Intracellular pH Distribution in *Saccharomyces cerevisiae* Cell Populations, Analyzed by Flow Cytometry

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Intracellular pH has an important role in the maintenance of the normal functions of yeast cells. The ability of the cell to maintain this pH homeostasis also in response to environmental changes has gained more and more interest in both basic and applied research. In this study we describe a protocol which allows the rapid determination of the intracellular pH of *Saccharomyces cerevisiae* cells. The method is based on flow cytometry and employs the pH-dependent fluorescent probe carboxy SNARF-4F. The protocol attempts to minimize the perturbation of the system under study, thus leading to accurate information about the physiological state of the single cell. Moreover, statistical analysis performed on major factors that may influence the final determination supported the validity of the optimized protocol. The protocol was used to investigate the effect of external pH on *S. cerevisiae* cells incubated in buffer. The results obtained showed that stationary cells are better able than exponentially grown cells to maintain their intracellular pH homeostasis independently of external pH changes. Furthermore, analysis of the intracellular pH distribution within the cell populations highlighted the presence of subpopulations characterized by different intracellular pH values. Notably, a different behavior was observed for exponentially grown and stationary cells in terms of the appearance and development of these subpopulations as a response to a changing external pH.

Intracellular pH (pH_i) is a crucial determinant of the overall physiology of *Saccharomyces cerevisiae* cells. Consequently, it appears to be of great importance for yeast cells to maintain pH_i homeostasis. In fact, it has been reported that yeast cells are able to maintain their pH_i around neutrality in order to sustain cellular processes, such as membrane transport, metabolic pathways (7), and biosynthetic pathways (15). Furthermore, pH_i homeostasis appears to be involved in the preservation of cellular equilibrium in response to several environmental factors and stress conditions such as hyperosmotic shock (27), heat shock (18) and the presence of weak organic acids (3). There is increasing interest in the measurement of the pH_i or, more generally, in the investigation of the yeast cell's ability to maintain pH_i homeostasis even in response to different stress conditions.

Several approaches to determine the pH_i of cells of *S. cerevisiae* and other yeasts have been described, including the distribution of radiolabeled weak acids (19), ^{31}P nuclear magnetic resonance spectroscopy (6), and the use of pH-dependent green fluorescent protein reporters (13). Since most of these methods require extensive or invasive manipulations of the cells, they appear unsuitable for measuring rapid pH changes occurring in vivo.

Further attempts to determine the pH_i have been made by using pH-dependent fluorescent probes. Obviously, such an approach is only valid if the probe is located in the cytoplasm and not in low pH organelles like the vacuoles. For example, a broad variety of fluorescein-derived reagents have been devel-

oped to obtain sensitive fluorescent pH indicators for a wide range of applications. However, the fluorescence intensity of such probes depends not only on the pH but also on the concentration of the probe inside the cell. Since the amount of the probe present in the cells is generally hard to control, a way has to be found to correct the signals for concentration differences. Fluorescein-derived reagents do not allow such a correction; therefore, pH determination on the single-cell level appears difficult. New fluorochromes have become available like seminaaphthorhodafluor (SNARF) probes, which show pH-dependent emission spectra and, notably, two inversely related emission signals at two different wavelengths. This allows a ratiometric pH calculation (3). It therefore becomes possible to calculate the pH through the determination of a ratio between the fluorescence intensities measured at the two wavelengths independently of the actual concentration of the fluorochrome. The best known probe belonging to this family is carboxy SNARF-1, which has been used to determine the pH_i in mammalian cells (12) according to a method that combines an in situ calibration and flow cytometry. The latter is a powerful tool for the investigation of several parameters at the single-cell level and has the advantage of processing a high number of individual cells at a high rate to obtain additional, accurate information on the distribution of one or more parameters within a heterogeneous cell population.

Until now, only a few attempts have been reported for the determination of the pH_i in *S. cerevisiae* cells with SNARF probes (1, 9, 10). Haworth et al. (10) have demonstrated by fluorescence microscopy that the SNARF probe is located in the cytoplasm of *S. cerevisiae* cells. However, fluorescence was usually detected by microscopy or spectroscopy, with the inherent disadvantages that only a limited number of cells could

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be analyzed or only a mean value for the entire population of cells could be obtained. Only recently, a protocol for pH_i measurement in the yeast *Candida boidinii* by using flow cytometry with a SNARF probe was reported (20). However, staining of the cells was reported to require incubation for 12 h at 20°C. Most studies of *in vivo* conditions appear to be impossible with such a treatment.

