

# Real-Time Detection of Hyperosmotic Stress Response in Optically Trapped Single Yeast Cells Using Raman Microspectroscopy

Gajendra P. Singh,<sup>†</sup> Caitriona M. Creely,<sup>†</sup> Giovanni Volpe,<sup>†</sup> Helga Grötsch,<sup>‡</sup> and Dmitri Petrov<sup>\*,†,§</sup>

ICFO—Institut de Ciències Fotòniques, Jordi Girona 29, Nexus II, 08034 Barcelona, Spain, IBMB—Institut de Biologia Molecular de Barcelona, Jordi Girona 18-26, 08034 Barcelona, Spain, and ICREA—Institut Catalana de Recerca i Estudis Avançats, 08015 Barcelona, Spain

**Living cells survive environmentally stressful conditions by initiating a stress response. We monitored changes in the Raman spectra of optically trapped *Saccharomyces cerevisiae* yeast cell under normal, heat-treated, and hyperosmotic stress conditions. It is shown that when glucose was used to exert hyperosmotic stress, two chemical substances—glycerol and ethanol—can be monitored in real time in a single cell.**

The cell stress response is the term for the reaction of a living cell to ambient changes that are potentially harmful, for example, an increase in temperature, pH, saline concentration, or the presence of toxins. In some instances, stress responses may eventually lead to stress tolerance as a long-term defense mechanism against cytotoxic agents.<sup>1</sup>

The biochemical methods and protocols available to monitor the response of living cells to external stresses are unable to monitor changes in situ. Furthermore, they are applied to a certain cell population and not to a single cell. Nonbiochemical methods, such as fluorescence or confocal microscopy, which are used to monitor real-time changes in single cells, normally require the use of exogenous fluorophores or stains. Additionally, only a small range of organelles can be visualized at any one time.

Raman microspectroscopy has been developed to the point where it allows one to study kinetic processes in living cells in real time (see, for example, refs 2–5), and recently, this technique has detected changes in the biochemical makeup of the cell at an earlier stage than is possible with microscopy alone.<sup>6</sup>

Optical tweezers (i.e., optical trapping of micrometer- and submicrometer-sized objects based on radiation pressure exerted

by a strongly focused light beam<sup>7,8</sup>) permit the manipulation of individual cells in their natural environment, with no need for fixing cells to substrates.<sup>9,10</sup> The tight focus required by both Raman microspectroscopy and optical tweezers makes combining these techniques straightforward.<sup>11–22</sup> This optical tweezers Raman microspectroscopy (OTRS) technique has been shown to be capable both of monitoring dynamic cellular processes induced by temperature change<sup>23</sup> and of detecting glutamate in optically trapped single nerve terminals.<sup>24</sup>

In this paper, we take this research forward and apply OTRS to study the fermentation process in a single *Saccharomyces cerevisiae* yeast cell. This cell is widely used as a model for fundamental studies of cell processes, including the cell stress response,<sup>25</sup> as many fundamental cellular processes are conserved from yeast to human cells and corresponding genes can often complement each other.<sup>26</sup> One response of *S. cerevisiae* yeast cells during hyperosmotic stress is to synthesize glycerol, which helps to balance the osmotic pressure across the cellular membrane. When a medium containing a high concentration of glucose is used, the cell switches to fermentation mode and produces ethanol

\* To whom correspondence should be addressed. E-mail: Dmitri.Petrov@icfo.es. Telephone: 0034 934137942, Fax: 0034 934137943.

<sup>†</sup> Institut de Ciències Fotòniques.

<sup>‡</sup> Institut de Biologia Molecular de Barcelona.

<sup>§</sup> Institut Catalana de Recerca i Estudis Avançats.

- (1) Storey, K. B.; Storey, J. M., Eds. *Cell and Molecular Response to Stress: Sensing, Signaling and Cell Adaptation*, Elsevier Science: Amsterdam, 2002.
- (2) Puppels, G. J.; de Mul, F. F.; Otto, C.; Greve, J.; Robert-Nicoud, M.; Arndt-Jovin, D. J.; Jovin, T. M. *Nature* **1990**, *347*, 301–303.
- (3) Schuster, K. C.; Reese, J.; Urlaub, E.; Gapes, J. R.; Lendl, B. *Anal. Chem.* **2000**, *72*, 5529–5534.
- (4) Uzunbajakava, N.; Lenferink, A.; Kraan, Y.; Volokhina, E.; Vrensen, G.; Greve, J.; Otto, C. *Biophys. J.* **2003**, *84*, 3968–3981.
- (5) Chadha, S.; Nelson, W. H.; Sperry, J. F. *Rev. Sci. Instrum.* **1993**, *64*, 3088–3093.

