

# Life at acidic pH imposes an increased energetic cost for a eukaryotic acidophile

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## Summary

Organisms growing in acidic environments, pH <3, would be expected to possess fundamentally different molecular structures and physiological controls in comparison with similar species restricted to neutral pH. We begin to investigate this premise by determining the magnitude of the transmembrane electrochemical H<sup>+</sup> gradient in an acidophilic *Chlamydomonas* sp. (ATCC<sup>®</sup> PRA-125) isolated from the Rio Tinto, a heavy metal laden, acidic river (pH 1.7–2.5). This acidophile grows most rapidly at pH 2 but is capable of growth over a wide pH range (1.5–7.0), while *Chlamydomonas reinhardtii* is restricted to growth at pH ≥3 with optimal growth between pH 5.5 and 8.5. With the fluorescent H<sup>+</sup> indicator, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), we show that the acidophilic *Chlamydomonas* maintains an average cytosolic pH of 6.6 in culture medium at both pH 2 and pH 7 while *Chlamydomonas reinhardtii* maintains an average cytosolic pH of 7.1 in pH 7 culture medium. The transmembrane electric

potential difference of *Chlamydomonas* sp., measured using intracellular electrodes at both pH 2 and 7, is close to 0 mV, a rare value for plants, animals and protists. The 40 000-fold difference in [H<sup>+</sup>] could be the result of either active or passive mechanisms. Evidence for active maintenance was detected by monitoring the rate of ATP consumption. At the peak, cells consume about 7% more ATP per second in medium at pH 2 than at pH 7. This increased rate of consumption is sufficient to account for removal of H<sup>+</sup> entering the cytosol across a membrane with relatively high permeability to H<sup>+</sup> (7×10<sup>-8</sup> cm s<sup>-1</sup>). Our results indicate that the small increase in the rate of ATP consumption can account for maintenance of the transmembrane H<sup>+</sup> gradient without the imposition of cell surface H<sup>+</sup> barriers.

Key words: acidophile, cytosolic pH, membrane potential, energetic cost, *Chlamydomonas* sp.

## Introduction

Extremely acidic aquatic environments, <pH 3, are rare in comparison to the neutral and slightly alkaline conditions that occur in open lakes and oceans (Wetzel, 1975; Palmer et al., 1998). The Rio Tinto, in Southwestern Spain, is one such extreme environment. The river extends 90 km, with a pH range of 1.7–2.5 over its entire length. The pH is heavily buffered with Fe<sup>3+</sup>/Fe(OH)<sub>3</sub> (pKa 2.5) and SO<sub>4</sub><sup>2-</sup>/HSO<sub>4</sub><sup>-</sup> (pKa 2.0) such that rainwater and more neutral pH tributaries do not change the local pH of the river (López-Archilla and Amils, 1999). Low pH dissolves heavy metals such as Fe, Cu, As, Zn, Ni and Ag that are only soluble in trace concentrations at neutral pH. Different regions of the river can reach 269–358 mmol l<sup>-1</sup> Fe, 3.5 mmol l<sup>-1</sup> Cu and 5.5 mmol l<sup>-1</sup> Zn (López-Archilla and Amils, 1999). Despite these seemingly toxic conditions, protists, fungi and bacteria (Amaral Zettler et al., 2002; Durán et al., 1999; López-Archilla et al., 2001), thrive in the river. In nature, acidic conditions, such as in the

stomach of higher organisms or within any of the many forms of acidic lysosomes, are used to denature and destroy macromolecules and to control specially made hydrolytic enzymes that speed the rate of molecular breakdown. It is, therefore, of fundamental biochemical and physiological interest to understand how these organisms are modified to be acid tolerant.

In order to identify the adaptations used to survive in acid, we need to consider first which components of acidophiles are in contact with low pH. For example, acidophilic bacteria have cell surface enzymes that have acid pH optima. A surface iron oxidase from bacterial strain TI-1 has a pH optimum of 3.0 (Takai et al., 2001) while a surface thiosulfate dehydrogenase from *Acidithiobacillus thiooxidans* has a pH optimum of 3.5 (Nakamura et al., 2001). Protistan extremophiles must also cope with acid conditions on the surface of the plasma membrane. Ion channels and transporters are in contact with

the low pH of the extracellular medium and this would minimally require molecular modifications as compared to similar proteins from closely related species growing at neutral pH. In considering the transmembrane  $H^+$  gradient, the internal conditions are as important. There are conflicting reports of the cytosolic pH from acidophiles. *Cyanidium caldarium* (Beardall and Entwisle, 1984; Enami et al., 1986) and *Dunaliella acidophilum* (Gimmler et al., 1989), grown at extracellular pH of 2.1 and 0–1.0, respectively, maintain cytosolic pH of 6.6 and 7.0, respectively. In contrast, *Picrophilus oshimae* (van de Vossenburg et al., 1998), *Bacillus acidocaldarius* (Thomas et al., 1976), *Sarcina ventriculi* (Goodwin and Zeikus, 1987) and *Euglena mutabilis* (Lane and Burris, 1981) grown at pH 0.8–4.0, 3.0, 3.0 and 2.8, respectively, are reported to maintain acidic cytosolic pH of 4.6, 5.5, 4.25 and 5.0–6.4, respectively. The methods used to determine the cytosolic pH of these later examples were a measure of total cellular pH, where the presence of acid-containing organelles could lead to more acidic estimates of cytosolic pH. Identification of cytosolic enzymes from these organisms that are optimally functional at acidic pH would support these measurements.

Maintenance of a neutral pH cytosol in an extracellular environment at pH 2 indicates that a  $10^5$ -fold  $[H^+]$  gradient must exist across the plasma membrane. Apart from acidophiles, there are only a few other reported examples of such large  $H^+$  gradients across cellular membranes, such as in the mammalian stomach ( $10^4$ - to  $10^6$ -fold) and the acidic vacuole in plant cells ( $10^5$ -fold). These  $H^+$  gradients are achieved by a combination of active transport and low permeability to  $H^+$  (Boron et al., 1994; Muller et al., 1996). Maintenance of large, transmembrane  $H^+$  gradients would be an energetically costly endeavor if the membrane were relatively leaky to  $H^+$ . Some measurements have shown that lipid bilayers are orders of magnitude less permeable to  $H^+$  at acidic pH than neutral pH (Gutknecht, 1984). Other measurements indicate that the difference at acidic pH is not so large. For example, the permeability of thermoacidophilic archeal membranes to  $H^+$  show less than an order of magnitude difference between pH 6 and pH 2.5 and are only an order of magnitude less permeable than egg phosphatidylcholine membranes at neutral pH (Komatsu and Chong, 1998). These measurements have been made on purified lipid and are not representative of cellular membranes that contain about 50% protein, including water-filled ion channels,  $H^+$ -cotransporters and other weakly acidic or weakly basic proteins that may increase permeability to  $H^+$ . Based on this information, we hypothesize that eukaryotic acidophiles have an increased energetic cost to survive at acidic pH due to the transmembrane  $H^+$  gradient, which may be orders of magnitude larger in the same unicellular organism growing at pH 2 vs pH 7.

In this study we characterize the electrochemical  $H^+$  gradient that exists across the plasma membrane of a eukaryotic acidophile at pH 2 and pH 7. Cytosolic pH was measured using the ratiometric fluorescent indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), and the plasma membrane potential difference was measured using

intracellular electrodes. We also monitored the relative metabolic activity of these organisms growing in environments at pH 2 and pH 7 by measuring  $O_2$  and ATP consumption in the dark to determine the energetic cost of living in acid.

