ELSEVIER

Biochimica et Biophysica Acta 1511 (2001) 74–79

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Factors underlying membrane potential-dependent and -independent fluorescence responses of potentiometric dyes in stressed cells: diS-C_3(3) in yeast

D. Gášková [a,*], R. Čadek [a], R. Chaloupka [a], J. Plášek [a], K. Sigler [b]

[a] *Institute of Physics, Charles University, Ke Karlovu 5, 121 16 Prague 2, Czech Republic*
[b] *Institute of Microbiology, Czech Academy of Sciences, 142 20 Prague 4, Czech Republic*

Received 27 September 2000; received in revised form 23 November 2000; accepted 29 November 2000

Abstract

The redistribution fluorescent dye diS-C_3(3) responds to yeast plasma membrane depolarisation or hyperpolarisation by $\Delta\psi$-dependent outflow from or uptake into the cells, reflected in changes in the fluorescence maximum λ_{max} and fluorescence intensity. Upon membrane permeabilisation the dye redistributes between the cell and the medium in a purely concentration-dependent manner, which gives rise to $\Delta\psi$-independent fluorescence responses that may mimic $\Delta\psi$-dependent blue or red shift in λ_{max}. These λ_{max} shifts after cell permeabilisation depend on probe and ion concentrations inside and outside the cells at the moment of permeabilisation and reflect (a) permeabilisation-induced $\Delta\psi$ collapse, (b) changing probe binding capacity of cell constituents (inverse to the ambient ionic strength) and (c) hampering of probe equilibration by the poorly permeable cell wall. At low external ion concentrations, cell permeabilisation causes ion outflow and probe influx (hyperpolarisation-like red shift in λ_{max}) caused by an increase in the probe-binding capacity of the cell interior and, in the case of heat shock, protein denaturation unmasking additional probe-binding sites. At high external ion levels minimising net ion efflux and at high intracellular probe concentrations at the moment of permeabilisation, the $\Delta\psi$ collapse causes a blue λ_{max} shift mimicking an apparent depolarisation.

Keywords: Membrane potential; Fluorescent probe; Probe binding to cell component; Permeabilization

1. Introduction

The method of monitoring changes in membrane potential ($\Delta\psi$) in suspensions of yeast cells by means of the redistribution fluorescent dye diS-C_3(3) developed in our group [1–6] is based on recording the time course of changes in the fluorescence of this dye (maximum fluorescence wavelength λ_{max} and fluorescence intensity at the λ_{max}). The $\Delta\psi$-dependent uptake of the dye into the cells, i.e., the process of dye redistribution between the intra- and the extracellular phase, is accompanied by a gradual red shift in the maximum fluorescence wavelength of the probe caused by an interaction of the intracellularly present dye with cytosolic components. This interaction is accompanied by a shift of the λ_{max} to longer wavelengths (λ_{max} of the free probe is at 569 nm, for the 'bound' probe at 582 nm), with a concomitant increase in fluorescence intensity. When the membrane potential changes, e.g., owing to the action

* Corresponding author. Fax: +420-2-296764;
E-mail: gaskova@karlov.mff.cuni.cz

0005-2736/01/$ – see front matter
PII: S0005-2736(00)00383-7

of environmental factors or substances (external pH change, glucose, KCl, protonophores such as CCCP, heat shock, etc.) the dye responds to the change by changing its concentration in the cells. Membrane depolarisation, accompanied by probe efflux, is signified by a blue shift in λ_{max}, hyperpolarisation causes probe uptake and a red shift in λ_{max}.

If the membrane potential is abolished as a result of membrane permeabilisation, what follows in most cases is again a red shift in λ_{max} of diS-C_3(3) fluorescence. This shift is, however, not associated with membrane hyperpolarisation and is no longer controlled by membrane potential; this independence of $\Delta\psi$ can be experimentally verified using CCCP [5]. In some situations, however, permeabilisation of the plasma membrane is accompanied by a blue shift in diS-C_3(3) fluorescence maximum. In situations involving cell damage of varying extent, both these shifts can be mistaken for probe responses to changes in membrane potential. They have been provisionally attributed to changes in the ionic relations inside the permeabilised cell [5]. This study elucidates the true mechanism of these shifts and explores their possible role in potentiometric probe responses.