- (6) Huang, Y.; Karashima, T.; Yamamoto, M.; Ogura, T.; Hamaguchi, H. *J. Raman Spectrosc.* **2004**, *35*, 525–526.
- (7) Ashkin, A.; Dziedzic, J. M.; Yamane, T. *Nature* **1987**, *330*, 769–771.
- (8) Grier, D. G. *Nature* **2003**, *424*, 810–816.
- (9) Xie, C.; Li, Y. *J. Appl. Phys.* **2003**, *93*, 2982–2986.
- (10) Greulich, K. O. *Micromanipulation by Light in Biology and Medicine: The Laser Microbeam and Optical Tweezers*; Birkhäuser Verlag: Basel, 1999.
- (11) Thurn, R.; Kiefer, W. *Appl. Spectrosc.* **1984**, *38*, 78–83.
- (12) Lankers, M.; Popp, J.; Kiefer, W. *Appl. Spectrosc.* **1994**, *48*, 1166–8.
- (13) Sasaki, K. *Mater. Sci. Eng.* **1997**, *B48*, 147–152.
- (14) Crawford, K. D.; Hughes, K. D. *J. Phys. Chem. B* **1998**, *102*, 2325–2328.
- (15) Ajito, K.; Torimitsu, K. *Lab Chip* **2002**, *2*, 11–14.
- (16) Xie, C.; Dinno, M. A.; Li, Y. *Opt. Lett.* **2002**, *27*, 249–251.
- (17) Cherney, D. P.; Conboy, J. C.; Harris, J. M. *Anal. Chem.* **2003**, *75*, 6621–6628.
- (18) Sanderson, J. M.; Ward, A. D. *Chem. Commun.* **2004**, *9*, 1120–1121.
- (19) Chan, J. W.; Esposito, A. P.; Talley, C. E.; Hollars, C. W.; Lane, S. M.; Huser, T. *Anal. Chem.* **2004**, *76*, 599–603.
- (20) Houlton, M. P.; Sjöstrom, C. M.; Uibel, R. H.; Kleimeyer, J. A.; Harris, J. M. *Anal. Chem.* **2002**, *74*, 4311–4319.
- (21) Ramser, K.; Logg, K.; Goksör, M.; Enger, J.; Käll, M.; Hanstorp, D. *J. Biomed. Opt.* **2004**, *9*, 593–600.
- (22) Gessner, R.; Winter, C.; Roesch, P.; Schmitt, M.; Petry, R.; Kiefer, W.; Lankers, M.; Popp, J. *ChemPhysChem* **2004**, *5*, 1159–1170.
- (23) Xie, C.; Li, Y.; Tang, W.; Newton, R. J. *J. Appl. Phys.* **2003**, *94*, 6138–6142.
- (24) Ajito, K.; Han, C.; Torimitsu, K. *Anal. Chem.* **2004**, *76*, 2506–2510.
- (25) Erasmus, D. J.; Van der Merwe, G. K.; Van Vuuren, H. J. *FEMS Yeast Res.* **2003**, *3*, 375–399.
- (26) Bassett, D. E.; Boguski, M. S.; Hieter, P. *Nature* **1996**, *379*, 589–590.

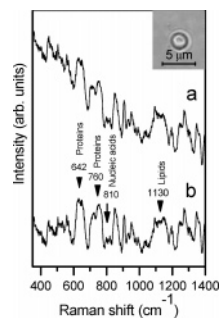
and carbon dioxide. A small amount of glycerol is also produced.<sup>25,27</sup> These products are very important for the industries utilizing the fermentation process.<sup>28</sup> Chromatographic techniques and biochemical assays are currently used to analyze the products of such fermentation and cell stress responses.<sup>27,28</sup> Four stages of fermentation are observed in the growth of yeast batch cultures—the lag phase, log (or exponential) phase, stationary phase, and death phase (phase of decline).<sup>29</sup> In the lag phase of fermentation, cells synthesize new proteins and membrane components, in particular the enzymes required to produce glycerol or ethanol.