## Materials and methods

### Cell culture

Clonal cultures of *Chlamydomonas* sp. (ATCC® PRA-125) from the Rio Tinto and *Chlamydomonas reinhardtii* (Carolina Biological Supply, Burlington, NC, USA) were grown in Modified Acid Medium (MAM; University of Toronto Culture Collection) consisting of (in  $mmol\ l^{-1}$ ): 3.8  $(NH_4)_2SO_4$ , 2.2  $KH_2PO_4$ , 2.0  $MgSO_4$ , 0.5 NaCl, 0.07  $CaCl_2$ , including trace metals (in  $\mu mol\ l^{-1}$ ): 46  $H_3BO_3$ , 9.1  $ZnSO_4$ , 1.6  $NaMoO_4$ , 0.8  $ZnSO_4$ , 0.3  $CuSO_4$ , 0.2  $CoCl_2$  with 30.0  $(Na_2)EDTA$  and vitamins (in  $\mu g\ l^{-1}$ ) 1.0 B12, 1.0 biotin and 200 thiamine-HCl. A set of buffers at a final concentration of 10  $mmol\ l^{-1}$  was used to maintain the different pH media. Sulfate served as a buffer at pH 1, 1.5 and 2; Hepes at pH 3, 7 and 7.5; DMGA (3,3-dimethylglutaric acid) at pH 4, MES at pH 5.5 and 6.5, and Bicine (*N,N*-Bis(2-hydroxyethyl)glycine) at pH 8.5. Media of neutral to alkaline pH were made by adding buffers and setting the pH with Tris base. Cultures were kept in an incubator under a 14 h:10 h light:dark photoperiod and maintained at 21°C with an irradiance of 56  $\mu E\ m^{-2}\ s^{-1}$ .

### Relative growth rate measurements

Instantaneous growth rates were acquired for both *Chlamydomonas* sp. and *Chlamydomonas reinhardtii* at different pH values in order to determine their pH tolerance. Instantaneous growth rate ( $r$ ) is calculated as the difference between natural logarithms of chlorophyll fluorescence ( $f$ ) at different points in time ( $t$ ) during exponential growth according to the following equation:

$$r = [\ln f(t_2) - \ln f(t_1)] / dt, \quad (1)$$

Chlorophyll was measured through excitation at 340–500 nm and emission >665 nm using a Turner Designs 10-AU fluorometer (Sunnyvale, CA, USA) with a red-sensitive photomultiplier tube. Culture tubes were gently vortexed to suspend cells uniformly before taking measurements each day near the end of the light cycle. 25 ml of fresh medium was inoculated with 0.5–1 ml of cultured cells to maintain cultures in exponential growth over a period of several weeks.

### Cytosolic pH measurements

The cell-permeant form of the ratiometric  $H^+$ -indicator, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) (Molecular Probes, Eugene, OR, USA), was used to measure cytosolic pH of *Chlamydomonas* sp. Cells were loaded with BCECF by incubation at pH 2 with 10–12  $\mu mol\ l^{-1}$  BCECF for 1 h, resulting in a final cytosolic concentration that gave a similar light intensity at the pH-insensitive wavelength as 6  $\mu mol\ l^{-1}$  dye held between two coverslips 10  $\mu m$  apart. Cells were rinsed 3 $\times$  with culture

medium, concentrated by centrifugation and resuspended in growth medium in order to remove the unloaded dye. A similar procedure was used to load the neutral growing *Chlamydomonas reinhardtii* (catalog no. WW-15-2041, Carolina Biological Supply, Burlington, NC, USA) as a positive control. *C. reinhardtii*, however, was loaded at pH 5 for the same amount of time. Imaging was performed on cells immobilized in 1% low temperature-gelling agarose (Boehringer Mannheim, Germany) on the bottom of small cultures dishes with a #0 coverglass bottom window. Images were acquired with a wide-field Attofluor RatioVision System (Rockville, MD, USA) coupled to a Zeiss 135 Axiovert inverted microscope. Images were collected through a  $\times 100$ , 1.3 N.A. lens using  $440 \pm 10$  nm and  $488 \pm 5$  nm excitation with  $525 \pm 12$  nm emission. The ratio of 488/440 was masked to remove background and out-of-focus light. At the BCECF excitation/emission wavelengths the chlorophyll contributed no autofluorescence. Image acquisition parameters were adjusted so that the small amount of autofluorescence contributed by the cells was no longer detected. The cytosolic pH is presented as the mean  $\pm$  S.E.M.

#### Calibration of fluorescent $H^+$ indicator

*In vitro* calibration of BCECF was performed by loading  $6 \mu\text{mol l}^{-1}$  BCECF in Fisher brand phosphate buffers pH 5–9 (Suwanee, GA, USA). A small volume of buffer,  $4.8 \mu\text{l}$ , was placed between one large #0 coverslip and one  $22 \times 22$  mm coverslip so that the depth of buffer including dye was  $10 \mu\text{m}$ , to simulate the thickness of the cells. Images were collected as described above and the average ratio of the central 90% of the image was used to calibrate the settings.

*In vivo* calibrations were performed to confirm that the cytosolic pH indicator responded to changes in cytosolic pH in the same manner as the Fisher brand buffers. Standard calibration procedures using the ionophores valinomycin and nigericin did not collapse transmembrane  $K^+$  or  $H^+$  gradients in this organism at either neutral or acidic pH. As an alternative, we used weak acids and a weak base to change and clamp the cytosolic pH at different known pH values. The weak acids and base diffuse through the plasma membrane in their uncharged form, thus releasing (acid) or gaining (base)  $H^+$  within the cytosol. The neutral form of the weak acid or weak base will reach equilibrium inside and outside of the cell so that the cytosolic pH will reach equilibrium with extracellular pH. Carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), a protonophore, was then used to eliminate small  $H^+$  gradients and charge build-up across the membrane. Acetic acid, pKa 4.76, was used at pH 5 and 6, *p*-nitrophenol, pKa 7.1, for pH 7 and ammonia pKa 9.2 for pH 8 and 9.

#### Membrane potential difference measurements

Plasma membrane potential differences were measured for cells growing in MAM at pH 2 and pH 7 without trace elements or vitamins. The cell holding chamber consisted of two  $24 \times 60$  mm coverslips spaced 3–3.5 mm apart by three standard sized microscope slides at each end, allowing culture

medium to be held between the two coverslips for several hours. The chamber allowed us to position a holding pipette and recording electrode parallel to the microscope stage. A large-tip holding pipette was fire polished to just under  $10 \mu\text{m}$  outer diameter. Culture media and cells were drawn into the holding pipette such that cells larger than  $10 \mu\text{m}$  could not enter and were held at the tip of the pipette. Plasma membrane potential differences were measured using 1.5 mm outer diameter theta glass. These electrodes were pulled to resistances of 60–90 M $\Omega$  when  $1 \text{ mol l}^{-1}$  KCl was used both as backfill and outside the pipette. Current–voltage (*I*–*V*) relationships were generated with these electrodes to ensure that the cells had been impaled. Intracellular recordings were acquired with Axoclamp 2B controlled with Pclamp 9 (Axon Instruments, Union City, CA, USA). The theta glass was backfilled with  $50 \text{ mmol l}^{-1}$   $K_2SO_4$  during recordings. Only measurements from cells that showed flagellar and contractile vacuole activity before and during impalement were used. To increase the chance of a successful impalement, larger cells were selected for these experiments by filtration through an  $8 \mu\text{m}$  Nuclepore filter (Pleasanton, CA, USA). Cells were resuspended from pH 2 medium into pH 7 medium, giving a final pH of 6.7. After impalement at pH 6.7, the holding pipette was removed and recordings were collected while the cell was impaled on the measuring electrode. An ISMATEC IPC 8 channel peristaltic pump (Glattbrugg-Zürich, Switzerland) was used to exchange pH 6.7 medium with pH 2 medium and then pH 7 medium. Membrane potential differences and *I*–*V* curves were collected at both pH 2 and pH 7. All membrane potential differences are presented as the mean  $\pm$  S.E.M.