Here, we set out to establish a rapid and noninvasive protocol for pH_i determination in *S. cerevisiae* cells. The protocol, based on flow cytometry, provides accurate information about the pH_i distribution within a given cell population. The method makes use of the pH-sensitive carboxy SNARF-4F which is a fluorinated derivative of carboxy SNARF-1. This probe shows a different maximum of pH sensitivity, better adapted to the typical cytosolic pH range of yeast cells.

MATERIALS AND METHODS

Strain and growth conditions. The *S. cerevisiae* strain used in this study was CEN.PK 113-5D (*MATa ura3*) (26). The yeast cells were incubated at 28°C in a 250-ml Erlenmeyer flask with 50 ml of minimal medium containing 6.7 g of yeast nitrogen base without amino acids (Difco) per liter, 20 g of glucose per liter, and 50 mg of uracil per liter. The medium was inoculated to an initial optical density at 660 nm (OD_{660}) of 0.004 with an exponential cell culture grown in the same medium. Growth was monitored by measuring the OD_{660} . The cells were harvested after 18.5 and 70 h, during the exponential (OD_{660} of 0.8) and stationary phases (OD_{660} of 4.1), respectively.

Chemicals and buffers. The stock solution of 5 mM carboxy SNARF-4F AM from Molecular Probes [SNARF-4F 5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate] was prepared in dimethyl sulfoxide. The stock solution of 9.7 mM amphotericin B from Sigma-Aldrich was obtained by dissolving 100 mg of the powder, which contains 45 mg of amphotericin B, in 5 ml of water. McIlvaine buffers (16) were made by the combination of appropriate volumes of 100 mM citric acid and 200 mM Na_2HPO_4 to obtain a buffer of the desired pH. The loading buffer was prepared by dilution of the carboxy SNARF-4F AM stock solution in McIlvaine buffer of the required pH to a final concentration of 20 μM ; the buffer was stored on ice and protected from the light.

Fluorescence spectra of the de-esterified probe. To prepare an *S. cerevisiae* cell extract, 5 ml of exponentially growing cells (OD_{660} of 0.85) was harvested by centrifugation at 4,000 rpm in a Heraeus Biofuge Pico centrifuge at 4°C for 5 min, washed with phosphate-buffered saline, pH 7.0, and resuspended in 150 μl of the same buffer. Then, the same amount of glass beads was added, and five cycles of mechanical cell breakage, consisting of a 15-s vortex and 15-s incubation on ice, were carried out. The supernatant containing the cell extract was collected after a centrifugation step at 13,000 rpm at 4°C for 5 min and transferred in a new tube. In order to obtain the de-esterified form of the probe (carboxy SNARF-4F), 10 μl of a 5 mM stock solution of the ester form (carboxy SNARF-4F AM) was incubated overnight at 28°C with 90 μl of the yeast cell extract previously prepared. The solution was then diluted 100-fold in McIlvaine buffers having pHs of 5.0, 6.0, 7.0, and 8.0. The fluorescence spectra of carboxy SNARF-4F in the buffers with different pHs were recorded with a Shimadzu spectrofluorophotometer F 4500 by using excitation wavelengths of 488 and 543 nm.

pH_i determination. (i) **Cell loading.** For every measurement a cell sample corresponding to 1 ml at 0.25 OD_{660} was collected by centrifugation at 13,000 rpm for 5 min and resuspended in 250 μl of the loading buffer. After incubation at 28°C for 11 min on a shaker, the cells were collected by centrifugation at 13,000 rpm for 5 min and resuspended in 250 μl of McIlvaine buffer, pH 3.0 if not otherwise stated. The samples were put on ice and immediately analyzed by flow cytometry. For the overall experiments, the samples were protected from light in order to guarantee the stability of the probe.