We have applied our dual OTRS system<sup>30</sup> to detect glycerol and ethanol in an optically trapped *S. cerevisiae* yeast cell under normal, heat-treated, and hyperosmotic stress conditions. The intention of this paper is to show that during hyperosmotic stress of a yeast cell two chemical substances known to be produced—glycerol and ethanol—can be detected in real time in a single cell. Despite the fact that so many chemical constituents change due to this stress, these two components could be successfully detected and monitored over time. The results were compared with the biochemical data reported in the literature.

## EXPERIMENTAL SECTION

**Sample Preparation.** *S. cerevisiae* yeast cells (haploid strain from Euroscarf, BY4741) were grown in Synthetic Defined media (SDC) with complete supplements under standard conditions. This medium was chosen to minimize the Raman peaks arising from the background spectrum while still providing sufficient nutrients for the duration of the experiment. Hyperosmotic stress was achieved by diluting cells (taken from a log phase culture) in a 100 g/L glucose solution made up in SDC.<sup>27</sup> This dilution brings the cells into a brief lag phase, where the yeast cells are biochemically active but not dividing.<sup>29</sup> The control cells were kept in SDC alone. In all Raman measurements, the cells were diluted such that a single cell could be trapped with no other cells in the surrounding medium.

**Confocal Raman Microscope and Optical Tweezers.** Our experimental setup has been described in detail elsewhere.<sup>30</sup> Briefly, a diode laser operating at 785 nm was used for the excitation of Raman spectra with an average power of <10 mW at the sample. Cells were trapped inside a custom-made holder using a 100- $\mu\text{m}$ -thick fused-silica coverslip. The holder was placed on an inverse Olympus IX 51 microscope equipped with a 100 $\times$  oil immersion objective, NA = 1.25. Backscattered light was collected by the objective and then passed through a holographic notch filter and a confocal system formed by two lenses and a 100- $\mu\text{m}$  pinhole. The spectrometer was a SpectraPro 2500-i from Acton containing a 600 lines/mm grating and incorporating a Princeton Instruments Spec-10 CCD, cooled to  $-100\text{ }^\circ\text{C}$ . A 1064-nm Nd:YAG laser operating at less than 2 mW of power at the sample was used as the auxiliary laser tweezers. A CCD camera attached to the microscope provided optical images during experiments. Raman spectra were recorded, with a spectral



**Figure 1.** Background-subtracted spectrum before baseline subtraction (a) and after baseline subtraction (b). The acquisition time was 180 s. Inset: image of a single trapped yeast cell.

resolution of  $8\text{ cm}^{-1}$ , over a range of  $1050\text{ cm}^{-1}$ , between 350 and  $1400\text{ cm}^{-1}$ . This range was chosen because glycerol and ethanol give strong Raman peaks in this region.

The system was aligned for optimum Raman spectra from a trapped spherical object by using a 5- $\mu\text{m}$ -diameter polystyrene bead. First the optical systems were aligned in such a way that the trap could be achieved at the same place inside the sample holder for both beams. Then the bead was trapped using the 785-nm laser, and the Raman spectrum was recorded in real time. In concordance with Xie and Li,<sup>9</sup> we found in our setup that the optimum alignment for obtaining the Raman spectra of a trapped object can vary greatly depending on its distance from the coverslip. By adjusting the position of the bead inside the sample holder and the orientation of the pinhole of the confocal system, we optimized the signal-to-noise ratio (SNR) for the trapped bead. The SNR for our system was large enough to be able to align the system in real time using a 0.2- or 0.1-s acquisition. After this measurement was made, the 1064-nm tweezers were switched on and used to trap and then move the particle out of the Raman laser beam waist in order to take a background measurement under the exact same experimental conditions.