2. Materials and methods

2.1. Yeast strains, growth and manipulation

The yeast strain *Saccharomyces cerevisiae* RXII was precultured as described previously [5] in a YEPG medium containing appropriate glucose concentrations (2% in high-glucose (HG) medium and 0.2% in low-glucose (LG) one) for 6–8 h and then main culture was performed in media with appropriate glucose concentrations for 16 h at 28°C using an initial concentration of 5×10^6 cells/ml ($OD_{600}=0.2$). The cells were then washed twice with double-distilled water and resuspended in a citrate–phosphate buffer of pH 6.0 to an $OD_{600}=0.2$ for fluorescence measurements.

For preparing cell lysate, the cells were suspended in 2 ml water and broken with glass beads by 6-fold 30-s vortexing with 30-s breaks at 4°C. Protein concentration in the lysate was determined according to Bradford. In experiments involving collection and fluorescence measurements in lysate supernatant, the lysate was first repeatedly washed by centrifugation to remove poorly sedimenting particles and ensure a low and reproducible ion level in the lysate.

2.2. Fluorescence measurements

The measurements were done on a spectrofluorimeter FluoroMax 2 (Jobin Yvon-Spex) with a xenon lamp. The excitation wavelength was 508 nm, fluorescence spectra were recorded in the range of 560–590 nm. One spectral scan took about 20 s. Scattered light was eliminated using a cut-off filter (orange glass) with a cut-off wavelength of 540 nm.

3. Results and discussion

3.1. Fluorescence signals from cells with different intracellular ion content: simulation by cell lysate

The ionic relations in cell interior before and after membrane permeabilisation were simulated by using cell lysate. The speed, extent and reversibility of probe binding to cell lysate were measured in citrate–phosphate buffer containing different ions. The uptake of the probe into cells was simulated by repeatedly adding the probe into a cuvette containing a fixed amount of cell lysate, either alone or in the presence of 150 mM KCl, NaCl or choline chloride (Fig. 1a). With increasing probe concentration the λ_{max} shifted to longer wavelengths. At a certain probe concentration (which strongly depended on the amount of lysate in the sample; Fig. 1b) this trend was reversed and the λ_{max} began to shift back to shorter wavelengths. This 'blue shift' is obviously due to the fact that, after saturation of the probe binding capacity of the lysate, any further probe addition brings about an increase in the signal from the free probe, i.e., the overall λ_{max} becomes steadily more dominated by the shorter wavelength λ_{max} of the free probe. It should be noted that this situation never arises with ion and probe concentrations used in our $\Delta\psi$-measuring experiments.

In contrast to this readily explained blue shift, the initial red shift in probe λ_{max} did not fit in with our notions of the probe behaviour. The λ_{max} in this part of the curve should be constant because, given a sufficient number of unoccupied probe binding sites

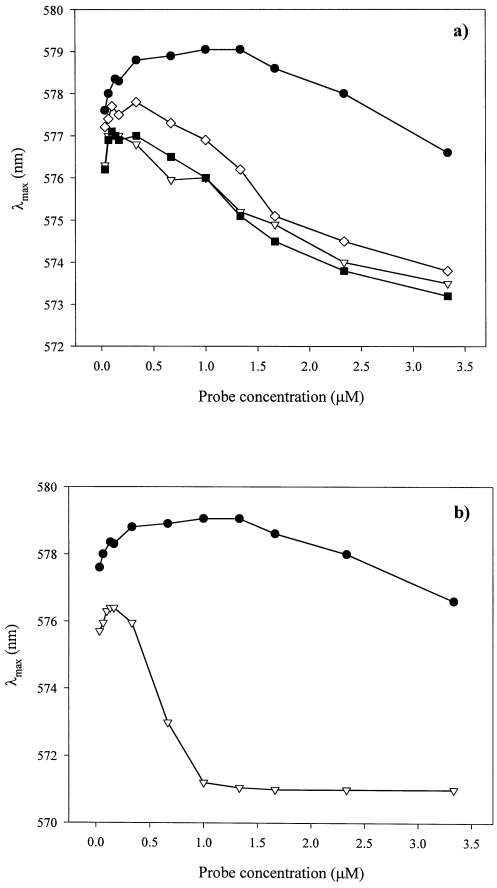

Fig. 1. (a) Change in the probe λ_{max} on increasing diS-C_3(3) concentration in a sample with a fixed cell lysate concentration. The sample contained 3 ml citrate–phosphate buffer (pH 6) and 50 μl lysate from *S. cerevisiae* RXII (2.4 mg protein/ml). Stock probe solution (0.1 mM diS-C_3(3) in ethanol) was added stepwise to reach indicated concentrations. ●, no ions added; ▽, 150 mM KCl; ■, 150 mM NaCl; ◇, 150 mM choline chloride. (b) Change in the probe λ_{max} on increasing diS-C_3(3) concentration in samples containing two different lysate concentrations. ●, 50 μl lysate from *S. cerevisiae* RXII (2.4 mg protein/ml); ▽, 10 μl lysate. Probe concentrations as in a.