#### Measuring $O_2$ consumption

$O_2$  consumption was measured using a miniaturized Whalen-style polarographic probe combined with spatial self-referencing, a signal-to-noise enhancement method. The self-referencing polarographic  $O_2$  electrode, described by Jung et al. (1999), consists of a recessed platinum reactive surface in the tip of a 2–3  $\mu\text{m}$  opening glass microelectrode. Oxygen is reduced at the Pt surface polarized between  $-0.6$  to  $-0.8$  V. In pH 2 medium the current used to maintain the  $-0.8$  V was so great that it saturated the amplifier. The current was brought into range by using a Pt wire reference electrode rather than the Ag/AgCl reference described in the original publication. The Pt wire reference was used at both acidic and neutral pH. Electrodes were polarized using an Ionview amplifier with a polarographic headstage running Ionview software (BRC, Woods Hole, MA, USA). Oxygen consumption could not be measured reliably from single cells so cells were immobilized in 1% low-temperature-gelling agarose (Boehringer Mannheim, Germany) in order to measure consumption from a population of cells at a density between  $10^{10}$ – $10^{11}$  cells  $\text{l}^{-1}$ . After mixing an equal volume of cells in medium with 2% agarose the mixture was taken up into a 1.5 mm diameter capillary glass and allowed to cool before pushing the molded agarose cylinder back into

the medium. Measurements were taken within an hour. Movement of gels between solutions of different pH resulted in pH gradients near the surface of the gels that disappeared within 30 min. This prevented us from measuring  $O_2$  consumption within 30 min after transfer to different pH medium but allowed the pH in the core of the gel to reach the pH of the bath. The agarose did not appear to harm the cells as they continued to divide, embedded within the agarose, for weeks. Measurements of  $O_2$  consumption were made on cells in a simplified culture medium consisting of MAM without trace elements or vitamins. Sulfate was used as the pH buffer at pH 2 while Hepes was used as the pH buffer at pH 7, as described above. Measurements were performed at ambient temperature, 23°C and in the dark to eliminate  $O_2$  efflux due to photosynthesis.

#### Monitoring ATP consumption

Cells normally cultured at pH 2 were transferred to pH 7 medium, concentrated, and washed 3 times with pH 7 medium. Azide-treated cells were incubated for 15 min in 159  $\mu\text{mol l}^{-1}$  azide, at pH 7. This resulted in 1  $\mu\text{mol l}^{-1}$  of cell-permeant azide, hydrazoic acid,  $pK_a$  4.7, based on the following equation  $AH = [A] / [(K_a / [H^+]) + 1]$ , a rearrangement of the  $K_a$  equilibrium expression, where AH is protonated weak acid and [A] is the total concentration of weak acid. A tenfold lower concentration of azide did not have an effect on ATP levels while a tenfold higher concentration resulted in greater than 50% loss of ATP. After loading, cells were rinsed 6 times to lower the extracellular hydrazoic acid concentration to less than 2  $\text{nmol l}^{-1}$  and then dispensed into pH 2 and pH 7 media so that the final concentration of cells matched the controls. Control samples were dispensed into pH 2 and pH 7 media from the first cell concentration step in pH 7 medium.

Total cellular ATP measurements were performed using the Promega ENLITEN kit (Madison, WI, USA) based on the ATP-dependent light output of the Luciferin/Luciferase reaction. Photons were counted with a Zylux FB12 Tube Luminometer (Zylux Corporation, Maryville, TN, USA) averaging counts for 10 s after a 2 s delay. We collected and averaged three 10 s readings from each sample. Luminometer dark counts averaged 15 relative light units (RLU) while counts due to the presence of the Luciferin/Luciferase alone gave a range of 50–400 RLU. Signals from samples ranged between  $10^4$ – $10^6$  RLU. Cell samples were processed by combining 250  $\mu\text{l}$  of cells in native medium with 250  $\mu\text{l}$  of 10% trichloroacetic acid (TCA) to lyse cells and inactivate ATPases. After brief vortexing the sample was left at room temperature (23°C) for 10 min before vortexing again. The sample was neutralized by adding 10  $\mu\text{l}$  of lysate to 40  $\mu\text{l}$  Tris-acetate buffer, pH 8.5. After brief mixing with the pipette tip, 10  $\mu\text{l}$  of this sample was combined with 90  $\mu\text{l}$  of enzyme and buffer provided in the kit, mixed by pipetting and immediately placed in the luminometer counting chamber. This method diluted the initial sample 100-fold, yielding measurements from  $10^5$ – $10^6$  cells.

## Results

### *Chlamydomonas sp. is not an obligate acidophile*

*Chlamydomonas sp.* and *Chlamydomonas reinhardtii* were placed in different pH media to determine their ability to adapt and grow at a range of external pH from 1.0 to 8.5. The *Chlamydomonas sp.* culture was maintained in MAM at pH 2 and pH 6.0 for at least 4 months while *C. reinhardtii* was maintained in pH 7 MAM before starting the experiment. *Chlamydomonas sp.* kept in long-term culture at pH 2 was used to inoculate freshly prepared pH 1, 1.5, 2, 3 and 4 MAM, while cells from long-term culture at pH 6.0 were used to inoculate freshly prepared pH 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 MAM. *C. reinhardtii* from pH 7 MAM was used to inoculate all culture media used. Fig. 1 shows the average instantaneous growth rate (change in chlorophyll fluorescence/day) during the exponential phase of growth for both *Chlamydomonas sp.* and *C. reinhardtii*, performed in triplicate. *Chlamydomonas sp.* did not grow at pH 1.0, 7.5, 8.0 or 8.5, but maintained relatively high growth rates between pH 1.5–6.0 with growth rates dropping off at pH 6.5 and 7.0. Peak growth was the same between pH 2.0–6.0. Cells growing at pH 1.5 for 1 week were transferred to pH 1.0 MAM without measurable growth. *C. reinhardtii*, grown in the same media, did not grow at pH 2 and showed highest growth between pH 5.5–8.5. This indicates that the medium was not toxic, at least to a neutral growing organism.