After the system was aligned for polystyrene beads, measurements were made on living cells. One accumulation of 180-s acquisition was taken for each spectrum. Fluorescence background was removed by fitting a baseline; all the spectra were normalized in power and finally smoothed by applying a Savitsky–Golay filter. For time-resolved measurements, the initial spectrum obtained after trapping the cell was subtracted from each subsequent spectrum to obtain the difference spectra as we were interested in seeing the changes occurring inside the cell when it is under hyperosmotic stress.

The power of the 1064-nm trapping beam was  $\sim 50$  times less than that used in ref 21, and the laser excitation power required was 5 times less than that used in ref 22. These conditions, together with using IR wavelengths for trapping, allow us to study living cells for long periods of time, obtaining kinetics for biochemical processes.

## RESULTS AND DISCUSSION

Figure 1 shows the background-subtracted spectra of a single trapped yeast cell, with tentative peak assignment as indicated in refs 23 and 31, before and after baseline subtraction. This cell

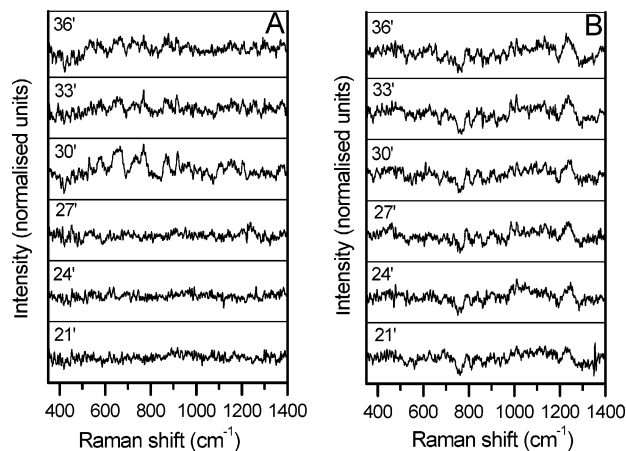
(27) Petrovska, B.; Winkelhausen, E.; Kuzmanova, S. *Can. J. Microbiol./Rev. Can. Microbiol.* **1999**, *45*, 695–699.

(28) Zhao, Y.; Lin, Y. H. *Biotechnol. Lett.* **2003**, *25*, 1151–1154.

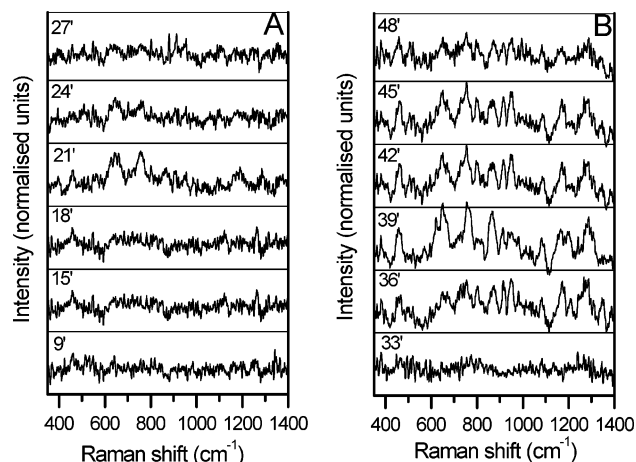
(29) Stanbury, P. F.; Whitaker, A.; Hall, S. J. *Principles of Fermentation Technology*; Pergamon: Oxford, 1984.

(30) Creely, C.; Singh, G. P.; Petrov, D. *Opt. Commun.* **2004**, *245*, 465–470.

(31) Nottingher, I.; Verrier, S.; Haque, S.; Polak, J. M.; Hench, L. L. *Biopolymers* **2003**, *72*, 230–240.



**Figure 2.** Difference spectra obtained under normal conditions (A) and after heat treatment (B). The numbers in the plots represent the time after beginning of each trapping experiment.



**Figure 3.** Difference spectra from the cell under hyperosmotic stress during the initial lag phase (A) and during the late lag phase (B). The numbers in the plots represent the time after the beginning of hyperosmotic stress.

was in SDC, under normal conditions (i.e., not under stress). The spectrum was taken from a cell trapped immediately after diluting the cells in a sample holder.