(ii) **Flow cytometric analysis.** Flow cytometric analyses were performed on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, N.J.). The probe was excited by using a 15-mW, 488-nm air-cooled argon ion laser, while the fluorescence emission was measured through a 585 ± 21 -nm-pore-size band-pass filter (FL2 parameter) and a 670-nm-pore-size long-pass filter (FL3 parameter). All data were acquired in a linear mode. Threshold settings were adjusted so that the cell debris was excluded from the data acquisition. A total of 10,000 cells were measured for every sample. Data analysis was performed afterwards with WinMDI 2.8 software, version 1.0 (Purdue University, Cytometry Labora-

TABLE 1. The factors and their respective high and low values of the factorial design of experiments

Factor	Low	High
Growth phase	Exponential	Stationary
pH of loading	3.0	7.0
pH of incubation	3.0	7.0

ories [http://facs.scripps.edu/software.html]). The ratio of fluorescence emission was calculated for every cell by dividing the emission signal at 585 nm by the emission signal at 670 nm. For any sample, the mean pH_i was calculated from the mean of the ratios of all cells by using the calibration described below.

(iii) **In situ calibration.** An *in situ* calibration was generated for each experiment. An appropriate quantity of cells was collected and, after loading according to the protocol previously described, divided into different tubes (250 μl in each tube). The pellets were collected by centrifugation at 13,000 rpm for 5 min and resuspended in 250 μl of McIlvaine buffers having different pH values. After the addition of amphotericin B to a final concentration of 30 μM , the cells were incubated at 37°C for 1 h on a shaker and then analyzed by flow cytometry. The calibration curve, constructed by plotting the fluorescence ratio of the different samples as a function of the pH of the buffer in which they were incubated, was fitted with a second-order polynomial function. The fitted data were used to generate an equation that converts the fluorescence ratio to pH_i values.

Statistical analyses. A factorial design of experiments was applied to evaluate the effects of different variables on the pH_i results. The principle of a factorial design of experiments is to evaluate the effects of several factors, usually at two levels, in a combined series of experiments and to calculate the mean effects of a change in any single factor on the final result, as well as the mean interacting effects. The three factors and the respective high and low values tested are presented in Table 1. The cells were grown and harvested in exponential and stationary phases as described above. Then the cells were loaded, following the protocol previously described, in McIlvaine buffers at pH 3.0 and 7.0, respectively. The loaded cells were then divided and incubated for 2.5 h in McIlvaine buffer at pH 3.0 or 7.0 after a washing step in the same buffer of incubation. The experiments were performed in duplicate and analyzed according to the method of Scheffler (22). Briefly, the main effects of each factor are the mean differences of the effects of this factor at its high and low values. The interacting effects are the mean differences of the effect of one factor, while the other factor is at high or low value.

Standard deviations and standard errors were calculated from the data of duplicate experiments.

Effect of external pH on pH_i . The cells were grown and harvested in exponential and stationary phases as described above. Then the cells were stained in McIlvaine buffer at pH 3.0 following the protocol previously described. The stained cells were then divided and incubated for 2.5 h in McIlvaine buffers of different pH values between 2.2 and 7.0 after a washing step in the same buffer of incubation. The experiment was performed in duplicate.

RESULTS

Molecular and spectral properties of the probe carboxy SNARF-4F. The noninvasive technique is based on the staining of the cells with the ester form of the probe carboxy SNARF-4F. Only the ester, which is not fluorescent, permeates the cell membrane. Once inside the cell, the ester is supposed to be hydrolyzed by ubiquitous intracellular esterases, releasing the proton-sensitive polyanionic probe which is fluorescent and unable to permeate the membrane, thereby being trapped inside of the cell (8).

Incubation of carboxy SNARF-4F AM with an *S. cerevisiae* cell extract confirmed the presence in this yeast of adequate esterases capable of hydrolyzing the respective ester. In order to record the pH-dependent emission spectra, the cell extract comprising the hydrolyzed probe was diluted in buffers of various pH values. Fluorescence emission was recorded at an excitation wavelength of 543 nm as previously described (14). Spectral analyses clearly showed the presence of two pH-sen-

FIG. 1. Effect of pH on the fluorescence emission spectra of carboxy SNARF-4F. Carboxy SNARF-4F AM was incubated with *S. cerevisiae* cell extract and then diluted in McIlvaine buffers with the indicated pH values. The emission spectra were recorded by using excitation wavelengths of 543 (a) and 488 (b) nm. The dashed lines in panel b mark the wavelengths at which fluorescence emissions were detected by the flow cytometer.

sitive emission peaks with opposite behavior in respect to pH changes (Fig. 1a), connected by an isostilbic (or isoemissive) point, representing the wavelength at which the intensity of fluorescence emission is pH independent.