### *In pH 2 Chlamydomonas sp. maintains near neutral cytosolic pH*

The cytosolic pH of the neutral growing *C. reinhardtii* and the acidophilic *Chlamydomonas sp.* were determined by monitoring the fluorescence intensity of the cell-permeant  $H^+$ -indicator, BCECF. The mean cytosolic pH of *C. reinhardtii*

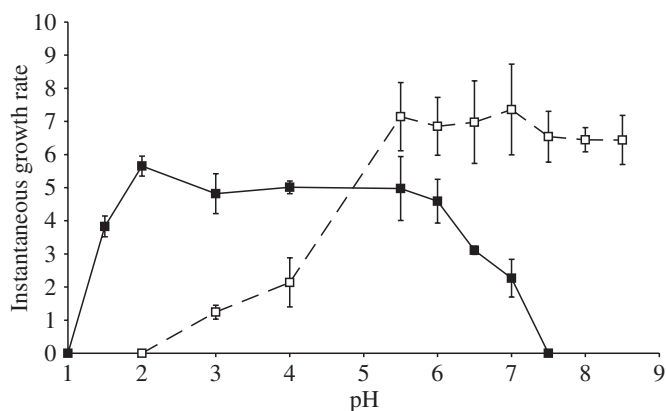


Fig. 1. Instantaneous growth rate of *Chlamydomonas sp.* (filled squares, solid line) and *Chlamydomonas reinhardtii* (open squares, broken line) at different pH values, measured as change in chlorophyll fluorescence per time elapsed; see Materials and methods. The acidophilic *Chlamydomonas sp.* does not grow at pH=1.0 or  $\geq 7.5$ . Growth at pH 2 is not significantly different from growth at pH 3, 4, 5.5 or 6.0 ( $t$ -tests,  $P > 0.2$  for all). *C. reinhardtii* does not grow at pH 2 and has lower growth at pH 3 and 4 but is growing faster than *Chlamydomonas sp.* at pH  $\geq 6.0$ . Values are means  $\pm$  S.E.M. ( $N=3$ ).

was found to be  $7.1 \pm 0.1$  ( $N=17$ ) in pH 7.0 medium. This value is similar to the cytosolic pH of *C. reinhardtii* reported by Braun and Hegemann (1999) growing at extracellular pH 7.4. The mean cytosolic pH of *Chlamydomonas* sp. growing at pH 2 is  $6.6 \pm 0.1$  ( $N=28$ ), range 6.2–7.3. The cytosolic pH range measured is within the linear range of BCECF, pH 6–8. Fig. 2A shows a transmitted light picture of the acidophilic alga. Fig. 2B displays a sample of fluorescence images from three different cells loaded with BCECF, with 488 nm as the  $H^+$ -sensitive wavelength and 440 nm as the  $H^+$ -insensitive wavelength. The dye distribution is not punctate in either the individual image pairs or the resultant ratio image, indicating that compartmentalization of the dye is not an obvious problem. The *in vitro* and *in vivo* calibration curves are shown in Fig. 3 with a histogram showing the average cytosolic pH of 28 cells of *Chlamydomonas* sp. growing in pH 2 medium. An example of an *in vivo* calibration recording is shown in Fig. 4. Initially, the cytosolic pH was measured at extracellular pH 2, the region of the curve between arrows A and B. The pH 2 medium was exchanged with pH 7 medium using a flow system. The beginning of medium exchange is indicated by arrow B. This did not have any effect on the cytosolic pH. Addition of the  $H^+$  ionophore CCCP at pH 7 (arrow C) led to a neutralization of the slightly acidic cytosolic pH. After reaching pH 7 there was a slight acidification before pH 5 medium was added. This is most likely due to the effects of

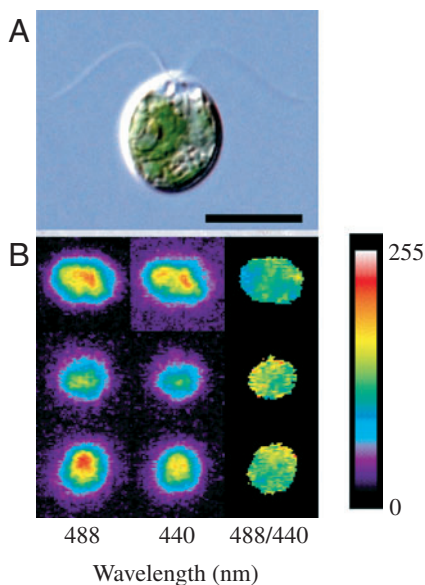


Fig. 2. (A) Transmitted light image of *Chlamydomonas* sp. isolated from the Rio Tinto. (B) Pseudocolored images of BCECF fluorescence in *Chlamydomonas* sp. The color bar to the right indicates relative pixel intensity. Imaging parameters were adjusted to eliminate low-level autofluorescence and to reduce exciting light intensity. The distribution of dye in the 488 nm ( $H^+$ -sensitive) is similar to the distribution of dye in the 440 nm ( $H^+$ -insensitive). Also the distribution of the dye is relatively homogenous. The ratio pairs show homogenous intensity except near the edges where the signal to noise ratio of the ratioed pixel intensity will decrease due to lower signals in the raw images. Scale bar, 10  $\mu\text{m}$ .

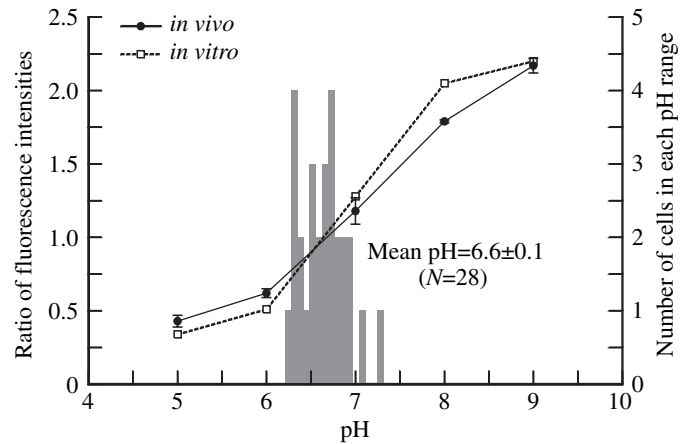


Fig. 3. Mean cytosolic pH histogram of *Chlamydomonas* sp., together with *in vivo* and *in vitro* calibration curves for BCECF, expressed as ratio of fluorescence intensities (pH sensitive/insensitive wavelengths). The calibration curves remain linear between pH 6 and 8. Weak acids and a weak base were used to clamp the cytoplasmic pH to ensure that the dye retained its  $H^+$  binding characteristics in the cells.

CCCP on mitochondria and release of  $H^+$  from acidic organelles into the cytosol. The medium was exchanged with pH 5 medium containing 10  $\text{mmol l}^{-1}$  acetic acid, beginning at arrow D. This caused the cytosolic pH to be clamped at pH 5 (see Materials and methods). Following the pH 5 clamp, the cytosolic pH was clamped at pH 9 when the pH 5 medium was

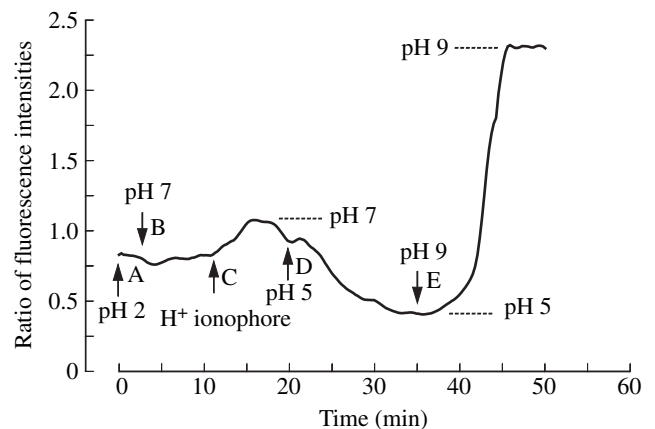


Fig. 4. An example of an *in vivo* BCECF calibration record, obtained by manipulation of cytosolic pH, expressed as ratio of fluorescence intensities (pH sensitive/insensitive wavelengths). A weak acid and weak base were used to clamp the cytosolic pH at 5 and 9 while a  $H^+$  ionophore was used to collapse the pH gradient at pH 7. The cytosolic pH is reported from a single *Chlamydomonas* sp. originally in pH 2 medium (arrow A) and then transferred to pH 7 medium starting at arrow B. A  $H^+$  ionophore was added (arrow C) to collapse the transmembrane pH gradient. A slight acidification occurred just before the medium was exchanged with pH 5 medium containing 10  $\text{mmol l}^{-1}$  acetic acid. Once the cytoplasmic pH had stabilized, the medium was exchanged with pH 9 medium containing 10  $\text{mmol l}^{-1}$  ammonia. This raised the cytoplasmic pH to 9 (arrow E), leading to a full-range calibration for the pH indicator *in vivo*.

exchanged with pH 9 medium containing  $10 \text{ mmol l}^{-1}$  ammonia, beginning at arrow E. These calibrations were repeated in five different cells with additional measurements acquired from two cells having the cytosolic pH clamped at 6 and 8, to complete the linear range of the dye as shown in Fig. 3.