in the lysate, the free/bound probe ratio should not change. A deviation from constant λ_{max} in this range reflects either a contribution of scattered excitation light or reabsorption of both free and, especially, bound probe fluorescence within the sample. The scattered excitation light (508 nm) was eliminated by the 540 nm cut-off filter (see Section 2). The true cause of the red shift was found to be the reabsorption of the probe-emitted fluorescence within the sample; this was documented by placing a cuvette with probe solution into the optical path beyond the sample.

In the presence of 150 mM KCl, NaCl or choline chloride the curve peaked at a lower concentration of added free probe, i.e., at a lower concentration of bound probe in the sample and, consequently, at shorter-wavelength λ_{max} (Fig. 1a). Hence, the probe-binding capacity of the lysate appears to be considerably decreased in the presence of ions. The decreased binding capacity is likely to reflect changes in the binding properties of lysate components (altered protein conformation, etc.) brought about by altered ambient ionic milieu. Fig. 2a,b also implicates ions as the cause of the decreased lysate probe-binding capacity. Ion-free lysate containing 3×10^{-8} M diS-C_3(3) was repeatedly supplied with 50-μl volumes

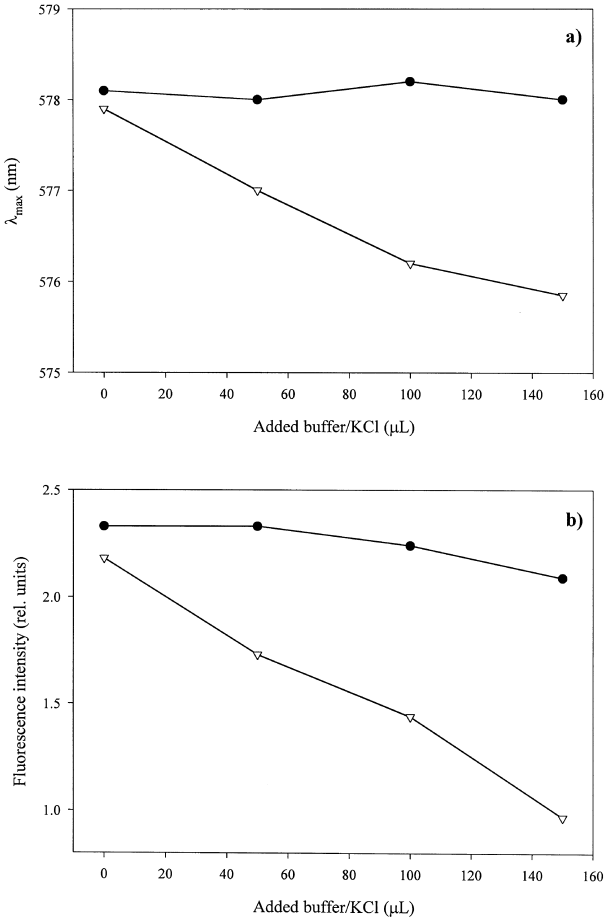

Fig. 2. Effect of increasing ion concentration on the probe-binding capacity of cell lysate. The sample contained 3 ml citrate–phosphate buffer (pH 6), lysate from *S. cerevisiae* RXII (2.4 mg protein/ml) and 3×10^{-8} M diS-C_3(3). Volumes of 50 μl buffer (●) or 3.3 M KCl (▽) were repeatedly added. (a) λ_{max}; (b) fluorescence intensity at λ_{max}.

of pure buffer or with 3.3 M KCl. With buffer additions both λ_{max} and fluorescence intensity remained approximately constant while KCl additions brought about a decrease in both λ_{max} and fluorescence intensity, signifying reduced probe binding.