In glucose minimal medium, the average doubling time of *S. cerevisiae* is 120 min at 30 °C.<sup>32</sup> As a control experiment, we trapped a single cell in SDC under normal conditions and recorded the Raman spectra over 2 h. The experiments were repeated, and the growth of a bud indicated that the trap was not preventing mitosis. It was observed that, on dilution, the cells take ~20 min to adapt to their new medium and start growing. This period is lag phase. It is also worth mentioning here that the growth of a yeast cell in an optical trap is observed to be slow compared to its growth in a free medium.

Figure 2A shows the change in difference spectra for a control yeast cell from 21 to 36 min after trapping. The initial spectrum, taken immediately after diluting the cells in the sample holder, was subtracted from subsequent data to get the difference spectra. Various peaks are observed after ~30 min of trapping. We tentatively assign the peaks at 578 cm<sup>-1</sup> to tryptophan/cytosine

and guanine, 918 cm<sup>-1</sup> to proline ring, the broad band centered at 660 cm<sup>-1</sup> to nucleic acids and proteins, and another broad band centered at 1150 cm<sup>-1</sup> to proteins.<sup>31,33</sup> The appearance of these peaks is related to cell growth while in the optical trap. Peaks are also observed at 738, 770, and 874 cm<sup>-1</sup>. A similar trend is seen for many other yeast cells tested as a control. The spectral difference seen in different cells for different times could be used in the future to identify a particular stage in the yeast cell cycle.

**Heat-Treated Cells.** For heat treatment, yeast cells were kept at 80 °C for 1 h. This treatment results in cell death.<sup>23</sup> Over the course of our experiments, it was interesting to note that heat-treated cells seemed to be more opaque and the signal intensity from them was generally higher compared to the cells that were under normal environmental conditions. This may be due to aggregation of denatured proteins present inside the cell. In recent work by Xie et al.,<sup>34</sup> Raman spectra were taken from yeast cells heated gradually from 25 to 80 °C in steps of 5 °C/5 min; they proposed that, after being heated, the microorganelles in the cell may have more chance to accumulate in the focused laser beam and generate large Raman intensities. Figure 2B indicates that the difference spectra of dead heat-treated cells do not show as large a change as the live control cell spectra do. However, they do show an appreciable rise in both the 1002- and 1234-cm<sup>-1</sup> peaks. Both these peaks are assigned to proteins.<sup>31</sup> Xie et al. also reported a rise in the phenylalanine peak (1002 cm<sup>-1</sup>) as a result of heating the cells to 80 °C, which the authors related to protein denaturation; however, no control experiment data were mentioned.<sup>23</sup> They used a power switching scheme and did not trap the cell in the Raman excitation laser for a long time.

We noted that when the cell is trapped for a long time in the Raman excitation laser beam, the intensity of the signal increases considerably, after some time of trapping the cell, and then it decreases. This effect was observed for all cells – living, dead, or under hyperosmotic stress while it was not observed when we trapped a polystyrene bead (5 μm) for more than 1 h. Since it also occurs for a dead cell, we can exclude the possibility of a biological process behind this. From an optical point of view, the light encounters a complex refractive index distribution inside the cell, because of the presence of the nucleus, vacuoles, mitochondria, and numerous nanometer-sized proteins, ribosomes, vesicles, etc. Theoretical analysis to model the behavior of cells of arbitrary shape in an optical trapping system has been reported,<sup>35</sup> showing that this can result in a torque being applied to the whole cell. This could cause the cell to move with respect to the beam waist. Due to the effect of the confocal aperture, the intensity variation seen due to cell movement is exaggerated. By postprocessing of the spectra obtained we compensated for this effect by spectral power normalization.

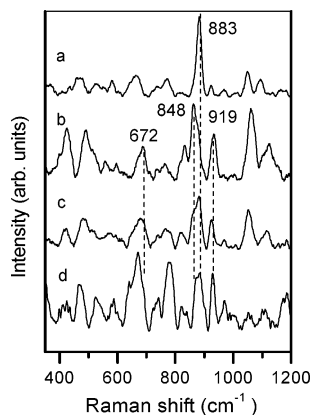
**Hyperosmotically Stressed Cells.** Hyperosmotic stress was achieved by diluting the cells in 10% glucose solution made up in SDC. We concentrated on the study of the stress response of cells that are in the lag phase of fermentation. We studied cells that were in the initial lag phase as well as cells measured 20 min

(32) Brewer, B. J.; Chlebowski-Sledziewska, E.; Fangman, W. L. *Mol. Cell. Biol.* **1984**, *4*, 2529–2531.