*Acidophile membrane potential difference is close to zero*

The transmembrane electrochemical  $\text{H}^+$  gradient normally comprises both concentration and electrical differences across the plasma membrane. The electrical component was characterized in the acidophile using intracellular recordings. Theta-glass, double-barreled microelectrodes were used to measure the membrane potential difference and also to clamp the membrane voltage to generate  $I$ - $V$  plots. When the backfilling solution was  $1\text{--}3 \text{ mol l}^{-1}$  KCl, impalements often led to ejection of flagella and cell swelling. The number of successful impalements increased when the backfill was changed to  $50 \text{ mmol K}_2\text{SO}_4$  as used in small fungi cells (Blatt and Slayman, 1983), with the pH set to 6.8. Larger cells growing at pH 2 were selected by filtration, suspended in a larger volume of pH 7 medium (final pH 6.7) and impaled. Cells showing flagellar activity were selected for impalement. At the higher magnification used for impalement, contractile vacuole activity could be identified. Only cells showing flagellar and contractile vacuole activity before and during impalement are reported. After impalement a flow system was used to change the pH of the medium to pH 2 and then back to pH 7 while monitoring the membrane potential difference. The membrane potential difference immediately after impalement at pH 6.7 was  $-3.3 \pm 2.6 \text{ mV}$ , and increased to  $+20.8 \pm 2.3 \text{ mV}$  upon exchange to pH 2 medium, returning to  $-3.6 \pm 1.8 \text{ mV}$  ( $N=8$ ) when cells were further exposed to pH 7 medium. The large change in pH affected the surface charge on the recording electrode glass and contributed an artifactual error to the measured potential difference. This error was measured by keeping the tip potential constant through immersion in pH 7 medium in a holding pipette and then exchanging the medium with a flow system over the immersed shaft of the recording electrode. Exchange of the medium from pH 7 to pH 2 gave rise to an increase of  $+22.2 \pm 0.6 \text{ mV}$  ( $N=4$  electrodes) in the measured potential despite the fact that the tip of the electrode was kept in the same medium. When this artifact is removed from the measurement of the membrane potential differences recorded from cells at pH 2, the measured membrane potential difference is  $-1.5 \pm 2.3 \text{ mV}$ .

The  $I$ - $V$  relationship of the cells was determined under these conditions to ensure correct placement of the microelectrode. At pH 7, cells showed smaller inward and outward currents compared to cells at pH 2. Fig. 5A,B shows representative current traces from a *Chlamydomonas* sp. at pH 7 and pH 2, respectively. Currents were recorded at voltages  $\pm 100 \text{ mV}$  about the measured potential difference. Fig. 5C shows the mean currents for seven cells, normalized for cell size, including the corrected voltage offset at pH 2. Impaled cells were best modeled as ovoid with an average length of  $12 \mu\text{m}$

and width of  $10 \mu\text{m}$ . Surface area due to flagella was not included. Cell conductance changes linearly at negative holding potentials but rises more rapidly with increasing voltage at positive holding potentials.

*O<sub>2</sub> consumption is not measurably different between pH 2 and pH 7*

We hypothesized that maintenance of the large transmembrane  $\text{H}^+$  gradient at pH 2 would require more energy than the smaller one at pH 7.0. Real-time  $\text{O}_2$  consumption in the dark was used as an indirect method for measuring energy

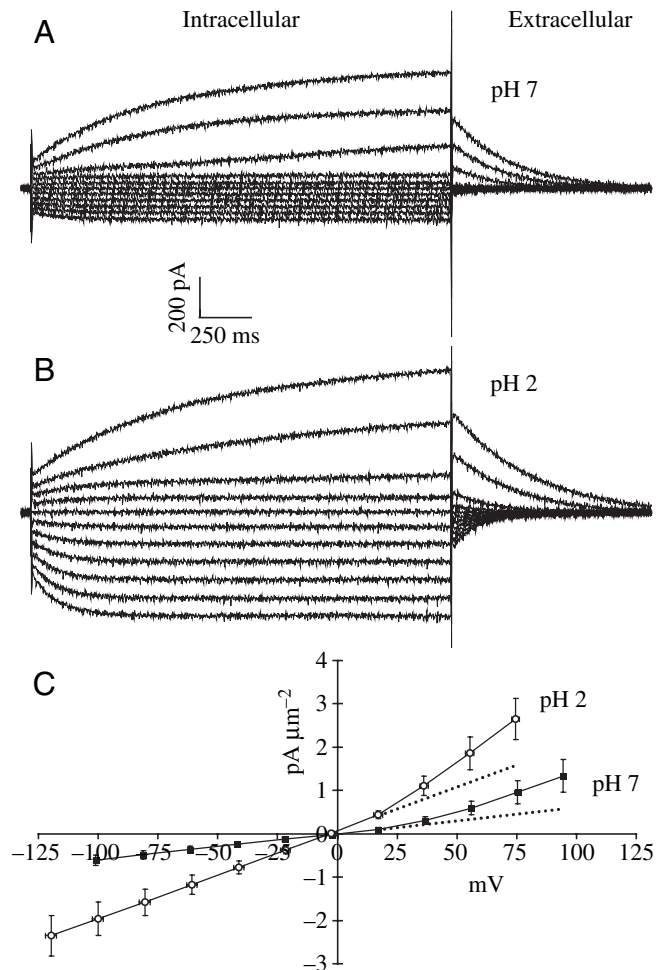


Fig. 5. Current-voltage ( $I$ - $V$ ) relationships acquired from acidophilic *Chlamydomonas* sp. at pH 7 and pH 2. (A,B) Current recordings obtained at  $\pm 100 \text{ mV}$ , acquired from the same acidophile at external pH 7 (A) and pH 2 (B). (C) Summary  $I$ - $V$  plot from seven acidophiles normalized to cell surface area. The currents at pH 2 (open circles) are shifted to more negative potentials to correct for the change in glass charge during the change in extracellular pH. Inward currents are linearly proportional to the change in membrane potential and are greater at pH 2 (open circles) than at pH 7 (filled squares). Outward currents rise more rapidly with voltage than inward currents, as displayed by the difference between the plotted lines and the dotted lines representing a continuation of the slope of the inward currents. Values are means  $\pm$  S.E.M.