3.2. Testing the dynamic character of probe 'binding' to lysate

On redistributing between the cell and the medium in response to $\Delta\psi$, part of the intracellular diS-C_3(3) interacts with cell constituents, whereupon its fluorescence increases and its λ_{max} is shifted to longer wavelengths. The gradually increasing contribution of this red-shifted fluorescence to the overall fluorescence spectrum is used as an indicator of probe uptake and its increasing intracellular concentration. If $\Delta\psi$ decreases, e.g., due to the action of stressors, the probe flows out of the cells. The free outflow of the probe requires that any interaction between the probe and cell constituents should be fully reversible. We again simulated this situation in cell lysate by using samples of pre-washed lysate containing 5×10^{-7} M diS-C_3(3). The sample was centrifuged, most of the supernatant containing the free probe was removed and replaced with pure buffer. The absence of lysate components in the supernatant was checked by measuring its fluorescence. The procedure was repeated several times. If the probe were truly bound to the lysate, then the repeated removal of free probe would cause an increasing dominance of the longer-wavelength fluorescence of bound probe and a progressive red shift in the overall λ_{max}. If the 'bound' probe interacted with the lysate reversibly and dynamically, the removal of free probe would cause a fast re-establishment of the free/bound probe ratio at the lower total probe concentration, which would be reflected in a constant λ_{max} but a lowered overall fluorescence intensity. As documented in Fig. 3, this was indeed the case. The drop in λ_{max} that occurs after repeated washing and probe removal is due to the lower effect of reabsorption at low probe concentrations (an effect opposite to the first part of the upper curve in Fig. 1a).

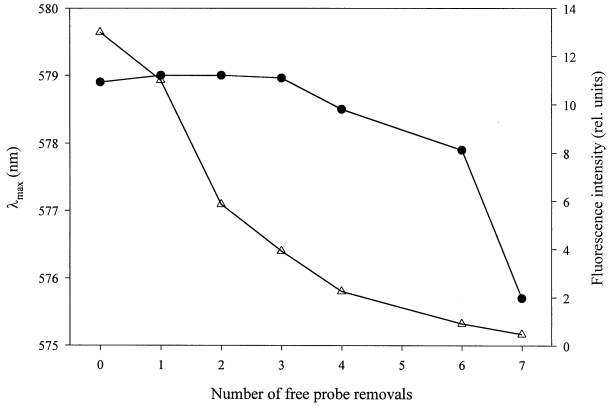

Fig. 3. Illustration of the dynamic equilibrium between free and lysate-bound probe. The sample contained 150 μl pre-washed lysate (cf. Section 2) in 3 ml citrate–phosphate buffer; initial probe concentration was 5×10^{-7} M. The sample was repeatedly centrifuged and 90% of the supernatant was removed and replaced with pure buffer. ●, dependence of λ_{max} on the number of free probe removals; △, dependence of fluorescence intensity on the number of free probe removals.

3.3. Denaturation of cell proteins caused by heat shock: effect on probe-binding capacity

The rate of probe uptake into cells permeabilised by heat may be affected by the heat denaturation of cell proteins, which affects the tertiary structure of proteins and may unmask additional probe binding sites. This unmasking can be inferred from the difference between the probe binding (i.e., λ_{max}) in a control lysate and in a heat-treated lysate (Fig. 4).

Based on the data obtained with cell lysate (Figs. 1–4) we can make the following statement: in probe-stained cells suspended in a medium containing different ion concentrations, membrane permeabilisation will bring about two events with mutually opposite effects: abolishment of membrane potential reflected in an outflow of accumulated probe accompanied by a blue shift in λ_{max}, and a weaker or stronger ion efflux causing an increase in the probe-binding capacity of cell constituents and a consequent probe inflow reflected in a red shift in λ_{max}. Depending on external ion concentration, the net result is then a red shift in λ_{max} in the fluorescence spectrum of the cell suspension, or its less or more pronounced blue shift. This blue shift should be less conspicuous in cells permeabilised by heat because of the increased probe-binding capacity of heat-treated cells. To prove this statement, both thick- and thin-walled cells were permeabilised by heat and in the absence and presence of ions in the medium.

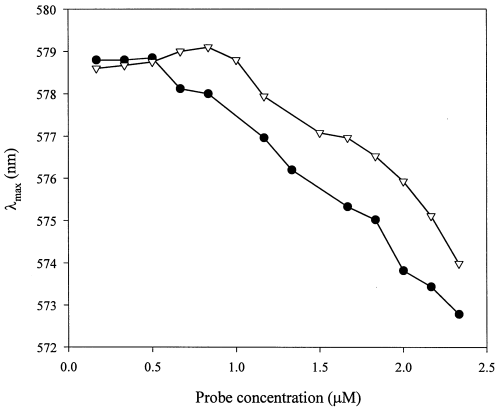

Fig. 4. Unmasking of additional probe-binding sites in cell lysate by heat treatment. The sample contained 3 ml citrate–phosphate buffer (pH 6) and 30 μl lysate (2.4 mg protein/ml). Stock probe solution (0.1 mM diS-C_3(3) in ethanol) was added stepwise to reach indicated concentrations. ●, native lysate; ▽, heat-treated lysate of the same concentration.