(33) Stone, N.; Kendall, C.; Smith, J.; Crow, P.; Barr, H. *Faraday Discuss.* **2004**, *126*, 141–157.

(34) Xie, C.; Goodman, C.; Dinno, M. A.; Li, Y. *Opt. Express* **2004**, *12*, 6208–6214.

(35) Grover, S. C.; Gauthier, R. C.; Skirtach, A. G. *Opt. Express* **2000**, *7*, 533–539.

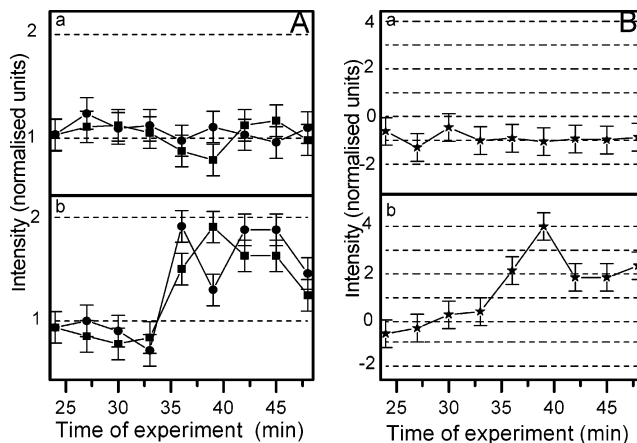


**Figure 4.** Spectra of 10% ethanol (a), 10% glycerol (b), mixture containing 5% glycerol and 5% ethanol (c), and a hyperosmotically stressed yeast cell which is in the late lag phase after 39 min of stress (d). Dotted lines indicate the positions of glycerol and ethanol peaks.

later, which should be in the late lag phase. To study the hyperosmotic stress response in cells in the initial lag phase, we trapped a yeast cell immediately after diluting it in hyperosmotic medium and recorded Raman spectra for  $\sim 30$  min. The difference spectra can be seen in Figure 3A. Well-pronounced peaks are observed after 21 min of trapping. We tentatively assign the peak at  $644\text{ cm}^{-1}$  to tyrosine and the peak at  $757\text{ cm}^{-1}$  to tryptophan.<sup>31,33</sup>

To probe the hyperosmotic stress response in cells in the late lag phase, we kept a yeast cell in hyperosmotic conditions for 21 min and then trapped the cell and recorded Raman spectra for the next 30 min. The difference spectra are as shown in Figure 3B. After  $\sim 36$  min of hyperosmotic stress conditions, well-pronounced peaks are observed in the spectrum for every consecutive measurement. We tentatively assign the peaks at  $758\text{ cm}^{-1}$  to tryptophan,  $866\text{ cm}^{-1}$  to proline,  $918\text{ cm}^{-1}$  to proline and glucose,  $954\text{ cm}^{-1}$  to cholesterol,  $1086\text{ cm}^{-1}$  to lipids and nucleotides, and  $1278\text{ cm}^{-1}$  to the amide III: $\alpha$ -helix.<sup>33</sup> Peaks are also observed at  $462, 654, 798, 1162,$  and  $1194\text{ cm}^{-1}$ . We performed multiple experiments on two kinds of cells, one in early and another in late lag phase. We also carried out experiments where one cell was trapped continuously for a long time under hyperosmotic stress conditions. The results obtained were similar. Numerous control experiments, where the cell does not experience hyperosmotic stress, were carried out by trapping cells for a long time and also by trapping different cells that had been in the same medium for a long time. No spectral changes of this magnitude were observed. Therefore, we believe that this large change is a direct result of the cell stress response.