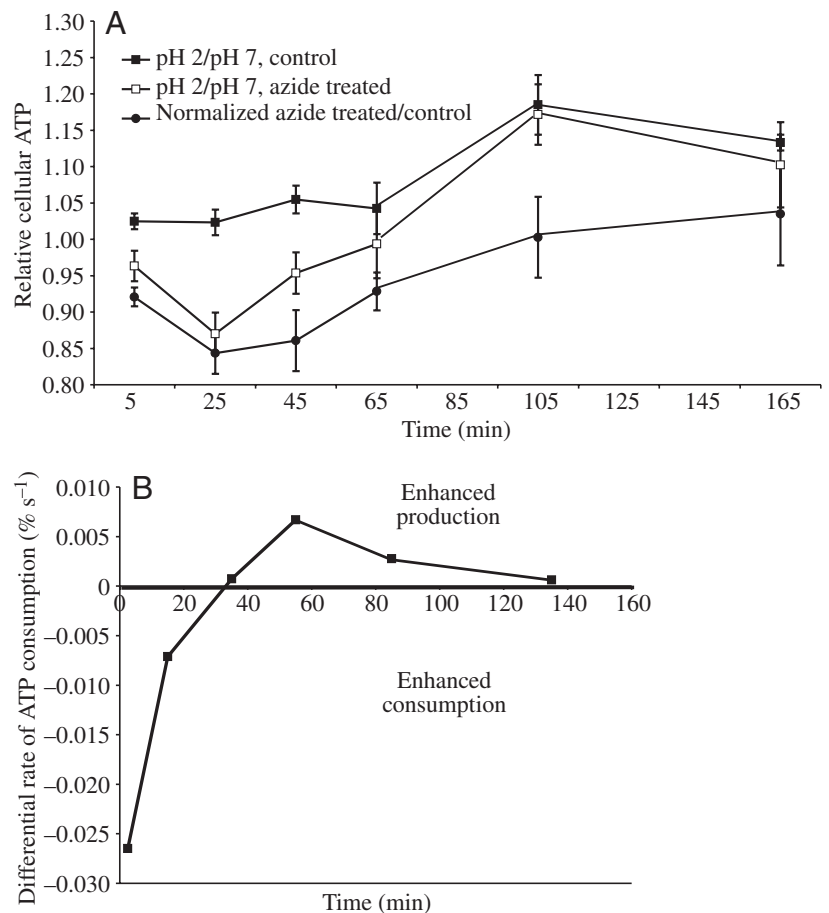
demands at different pH. Measurements were the average of 5 min of O<sub>2</sub> consumption. Using the measurement of the O<sub>2</sub> concentration between two points along a path orthogonal to the agarose cylinder, we calculated the O<sub>2</sub> flux  $J$  according to the Fick equation,  $J = -D(dC/dx)$ , where  $D$  is the diffusion coefficient of O<sub>2</sub> ( $2.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ),  $C$  is the concentration difference between the two points ( $x$ ) 30  $\mu\text{m}$  apart. The differential concentration is calculated as the differential current  $I$  measured between the two points of excursion by the acquisition software and divided by the slope  $S$  of the calibration curve,  $dI/S$  yielding the working Fick equation  $J = -D(dI/Sdx)$ . There was no significant difference in O<sub>2</sub> consumption for a population of cells in pH 2 medium ( $2.8 \pm 0.2 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ) compared to the same population in pH 7 medium ( $3.1 \pm 0.2 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ; paired  $t$ -test,  $P > 0.07$ ,  $N = 12$ ). There was also no difference 1 h later when cells had been transferred back to pH 2 medium ( $3.2 \pm 0.2 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ) from pH 7 ( $3.5 \pm 0.1 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ; paired  $t$ -test,  $P > 0.15$ ,  $N = 6$ ). Integrating the O<sub>2</sub> influx over the surface of the agarose log from a known density of cells we calculated that the average O<sub>2</sub> consumed by a single cell at pH 2 is  $5.7 \times 10^{-18} \text{ mol O}_2 \text{ s}^{-1}$ .

#### Acidic conditions increase ATP consumption

Measuring O<sub>2</sub> consumption as a relative indicator of metabolic cost was a coarse method, as cells can generate ATP by other means without consuming O<sub>2</sub>, and other cellular

enzymes consume O<sub>2</sub> without producing ATP. A more sensitive method for determining the differences in metabolic cost was to measure the rates of ATP consumption at pH 2 and pH 7. As the total cellular ATP is the difference between ATP production and ATP consumption, we loaded cells with sodium azide, an electron transport inhibitor, to arrest ATP production, and then followed the rate of ATP consumption by cells at pH 2 and pH 7. According to our hypothesis, cells in pH 2 medium should consume their cellular ATP pool more quickly than cells growing in pH 7 medium. After incubating cells in azide for 15 min, then rinsing away the azide, cells were moved to fresh medium buffered at pH 2 and pH 7 and the total cellular ATP levels were measured over time. Both groups of azide-treated cells showed an average 30% reduction in the total amount of ATP compared to the levels in untreated controls perhaps due to cell death within the population. The ATP standard curve gave an average 8.3  $\pm$  0.4-fold increase in light emission per order of magnitude increase in ATP concentration. We found that, on average, cells growing at pH 2 contain  $5.2 \times 10^9$  molecules ATP/cell ( $N = 10$  populations of cells). A summary of the time course of changes in cellular ATP levels is displayed in Fig. 6. All data are shown as the ratio between paired groups to eliminate variation between experiments due to the loss of enzyme activity over time and differences in cell density. The first time point is taken just less than 5 min after the shift to pH 2 and pH 7 medium. The

Fig. 6. (A) Relative amounts of cellular ATP over time. Control measurements (filled squares) are displayed as the ratio of cellular ATP in cells placed at pH 2 and pH 7. There is no significant difference in ATP between the cells for at least 1 h. After 105 and 165 min, there is a 17% and 10% increase in the ATP ratio, respectively, which is due to a decrease in ATP levels in the cells at pH 7. Open squares, ratio of the relative amount of ATP in azide-treated cells. Filled circles, normalization of the relative amount of cellular ATP in azide-treated and control cells. There is a significant decrease in cellular ATP in azide-treated cells in pH 2 medium at 5, 25, 45 and 65 min. At 105 and 165 min, ATP levels have recovered in the cells growing in pH 2 so that there is no longer a significant difference between the cells growing at pH 2 and pH 7. (B) Rate of change of the relative amounts of ATP in azide-treated cells at pH 2 and pH 7. The difference is plotted as the percent of the total cellular ATP pool at the middle time point between the two measuring points in A (filled circles). Relatively higher ATP consumption occurs for nearly 40 min, after which time the cells in pH 2 medium produce relatively more ATP. At 135 min the rate of ATP change is nearly the same in cells growing at pH 2 and pH 7. The greatest rate of consumption was 0.027% of the total cellular ATP pool per second. This was the average for the first 5 min of measurement in A (filled circles), plotted here at 2.5 min.



control curve (filled squares) in Fig. 6A is the ratio of the ATP-dependent light emission from cells in pH 2 medium to cells in pH 7 medium. Neither control group was treated with azide. There was no significant difference in ATP levels between cells growing in pH 2 compared to pH 7 for the first 65 min after moving to a different pH ( $P > 0.2, 0.5, 0.4, 0.2$  for 5, 25, 45 and 65 min, respectively,  $N=10$ ). However, at 105 min and 165 min there was, relatively, 17% and 10% more ATP measured from cells in pH 2 than pH 7. The raw data show that this is due to a decrease in ATP levels in cells at pH 7. The second curve (open squares) in Fig. 6A shows the ratio of the ATP levels of cells at both pH treated with azide while the third curve (circles) is the normalization of the ratio of azide-treated cells to their control pH group for each trial.