3.4. *Probe fluorescence response of heat-shocked cells in media with and without Δψ-affecting and Δψ-inert ions: thick-walled cells*

In cells cultured at conventional (2%) glucose concentrations and exposed to heat shock after a 60-min staining with 3×10^{-7} M diS-C_3(3) in the absence of ions and in the presence of 150 mM KCl or NaCl (Fig. 5a), the character of the fluorescence response after the heat shock is clearly determined by the speed of probe penetration into the cell interior. As shown previously [5], normal cell wall severely slows down the speed of probe uptake. Hence, it may bring about a strong 'understaining' of cell interior, i.e., a lower intracellular probe concentration relative to the ambient one, which is further aggravated in the presence of extracellular Δψ-depolarising K^+. A similar slowing down of probe uptake is observed in the presence of external Na^+. Although Na^+ does not cause Δψ depolarisation in intact *S. cerevisiae* cells [7], its presence seems to reduce the permeability of the cell wall for the probe. Consequently, a large red shift (caused by fast probe uptake into the heavily understained cells) occurs on heat permeabilisation of thick-walled cells in the presence of K^+ or Na^+.

3.5. *Probe fluorescence response of heat-shocked cells in media with and without Δψ-affecting and Δψ-inert ions: thin-walled cells*

Whereas cells cultured at the commonly used 2% glucose have a thick and poorly permeable cell wall that hampers probe uptake, cells cultured at 0.2% glucose exhibit a much faster probe uptake [5]. They are therefore stained very rapidly to the true Nernstian equilibrium and, on heat permeabilisation, do not display any marked red shift in probe λ_{max} (Fig. 5b). In fact, the heat shock in the presence of extracellular Na^+ brings about a blue shift in λ_{max}. This is caused by the following events: before cell permeabilisation, the probe is drawn into the cells over its concentration equilibrium by a full-sized Δψ (Na^+ has no depolarising effect on Δψ [5]). Then, when the heat shock abolishes membrane potential, the probe flows out of the cells to reach its Δψ-independent concentration equilibrium while its inflow and increased interaction with cell constitu-

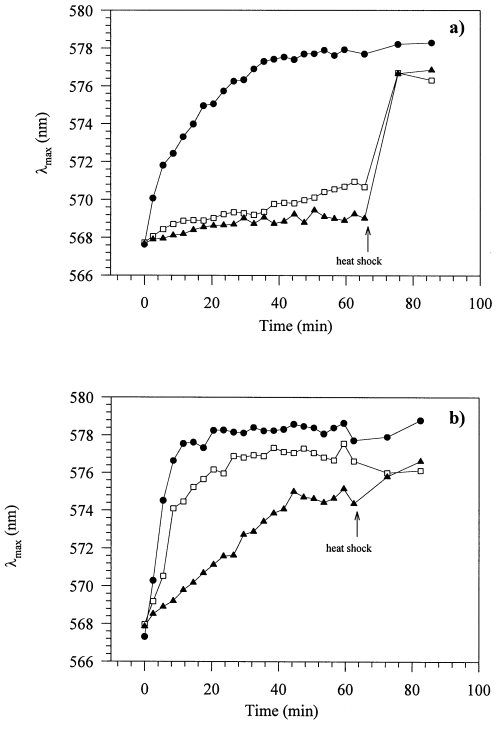

Fig. 5. Effect of heat shock applied 60 min after cell staining with diS-C_3(3) on thick-walled (a) and thin-walled (b) cells. Concentration of *S. cerevisiae* RXII cells 5×10^6 cells/ml, probe concentration 3×10^{-7} M. ●, cells stained in buffer; □, staining in 150 mM NaCl; ▲, staining in 150 mM KCl.

ents is prevented by the persisting intracellular ionic strength ensured by the high external Na^+.

The small red shift in probe λ_{max} observed after heat shock in the presence of $\Delta\psi$-depolarising external K^+ reflects the effect of denaturation (see above).