To investigate whether we could elucidate the changes in concentrations of glycerol and ethanol that arise as a consequence of the glucose hyperosmotic stress, from the spectra, which also included contributions from different cell constituents, we recorded the Raman spectra of pure glycerol and ethanol with dilutions approaching intracellular concentrations for comparison. Figure 4 compares the spectra of 10% ethanol, 10% glycerol, a mixture containing 5% glycerol and 5% ethanol, and the original spectrum corresponding to the 39-min spectrum of a hyperosmotically stressed cell in the late lag phase. This time was chosen because the greatest change appears at this time, as seen in Figure 3B. For the Raman spectra of pure glycerol and ethanol mixtures, it was observed that the presence of a strong glycerol peak at



**Figure 5.** (A) Plots of the normalized intensities of the two peaks of glycerol at  $672\text{ cm}^{-1}$  (■) and  $919\text{ cm}^{-1}$  (●) versus time for a control cell (a) and for a hyperosmotically stressed cell in the late lag phase (b). (B) Plots of the difference in normalized intensities of the ethanol peak at  $883\text{ cm}^{-1}$  and the glycerol peak at  $848\text{ cm}^{-1}$  versus time for a control cell (a) and for a hyperosmotically stressed cell in the late lag phase (b). Error bars represent a confidence interval  $[-2\sigma, +2\sigma]$  corresponding to a probability of 0.95 of finding the signal inside it.

$848\text{ cm}^{-1}$  close to a strong ethanol peak at  $883\text{ cm}^{-1}$  affects the spectra of the mixture of glycerol and ethanol. Similarly, the presence of proteins and lipids also influences the glycerol and ethanol spectra. This influence is greater in the region beyond  $1200\text{ cm}^{-1}$ .

First we tracked the intensities of the peaks near  $672$  and  $919\text{ cm}^{-1}$  in the spectra of hyperosmotically stressed cells in the late lag phase and in the control cells. These peaks correspond to peaks of glycerol, which do not overlap with any ethanol peaks. Figure 5A shows an increase in the normalized intensities of these peaks after 33 min of hyperosmotic stress and is evidence for glycerol production in the cell under stress. No changes were seen for the corresponding control cell spectrum. We could also see a rise and fall in the intensities of glucose peaks. This corresponds to the entering of glucose in the cell and its subsequent biochemical conversion.

To probe the production of ethanol from a stressed cell, we plotted the difference in the normalized intensities of the peaks at  $883\text{ cm}^{-1}$  (ethanol peak) and at  $848\text{ cm}^{-1}$  (glycerol peak) from the spectra of hyperosmotically stressed cell and control cell. As seen in Figure 5B, the stressed cell produces more ethanol than glycerol, as expected in the fermentation process. Error bars shown take into account the errors due to usual error sources such as the noise of electronic equipments (shot noise, dark current noise, and processing noise)<sup>4</sup> as well as the error specific to our experiment such as Brownian motion of a trapped object.

The volume of the detection area of our confocal microscope is  $\sim 1\text{ fL}$ . The increments in the quantities of glycerol and ethanol in the detected volume, as measured at 39 min of hyperosmotic stress, are about 300 and 700 amol, respectively. These values were estimated from the peak heights of the normalized spectra and comparison with the known concentration solutions (5% ethanol is  $\sim 0.86\text{ M}$  and 5% glycerol is  $\sim 0.69\text{ M}$ ). This amounts to the detection of approximately  $2 \times 10^8$  glycerol molecules and  $4 \times 10^8$  ethanol molecules after 39 min of hyperosmotic stress within our confocal volume. This procedure was repeated for other cells

under hyperosmotic stress getting similar results (data not shown).

## **CONCLUSION**

Using Raman microspectroscopy together with Optical tweezers we monitored real-time changes known to occur during the hyperosmotic stress in a single cell. Even using a low excitation power and near-IR laser, the production of glycerol and ethanol could be tracked over time. This shows the ability of this technique to detect real-time changes in cell biochemistry and the potential for elucidating the biochemical processes in living cells at a single-cell level. One of the potential applications lies in studying the effect of drugs and toxins on the biochemistry of living cells.

Further investigation is being carried out on the *S. cerevisiae* cell stress responses.

## **ACKNOWLEDGMENT**

This research was carried out in the framework of ESF/PESC (Eurocores on Sons), through grant 02-PE-SONS-063-NOMSAN, and with the financial support of the Spanish Ministry of Science and Technology. We gratefully acknowledge the ongoing collaboration with Dr. T. Thomson and Marta Soler of the CSIC, Barcelona.

Received for review November 5, 2004. Accepted January 26, 2005.

AC048359J