We now consider only the normalized ATP curve (circles). There is an 8% decrease in the total cellular ATP of azide-treated cells at pH 2 compared to azide-treated cells at pH 7 between 0 and 5 min ( $P < 0.001$ ; paired  $t$ -test,  $N=7$ ). ATP levels decrease further in pH 2 at 25 min after transfer, where cells have 16% less ATP than cells growing at pH 7 ( $P < 0.002$ ). Recovery of ATP levels begins to occur at 45 min and is obvious by 65 min after treatment with azide, when ATP levels are only 14% and 7% less than in cells growing at pH 7 ( $P < 0.02$  and  $0.03$ , respectively). ATP levels have fully recovered in cells growing at pH 2 by 105 and 165 min to the extent that there is not a significant difference in ATP between cells in pH 2 and pH 7 ( $P > 0.3$  for both). Fig. 6B shows the rate of change of ATP levels, which is the slope of the normalized curve in Fig. 6A. We assume that there is no difference in the amounts of cellular ATP for azide-treated cells at  $t=0$  such that the normalized curve is equal to 1 at  $t=0$ . In Fig. 6B the slopes of the individual segments of the normalized curve in Fig. 6A are plotted midway between the original time points. Negative values indicate a faster rate of ATP depletion while positive values indicate a faster rate of ATP production. The greatest rate of ATP depletion occurred as soon as the azide-treated cells were moved to pH 2. ATP decreased until about 30 min, after which time it slowly increased until the ATP levels in the pH 2 grown cells matched the ATP levels in the pH 7 cells. As azide is a weak acid it begins diffusing out of the cells as soon as it has been removed from the extracellular medium. The loss of azide from the cytosol makes it possible for the cells placed in pH 2 medium to recover their cellular ATP levels. This implies that the most accurate measurement of the difference in ATP consumption occurs at the beginning of the recording, when ATP production is most effectively inhibited.

### Discussion

Unlike previously characterized eukaryotic acidophiles such as *Cyanidium caldarium* (pH 0–4; Doemel and Brock, 1971) or *Dunaliella acidophila* (pH 0.4–2.0; Fuggi et al., 1988), *Chlamydomonas* sp. is able to grow over a wide range of pH from 1.5–7.0 (Fig. 1). The permissive pH range ends abruptly below pH 1.5 and above pH 7.0. Below pH 1.5 the osmotic concentration of the medium may become an important factor.

Cells plasmolyze when shifted from medium at pH 2 to pH 1. Growth still did not occur when cells that had acclimated to growth at pH 1.5 were inoculated into medium at pH 1.0. *C. reinhardtii*, commonly grown in neutral pH, did not grow at pH 2, and optimal growth occurred at pH  $\geq 5.5$ .

There could be many reasons for the lack of growth of *Chlamydomonas* sp. above pH 7.0, including restrictions due to the working pH range of cell wall enzymes or even reversal of the electrochemical  $H^+$  gradient. For example, the cell-wall-removing enzymes of *C. reinhardtii* have relatively no activity outside of their working pH ranges. The vegetative lytic enzyme has relatively no activity below pH 7 (reported by Jaenicke et al., 1987) or pH 5 (reported by Matsuda et al., 1995), while the gametic lysin does not work below pH 3 or above pH 12 (Jaenicke et al., 1987). The reduced growth of *C. reinhardtii* below pH 5.5, measured here, is consistent with the more acidic working pH range of the autolysin enzyme reported by Matsuda et al. (1995). With respect to the  $H^+$  gradient, the electrochemical driving force reverses at extracellular pH 6.6, due to the average cytosolic pH of 6.6 and near zero membrane potential difference, indicating that transport processes coupling  $H^+$  influx to nutrient uptake would no longer occur. One possible explanation for this wide range of tolerance is that single cells must be able to deal with the acidic pH of the river. However, cells growing at high density, such as mats, may be able to neutralize the pH of the local environment, as has been demonstrated with *C. reinhardtii*, the nonacidophilic species used in these studies, which was able to neutralize the culture medium originally set at pH 4 and pH 6 (Lustigman et al., 1995).

*Chlamydomonas* sp. maintains a mean cytosolic pH of 6.6 in extracellular medium of pH 2.0, so it is 3.2 times more acidic than *C. reinhardtii*, which maintains a mean cytosolic pH of 7.1 in an extracellular pH 7.0. The neutral cytosolic pH is similar to other acidophilic algae like *Cyanidium caldarium* (Beardall and Entwisle, 1984; Enami et al., 1986) and *Dunaliella acidophilum* (Gimmler et al., 1989). The cytosolic pH of the acidophile does not change dramatically between extracellular medium at pH 2 or pH 7. This indicates that the cytosolic milieu of these acidophilic algae are not too different from similar organisms growing under neutral conditions. It also indicates that the cells are preferentially maintaining a slightly acidic cytosol even under more neutral conditions, reversing the electrochemical driving force on  $H^+$ . We did measure a higher variation in cytosolic pH from cells growing in medium at pH 2, i.e. a range of nearly 1 order of magnitude. This may be a unique property of the acidophile. While lighting conditions change cytosolic pH in *C. reinhardtii* (Braun and Hegemann, 1999), the conditions used for imaging *Chlamydomonas* sp. and *C. reinhardtii* in this paper were identical.

The electrochemical  $H^+$  gradient of the acidophile is lower than predicted based on the negative resting potential difference of other protists. A weakly negative membrane potential difference was measured using theta-glass microelectrodes with 50 mmol  $K_2SO_4$  backfill,  $-1.5 \pm 2.3$  mV at



pH 2 and  $-3.6 \pm 1.8$  mV at pH 7. Tip potentials measured between pH 7 MAM and  $100 \text{ mmol l}^{-1}$  KCl were +5 mV (data not shown) for the theta-glass electrodes backfilled with  $50 \text{ mmol K}_2\text{SO}_4$ . It is possible that the near zero membrane potential difference could be due to excessive damage during impalement, although we do not think that this was the case. Excessive damage during impalement led to ejection of flagella, perhaps in a  $\text{Ca}^{2+}$ - or  $\text{H}^+$ -dependent manner, similar to *C. reinhardtii* (Quarby and Hartzell, 1994). If damage occurred when impaled at neutral pH, flagellar ejection would have occurred when the pH was lowered to 2. We employed rigorous impalement criteria to keep poor impalements out of the analyzed data. The  $I-V$  plots also help confirm that the microelectrodes are in fact in the cell. Interestingly the  $I-V$  plots acquired from the acidophile look similar to  $I-V$  plots acquired from *Neurospora* under acid load (Sanders et al., 1981). Specifically, membrane conductance is greater during *Neurospora* acid load, a phenomenon observed in the current study when *Chlamydomonas* sp. was in acidic conditions. Further study of ionic conductances in *Chlamydomonas* sp. may lead to the first characterization of ion channels in a eukaryotic acidophile.