Figure 1b shows the emission spectra of carboxy SNARF-4F (in cell extract) at different pH values at an excitation wavelength of 488 nm, the spectral line of the argon ion laser commonly used in flow cytometers. Notably, the isostilbic point is shifted, as previously reported for carboxy SNARF-1 (8), and the amplitude of the second pH-dependent peak is significantly reduced; nevertheless, the principle of the spectral properties (two peaks, with inverse correlation to the surrounding pH) is also retained at this wavelength. It is known that cellular constituents can influence the spectral properties of the fluorochromes through quenching phenomena. The data reported here demonstrate that the pH dependence of the fluorescence emission of carboxy SNARF-4F is retained in the presence of cell extracts. The compound therefore appears useful for pH_i determination in *S. cerevisiae* cells.

In Fig. 1, the dashed lines mark the wavelengths corresponding to the channels by which the fluorescence was detected by the flow cytometer. Due to the opposite behavior of the two pH-sensitive emission peaks, it is possible to calculate the ratio of the fluorescence recorded at these two channels and to correlate that ratio directly to the pH. The use of the ratio instead of a single fluorescence signal allows a pH determination that is independent of the actual probe concentration and, furthermore, leads to more accurate values. In particular, by employing flow cytometry, the analysis of thousands of cells will be possible, even assuming that the dye concentrations in different cells will not be exactly identical. Since the obtained ratio of the signals is only correlated to the pH, the results will be directly comparable. The spectra show the suitability of the carboxy SNARF-4F AM probe for the method under study.

Protocol for pH_i determination. The first step for the measurement of the pH_i is the loading of the cells with the probe. Ideally, this should take place during the cultivation, that is, without disturbance of the culture. This proved to be impossible due to the instability of the probe in the presence of amine groups, such as amino acids and vitamins, which cleave the ester and thereby prevent the loading of the cells (data not shown).

With the aim of obtaining a pH_i determination as close to the situation of interest as possible, we optimized the loading protocol based on a short incubation time of only 11 min at 28°C. Loading was performed in the biologically compatible McIlvaine buffer.

Among the pH values tested, pH 7.0 was considered close to the expected cytosolic range and pH 3.0 was considered closer to the growth condition of the yeast, since the pH typically decreases during a shake flask cultivation of *S. cerevisiae*. Statistical analyses, which are described in detail below, indicated that the loading pH had no significant effect on the measured pH_i; thus, McIlvaine buffer at pH 3.0 was chosen as loading buffer for all the experiments.

For the final conversion of the acquired fluorescence ratios into pH_i values, a calibration of the system was necessary. To be as close to the natural situation as possible and to take into account the possible further influence of cellular constituents on the fluorescence spectrum, we decided to use an in situ calibration curve that has to be determined independently for each experiment. The in situ calibration procedure consists of the loading of the cells with the probe and subsequent permeabilization and incubation of the cells at various pH values in order to obtain a series of cell populations with a precisely manipulated intracellular proton concentration (1, 4, 5).

Figure 2 shows a typical example of an in situ calibration curve, where the determined values correlate to a polynomial curve of second order with the pH. The fitted equation best describing the curve has a correlation coefficient r of 0.9971.

Only permeabilization of the cells with amphotericin B (25) gave optimal and highly reproducible results. Amphotericin B is an antifungal drug that is able to form complexes with membrane sterols that associate into transmembrane channels through which the free diffusion of many components (including protons) can occur. The ionophore nigericin, typically used for mammalian cells to calibrate the fluorescence response of cytosolic pH probes (4), and Triton X-100, reported in the literature as a compound able to permeabilize yeast cells (1),

FIG. 2. In situ calibration curve of carboxy SNARF-4F. Loaded *S. cerevisiae* cells were permeabilized with amphotericin B in McIlvaine buffers of different pH values. The ratios of fluorescence detected at 585 and 670 nm are plotted versus the corresponding pH. The shown polynomial curve of second order fitted best to the determined values.

