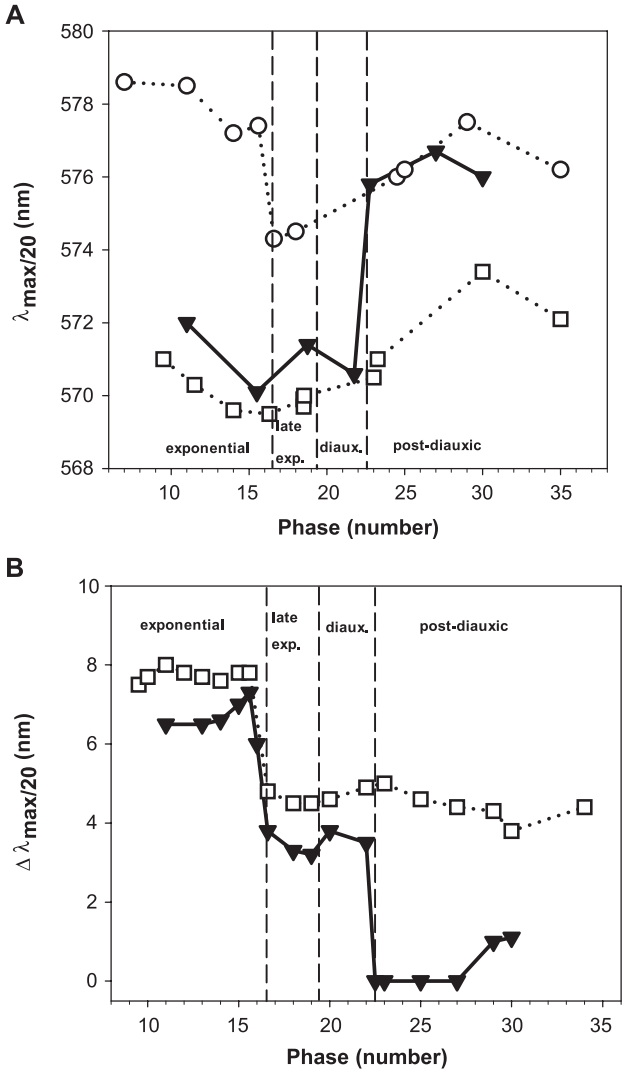

Fig. 5. The growth phase-dependence of the staining level (A) and pump activity (B) of the wild-type IL125-2B strain. (A) The level of staining ($\lambda_{max/20}$ value) of IL125-2B cells (▼); values for reference strains AD1-3 (○, dotted) and US50-18C (□, dotted) taken from Fig. 2B. (B) Pump activity ($\Delta\lambda_{max/20}$) in wild-type IL125-2B (▼). The $\Delta\lambda_{max/20}$ values were obtained by subtracting the $\lambda_{max/20}$ values for IL125-2B from corresponding $\lambda_{max/20}$ values of the pump-free strain AD1-3 used as negative control. The $\Delta\lambda_{max/20}$ curve for US50-18C (□, dotted) from Fig. 3 is shown for comparison.

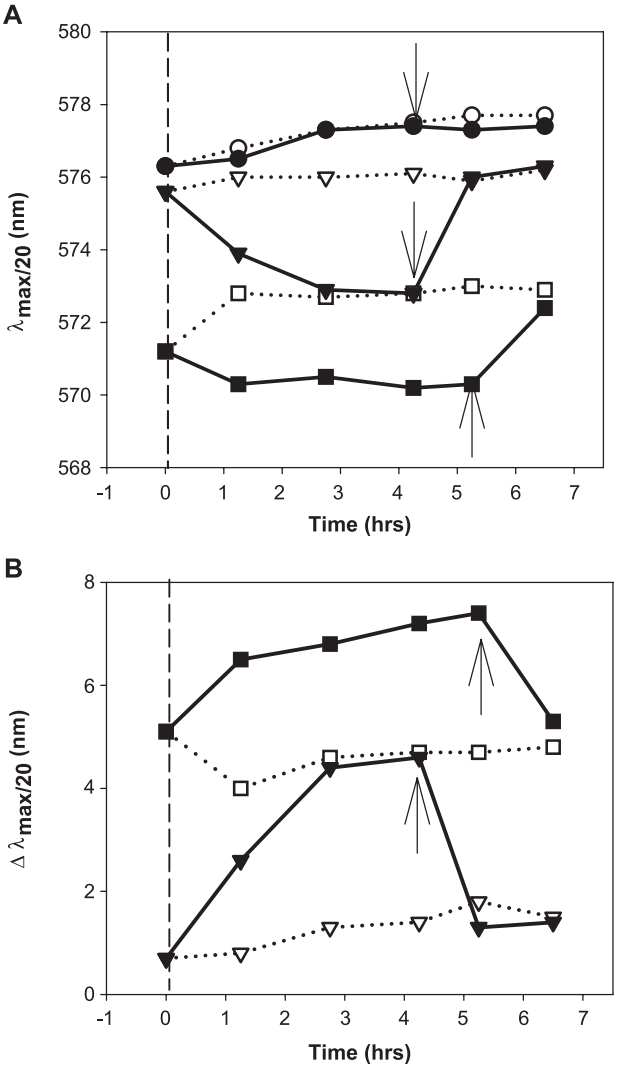

Fig. 6. (A) Effect of glucose added to cells in the diauxic/post-diauxic transition (phase point 22.5) on staining levels ($\lambda_{max/20}$ values) during subsequent growth. Glucose-free control (○, dotted) and glucose-supplied cells (●) of pump-free AD1-3, glucose-free (▽, dotted) and glucose-supplied cells (▼) of wild-type IL125-2B, glucose-free (□, dotted) and glucose-supplied cells (■) of pump-overproducing US50-18C. (B) Changes in the activity of Pdr5p and Snq2p pumps ($\Delta\lambda_{max/20}$) in wild-type IL125-2B (▼) and pump-overproducing US50-18C (■) cells following glucose addition. Dotted lines denote glucose-free controls in wild-type IL125-2B (▽) and in pump-overproducing US50-18C (□). Dashed line indicates the moment of glucose addition, arrows show the moment of glucose depletion from growth medium. The $\Delta\lambda_{max/20}$ values were obtained by subtracting the $\lambda_{max/20}$ values for individual strains from corresponding $\lambda_{max/20}$ values of the pump-free strain AD1-3 used as negative control.

the wild-type strain but is only marginal in the pump-hyperproducing strain carrying the *PDR1-3* allele.

The rapid restoration of a high MDR pump activity in post-diauxic-phase cells caused by a renewed supply of a fermentable substrate shows that the activity of ABC pumps is strongly affected not only by the transition from fermentative to respiratory, but also from respiratory to fermentative metabolism. Rapid adjustment of MDR pump efficiency to low- and/or high-energy conditions may enable, e.g. cells in natural setting to respond adequately to sudden changes in the environment. The close association of the increased pump activity with the presence of glucose points to a more general factor—a strong dependence of ABC pump activity on the type of the carbon source. A similar dependence has been noted, e.g. for the yeast MFS-type efflux pump Sge1p [24]. Study of the mechanism(s) of these phenomena is underway.

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