Microelectrodes were used to measure the membrane potential difference of another acidophile, *Dunaliella acidophila* (Remis et al., 1992). Here  $1 \text{ mol l}^{-1}$  choline chloride was used as the backfilling solution to measure an average membrane potential difference of +48.5 mV at pH 1.0. However a  $-36.5$  mV membrane potential difference was measured at pH 7. This may reflect different mechanisms of maintaining cytosolic neutrality, for example  $\text{H}^+$  efflux through a plasma-membrane-bound pump vs  $\text{H}^+$  sequestration via an intracellular  $\text{H}^+$  pump on cytoplasmic organelles such as the V-type  $\text{H}^+$  ATPase pump associated with the contractile vacuole. The contents of the contractile vacuole of *Paramecium multimicronucleatum* are more acidic (pH 6.4) than the cytosol (pH 7.0) when grown in medium at pH 7.0 (Stock et al., 2002). Of the two compartments of the contractile vacuole, the decorated spongione labels with antibodies to the V-ATPase  $\text{H}^+$  transporter while the smooth spongione that fuses with the plasma membrane does not (for a review, see Allen and Naitoh, 2002). This indicates that  $\text{H}^+$  can be taken up into an intracellular compartment and released without the use of a plasma-membrane-bound  $\text{H}^+$  transporter. *Dunaliella acidophila* possesses a well-characterized P-type  $\text{H}^+$  ATPase to maintain cytosolic neutrality (Sekler et al., 1991). The  $\text{H}^+$ -ATPase transcripts increase with decreasing extracellular pH (Weiss and Pick, 1996). *D. acidophila* also contains two contractile vacuoles, but they may not be very active due to the hypersaline environments in which the organism is found. *Chlamydomonas* sp. also has at least two contractile vacuoles that may help to neutralize cytosolic pH; it is grown in more dilute conditions than *D. acidophila* so may rely more heavily on  $\text{H}^+$  extrusion via the contractile vacuole rather than a plasma-membrane-bound  $\text{H}^+$  transporter. Use of a  $\text{H}^+$  transporter that never comes into contact with the harsh

extracellular conditions may explain the ability of *Chlamydomonas* sp. to survive such a wide pH range.

Transmembrane ionic gradients are maintained by low permeability to ions and ionic transporters that pump ions against their electrochemical gradients. The greater the permeability to  $\text{H}^+$  the more energy the cell must expend in order to maintain the gradient. This could occur either by direct ATP-dependent transport of  $\text{H}^+$  out of the cytosol, or via coupled export, where a symporter or antiporter uses the driving force of a different ion to remove  $\text{H}^+$  from the cytosol. In the latter case, energy would be needed to reset the gradient of the coupled ion. In order to test the hypothesized increase in energy demand we compared the rates of  $\text{O}_2$  consumption and ATP consumption in cells growing at pH 2 with those growing at pH 7. Initially we found that the rates of  $\text{O}_2$  consumption, measured using an  $\text{O}_2$  electrode, were not different in cells growing at acidic pH. Also, under normal conditions, the cellular ATP levels are similar at pH 2 and pH 7, at least for 1 h after transfer to medium of different pH. However, upon reduction of ATP production by an electron transport inhibitor we were able to measure a relatively high rate of ATP consumption by cells in medium at pH 2. The inhibitor is a weak acid, which diffuses out of the loaded cells over time, so that the relatively high rate of ATP consumption dropped to zero within 40 min and then reversed, yielding relatively high production after 40 min (Fig. 6B); cells therefore recovered near normal amounts of ATP after around 135 min. The most accurate measurement of the difference in the rate of ATP consumption is thus during the first few minutes, when the greatest inhibition of ATP production occurs. Cells at pH 2 consume, on average, nearly 0.03% more of the total cellular ATP pool per second than cells at pH 7. For comparison, we calculate that the cells at pH 2 consume  $\text{O}_2$  at a rate that could produce nearly 0.4% of the total cellular ATP pool  $\text{s}^{-1}$  if all of the consumed  $\text{O}_2$  was used to generate ATP. Considering these two values we find that the cells at pH 2 consume about 7% more of the ATP produced each second than cells at pH 7; i.e. cells at pH 2 are working 7% harder than cells at pH 7. This small difference in ATP consumption could account for our inability to detect differences in the rate of  $\text{O}_2$  consumption, as the standard error of the measurements was just over 6% of the basal  $\text{O}_2$  consumption.

If we assume that the increase in ATP consumption is primarily used to maintain the transmembrane  $\text{H}^+$  gradient we can estimate the permeability of the plasma membrane to  $\text{H}^+$  using the relationship that net  $\text{H}^+$  flux across the plasma membrane ( $J_{\text{H}^+}$ ) is the difference between the passive influx ( $P_{\text{H}^+}dC$ ) and the active efflux (relatively higher rate of ATP consumption/cell surface area), where  $P_{\text{H}^+}$  is the cellular permeability coefficient for  $\text{H}^+$  and  $dC$  is the electrochemical  $\text{H}^+$  gradient:

$$J_{\text{H}^+} = (P_{\text{H}^+}dC) - (\dot{V}_{\text{ATP}}/A).$$

$\dot{V}_{\text{ATP}}$  is the differential rate of ATP consumption and  $A$  is cell surface area. It is assumed that  $J_{\text{H}^+}$  is essentially zero. If ATP

production was inhibited sufficiently, there could be H<sup>+</sup> influx leading to cytosolic acidification and our present assumption would lead us to underestimate the permeability to H<sup>+</sup>. The electrochemical H<sup>+</sup> gradient is nearly a 40 000-fold difference comprising only the H<sup>+</sup> concentration difference.  $\dot{V}_{\text{ATP}}$  can be converted to H<sup>+</sup> transport knowing the stoichiometry of ATP consumption to H<sup>+</sup> transport. The P-type H<sup>+</sup> transporters found in other acidophilic algae (Ohta et al., 1997; Weiss and Pick, 1996) use one ATP molecule to pump out one H<sup>+</sup> ion. With a 10<sup>4.6</sup>-fold [H<sup>+</sup>] gradient and an average cell diameter of 10 μm we find that the permeability to H<sup>+</sup> would be about 7.0×10<sup>-8</sup> cm s<sup>-1</sup>. This value is slightly lower than the 10<sup>-4</sup>–10<sup>-7</sup> range found in lipid membranes at neutral pH (Deamer and Akeson, 1994), but could be an underestimate, as contractile vacuole H<sup>+</sup> extrusion employs V-type H<sup>+</sup> transporters that pump 2–3 H<sup>+</sup> ions per ATP molecule hydrolyzed (for a review, see Sze et al., 1999). It could also be an underestimate as the azide was rinsed from the cells at neutral pH before the cells were transferred to pH 2, allowing more time for azide to leave the cells. Nonetheless, this calculation indicates that a small increase in the rate of ATP consumption could maintain a large H<sup>+</sup> gradient for a membrane that is relatively permeable to H<sup>+</sup>.

Our results show that *Chlamydomonas* sp. survives a wide range of extracellular pH, maintains a relatively neutral cytosolic pH, and burns ATP at a faster rate when living in acidic than in neutral conditions. The protection mechanism could be entirely due to active H<sup>+</sup> extrusion into a cytosolic vacuole, a hypothesis based on the relatively higher rate of ATP consumption, near zero membrane potential difference and tolerance to a wide range of extracellular pH. The contractile vacuoles of *Chlamydomonas* sp. may be able to help maintain near neutral cytosolic pH without H<sup>+</sup> transporters or exchangers being exposed to the harsh extracellular environment. This implies that the primary structure of H<sup>+</sup> transporters in this acidophile may be no different from those found in neutral growing protists. The plasma membrane and cell wall are still in contact with the extracellular environment and certainly there is a higher conductance in the plasma membrane under acidic conditions. Two classes of enzymes are thus identified that may have evolved to function at acidic pH: plasma membrane channels and cell wall lysins.

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