were also tested but did not allow a reproducible change of the pH_i (as change in fluorescence ratios) dependent on the surrounding proton concentration. We also tested the influence of different buffers (200 mM Tris-HCl, 200 mM morpholinethanesulfonic acid [MES], and McIlvaine buffer) for the adjustment of the pH_i on the quality of the in situ calibration curve. Permeabilization of the cells in MES buffers at pH values lower than pH 6.0 resulted in a change on the side scatter signal, which generally correlates with the cell granularity and internal complexity of the cell (data not shown) (23). Due to this unclear effect of MES on the morphological properties of the cells, we excluded this buffer from further use. The other buffers tested gave comparable results.

FIG. 3. Effects of growth phase, loading pH, and incubation pH on pH_i of *S. cerevisiae* cells after a 2.5-h incubation in buffer. The sizes of the histogram bars represent the mean effects on pH_i (ΔpH_i) of a change from low to high of the respective factors (exponential versus stationary growth phase and pH 3.0 versus pH 7.0 for either loading or incubation) (Table 1). A positive effect means that the mean pH_i was higher at the high value of the respective factor, whereas a negative effect denotes that the mean pH_i was lower at the high value. Above the single effects of each factor, the three two-factor interacting effects and the three-factor interacting effect are displayed. The dotted lines represent the standard deviation (± 0.21 pH units) over this experiment.

FIG. 4. Effect of the external pH on pH_i of *S. cerevisiae* cells. The cells were harvested in exponential (■) and stationary phase (□), loaded with the fluorochrome, and incubated in McIlvaine buffers of different pH values. The mean values of pH_i obtained from two independent experiments are plotted versus the external pH. Standard deviations are indicated.

McIlvaine buffer was finally chosen as the standard buffer for our experiments due to its extended buffering capacity range and its optimal suitability for biological systems.

Statistical analyses: influence of loading pH on the final result. Figure 3 illustrates the results of the factorial design of experiments to evaluate the effects of growth phase, loading pH, and incubation pH on the pH_i determination. Interestingly, the comparison of exponentially grown to stationary cells shows that the growth phase has a significant effect on the results. In fact, after 2.5 h of incubation in McIlvaine buffers, the mean pH_i of stationary cells was lower by 0.6 units. The effect of the loading pH is hardly significant, relative to the standard deviation, while the average effect of the incubation pH was to increase the pH_i by 1.1 units. This shows that the loading pH influences the result only slightly, if at all, while the subsequent incubation in buffer exhibits the strongest influence on pH_i .

The interacting effect between growth phase and incubation pH was significant and negative, indicating that the effect of the incubation pH should be lower in stationary cells. The other interacting effects were not significant.

Ability of exponentially grown *S. cerevisiae* cells versus stationary cells to maintain the pH_i at changing external pH values. The described protocol for pH_i determination was employed to analyze the effect of the external pH on the yeast *S. cerevisiae*.

Cells were harvested in the exponential or stationary phase and stained as described in Material and Methods. Both types of cells show similar pH_i values of 6.5 (exponential) or 6.4 (stationary). The loaded cells were then incubated for 2.5 h in McIlvaine buffers of pH values ranging between 7.0 and 2.2. Since the buffers lack any energy source and certain ions that might be important for cellular equilibrium, the cells are maintained under starvation conditions.

Figure 4 shows the mean values of two independent experiments. Interestingly, the cells derived from different growth phases behave significantly differently under the chosen condition at different pH values. By decreasing the external pH

FIG. 5. Investigation of the pH_i distribution of *S. cerevisiae* cell populations after incubation in McIlvaine buffers of different pH values. Both exponentially grown cells and stationary cells were analyzed. Each histogram represents the distribution of the pH_i values of a defined cell population after incubation at the pH indicated in the central column.

from 7.0 to 2.2, a progressive reduction of the pH_i from 7.1 to 5.1 was observed in exponentially grown cells. In contrast, stationary cells, which were able to maintain the pH_i constant at around 6.1 when the external pH was in the range of 7.0 to 5.5, experienced a drop in pH_i to 5.5 as a consequence of a

reduction of the external pH to 5.0. Further reductions in the external pH did not have any effect; in fact a pH_i of 5.5 was maintained by the stationary cells even with decreasing pH values from 5.0 to 2.2.