In conclusion, we proved that a red shift in λ_{max} can be observed also in cells that have lost their membrane potential, provided these cells are 'understained' for some reason (insufficient equilibration time [5], impermeability of cell wall) and are immersed in a medium without ions that could compensate the ionic milieu inside the cell after permeabilisation.

3.6. Cautionary remarks

Spectral shifts due to the interaction of a fluorochrome with cell macromolecules do not seem to be a feature specific for diS-C_3(3). They have also been found with many other fluorescent probes, e.g., diS-C_3(5) or diO-C_3(5) [8] and other redistribution fluorescent probes [9], with the electrochromic dye di-4-ANEPPS [10], the pH-sensitive probe BCECF [11] and the bromocresol purple (BCP) used for determining dead cell count in yeast suspensions [12]. The last probe, or the equally impermeant propidium iodide [13], can be used for distinguishing permeabilised from non-permeabilised cells. Our recent results (unpublished) indicate an existence of a similar interaction for the membrane fluidity probe diphenylhexatriene (DPH). Although the nature of these interactions is not clear, they seem to be non-ionic because BCP and DPH are uncharged molecules.

These interactions may cause some concern about the suitability of using fluorescent probes for the measurement of membrane potential and other cell characteristics. However, careful in-depth analysis shows that artefacts that appear as the main shortcoming of the use of fluorescent probes, actually mostly reflect a failure to find optimum conditions (optimum cell and probe concentration, optimum range of ambient ion concentrations, growth conditions ensuring reasonable probe uptake into the cultured cells) ensuring an artefact-free result. The effort invested into carefully defining this optimum 'experimental window' (which may differ from one cell type or even one yeast strain to another) may bring considerable experimental benefits. It may enable the experimenter to measure with a single probe not only membrane potential but also, e.g., damage to the cell membrane too subtle or transient to be revealed by vital staining or a plating test, permeability properties of the wall of mutant or differently grown cells, possible 'clogging' of cell wall by medium components or other added substances, etc.

Acknowledgements

We thank Professor M. Höfer, University of Bonn, for valuable discussions on the manuscript. The work was supported by Grant Agency of the Czech Republic (grants 204/99/0488, 204/00/0629 and 202/99/0186), Czech Ministry of Education (grant ME 315) and a BMBF grant TSR-113-97.

References

[1] J. Plášek, R.E. Dale, K. Sigler, G. Laskay, Biochim. Biophys. Acta 1196 (1994) 181–190.
[2] B. Denksteinová, D. Gášková, P. Herman, J. Malínský, J. Plášek, K. Sigler, J. Vecer, Phys. Med. (Trieste) 13 (1) (1997) 269–271.
[3] D. Gášková, B. Denksteinová, P. Herman, J. Vecer, J. Plášek, K. Sigler, in: J. Slavík (Ed.), Fluorescence Microscopy and Fluorescent Probes, Plenum, New York, 1997, pp. 147–153.
[4] D. Gášková, B. Brodská, P. Herman, J. Vecer, J. Malínský, K. Sigler, O. Benada, J. Plášek, Yeast 14 (1998) 1189–1197.
[5] D. Gášková, B. Brodská, A. Holoubek, K. Sigler, Int. J. Biochem. Cell Biol. 31 (1999) 575–584.
[6] M. Eminger, D. Gášková, B. Brodská, A. Holoubek, K. Sigler, Folia Microbiol. 44 (1999) 283–288.
[7] K. Sigler, D. Gášková, B. Brodská, Folia Microbiol. 44 (1998) 236.
[8] R. Chaloupka, T. Obšil, J. Plášek, F. Sureau, Biochim. Biophys. Acta 1418 (1999) 39–47.
[9] D. Novo, N.G. Perlmutter, R.H. Hunt, H.M. Shapiro, Cytometry 35 (1999) 55–63.
[10] R. Chaloupka, J. Plášek, J. Slavík, V. Siglerová, K. Sigler, Folia Microbiol. 42 (1997) 451–456.
[11] J. Plášek, J.J. Ter Horst, M. Ameloot, P. Steels, in: J. Slavík (Ed.), Fluorescence Microscopy and Fluorescent Probes, Plenum, New York, 1996, pp. 120–125.
[12] H. Kurzweilová, K. Sigler, Yeast 9 (1993) 1207–1211.
[13] M. Kowalski, K. Hannig, G. Klock, P. Gessner, U. Zimmermann, G.A. Neil, D.W. Sammons, Biotechniques 9 (1990) 322–341.