Statistical analyses showed that the standard deviation of the

overall experiment was 0.27. More detailed analysis of the data showed that the standard deviation of the samples with exponentially grown cells was much smaller (0.18) than that of stationary cells (0.35).

Furthermore, the analysis of the distribution of the pH_i within the cell populations yielded very striking results. Figure 5 shows the pH_i distribution of the analyzed cell populations. While some populations are characterized by a homogeneous pH_i distribution, others are evidently divided into two subpopulations with significantly different pH_i values. A gradual change of pH_i distribution was observed for both cell types after incubation in buffers with decreasing pH values. However, for cells harvested in different growth phases, different behaviors were observed.

In exponentially grown cells a subpopulation characterized by low pH_i appeared after incubation in buffer of pH 5.0 and became more pronounced with a further reduction of the pH. After incubation in a buffer with pH 2.2, the population with higher pH_i completely disappeared.

The population of stationary cells comprised cells of low pH_i in all the incubation buffers tested. Similar to that among exponentially grown cells, the subpopulation characterized by low pH_i became more pronounced with the reduction of the incubation pH. However, both subpopulations are present even at the lowest external pH.

Another noteworthy phenomenon is the behavior of the subpopulation with the higher pH_i . While in stationary cells the pH_i is maintained independently of the incubation pH, in exponentially grown cells the pH_i of the subpopulation gradually decreases with decreasing external pH.

DISCUSSION

In the present work we describe a new protocol for flow cytometric determination of the pH_i of *S. cerevisiae* cells based on the fluorescent probe carboxy SNARF-4F AM and in situ calibration of the system. In order to obtain the most accurate information possible about the physiological state of the cells, we optimized a fast protocol to minimize the perturbation of the system under study. For the analysis of the validity of the protocol, we checked the effects of several factors, particularly the loading pH, on the final result. Statistical analyses proved that a change of the loading pH has no significant effect on the final result, in contrast to, e.g., the growth phase. In fact, we could show that the pH_i of stationary cells is prevalently lower than the pH_i of exponentially grown cells after incubation for 2.5 h under starvation conditions.

Further, we investigated the ability of *S. cerevisiae* cells to maintain the pH_i when exposed to changes of the external pH. The incubation of exponentially grown or stationary cells in buffers of different pH values showed a marked difference in the behavior of these two types of cells. While exponentially grown cells show a constant decrease of the pH_i as a consequence of the reduction of the external pH, stationary cells, after an initial drop, maintain a more or less constant pH_i when the external pH is decreasing. Generally, these results are in good agreement with prior reports of results from different technologies showing that the pH_i of yeast changes as a consequence of an incubation of the cells in buffers of different pH values (9, 11). However, to the best of our knowledge, there is

as yet no information about the influence of the growth state of the cells on their behavior in response to the external pH. The observed difference appears even more striking if one considers that the cells were incubated in buffers lacking any energy source and certain ions that are extremely important for the overall equilibrium of the cells.

Another point to consider is the reported fact that stationary cells are generally less susceptible to several environmental (stress) factors than exponentially grown cells (17, 28). Not only is the incubation of the cells at low pH under starvation conditions a form of stress, but also pH_i regulation has to be considered physiologically directly connected to the response of the cells to different stress conditions (2, 27). The demonstrated fact that stationary cells are better able to maintain their pH_i homeostasis is therefore in good agreement with descriptions in the current literature.

The technique described allowed us, furthermore, to identify subpopulations characterized by different pH_i values. There are some reports on the heterogeneity of stress resistance in yeasts (reference 24 and references therein), but to the best of our knowledge, the appearance of two well-defined subpopulations with different pH_i values in a yeast cell sample has not previously been described. Moreover, the well-defined and different behavior of exponentially grown versus stationary cells was observed in terms of appearance and development of these subpopulations as a response to a changing external pH.

In conclusion, the results confirmed the suitability of the protocol for pH_i determination in *S. cerevisiae* cells. The protocol is useful to investigate the effect of the exposure to various environmental conditions on the pH_i and provides the possibility of being combined with other physiological analyses by using flow cytometry. Correlated experiments demonstrated the possibility of applying the protocol also to other yeast species, for example, *Pichia pastoris* (21).

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