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The acid stress response of the cyanobacterium Synechocystis sp. strain PCC 6308

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Abstract The cyanobacterium *Synechocystis* sp. strain PCC 6308 has been shown to exhibit predictable physiological responses to acid stress. Originally isolated from a Wisconsin lake, this cyanobacterium grows optimally under alkaline conditions in the laboratory. After acid stress at a pH of between 4.4 and 7.7, cells return to exponential growth following a lag phase. The organism's response to this tolerable acid stress involves cell concentration-dependent neutralization of the external medium to pH 6 or above within 5 min, maintenance of a transmembrane pH gradient, and maintenance of photosystem II efficiency. Lethal acid stress, at a pH below 4.4, results in the formation of aggregates of denatured proteins observed as granules near the cell periphery, the disruption of the transmembrane pH gradient, cell color change to blue, and damage to photosystem II.

Keywords Cyanobacteria · Acid stress · pH effects

Introduction

The flexibility of the metabolism of microbes enables them to respond and adapt to environmental changes outside their range for optimal growth (Poole 1999). Research on environmental stresses, such as nutrient starvation, temperature stress, salt stress and pH stress, has revealed several mechanisms that enable bacteria to survive under adverse conditions (Allen et al. 1980; Olson 1993; Foster 1992). Acid stress responses have been studied in a variety of microorganisms, such as *Escherichia coli* (Gale and Epps 1942), *Salmonella typhimurium* (Foster, and Hall 1990), *Streptococcus mutans* (Hamilton 1991), *Helicobacter pylori* (Mooney et al. 1990), *Listeria monocytogenes* (O' Driscoll et al. 1996) and *Lactococcus lactis* (Rallu et al. 1996).

The strategies used to survive acid stress vary among the microorganisms observed. Some microbes utilize amino acid decarboxylases to neutralize the external environment (Gale and Epps 1942), while others utilize arginine catabolism to increase the pH of the cell interior (Casiano-Colon and Marquis 1988; Rallu et al. 1996). Other acid tolerance mechanisms include decreased permeability of the cell envelope to protons caused by changes in lipid composition, such as elevated levels of cyclopropane fatty acids (Jordan et al. 1999; Brown et al. 1997), so that cells have an improved ability to maintain internal pH. *S. typhimurium* (Foster 1993, 1995) and *Clostridium perfringens* (Villarreal et al. 2000) exhibit acid tolerance responses (ATRs) that enable cells pre-exposed to acidic pH to survive more severe acid challenges (<pH 4) better than cells that were not pre-exposed. ATR, as well as immediate response to acid stress, involves the induction of acid-shock proteins and depends on several regulatory systems (Foster and Moreno 1999).

There have been relatively few studies showing how cyanobacteria tolerate acid stress, although their responses to nutrient stress, heat shock and salt stress have been studied extensively (Inoue et al. 2000; Joset et al. 1996). While cyanobacteria are found in nearly all ecosystems (Tandeau de Marsac et al. 1993), ecological observations have shown that cyanobacteria have a preference for alkaline conditions (Kratz and Myers 1955). From studies of the distribution of cyanobacteria in 22 lakes and natural thermal and non-thermal gradients in Yellowstone National Park in the pH range 1.9–8.6, and from studies with acidified lake water enrichment culture experiments, Brock (1973) found no cyanobacteria at pH lower than 4–5, although eukaryotic algae are present. In contrast, in a study of 400 lakes on the Swedish West Coast, Almer et al. (1974) found that cyanobacteria were always present at pH as low as 3.7, although there was reduced phytoplankton biomass and diversity. Cyanobacteria were completely absent in picoplankton if the pH fell below 4.5 in ten lakes (Steinberg et al. 1998).

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It has been shown that acid-tolerant cyanobacteria maintain a neutral cytoplasmic pH, although how they maintain a strong transmembrane gradient is unknown (Steinberg et al. 1998). Kallas and Castenholz (1982), using ^{31}P-NMR spectroscopy, showed that the maintenance of alkaline internal pH was destroyed by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, suggesting an active-transport process was involved. A strong light- or energy-dependent buffering of the cytoplasm over a range of external pH was also shown (Kallas and Dahlquist 1981).

The major hypothesis tested in this work was that a cyanobacterium would respond to low pH stress in a determinable physiological fashion and that these responses could then be predictable for the cells' survival. This communication describes acid stress responses of the cyanobacterium *Synechocystis* sp. strain PCC 6308 and identifies several characteristics of the cells' response to both tolerable and lethal pHs. Studies of growth, pigmentation, internal pH (pH_i) and external pH (pH_o), and of intracellular granules formed during pH stress were carried out in order to characterize these predictable responses.

Materials and methods

Strain and growth conditions

Synechocystis sp. strain PCC 6308 is a unicellular, non-nitrogen-fixing cyanobacterium that produces cyanophycin when environmentally stressed (Allen et al. 1980). This bacterium was originally isolated from a Wisconsin lake by G.P. Fitzgerald (described in Allen 1966), and grows optimally at around pH 8 in the laboratory. Cultures were maintained in flasks of BG-11 (minimal salts) medium (Allen 1968) on a shaker (100 rpm) at room temperature under 98 μmol photons m^{-2} s^{-1} of light. Under these conditions, the minimum exponential generation time of this species was 30 h.

Acidic pH stress

Exponentially growing *Synechocystis* sp. strain PCC 6308 cells were pH-stressed by centrifuging the cell cultures and resuspending the cell pellets in pH-adjusted BG-11 medium. BG-11 medium was acidified using HCl. Centrifuging alone did not inhibit growth (data not shown). The pH of the medium was measured before inoculation, using a Corning pH meter, after the medium had been autoclaved and had cooled. Cell growth was measured using a Klett colorimeter with a red filter or a Hitachi spectrophotometer at 750 nm. Cell pH_o during pH stress was followed over time using a Corning pH meter to measure the supernatant pH. Experiments at each pH were done in duplicate at least three times.

Acid tolerance response growth experiments

Exponentially growing cells (OD_{750nm} 0.96) were diluted to an OD_{750nm} of 0.45 with BG-11 medium. Cultures were pre-stressed at pH 5.1 for 1 h by centrifuging 90 ml of culture in a Sorvall centrifuge at 10,400×*g* for 10 min, and resuspending the cell pellet in BG-11 medium at pH 5.1. The cultures were placed on a shaker for 1 h under otherwise standard growth conditions. These cultures were then re-stressed by resuspending them in BG-11 medium at pH 3.5. The growth of the re-stressed cultures was compared to the growth of control cultures and cultures pH-stressed at pH 3.5 with no pre-stress treatment. Similar growth experiments were carried out with cells that were stressed at pH 3.5 after pre-stress at pH 5.5 for 24 h, and with cells stressed at pH 4.3, 2.9 and 3.5 after a 1-week pre-stress at pH 4.3.

Determination of cell viability using SYTOX Green dye

Stock solutions of SYTOX Green (Molecular Probes) fluorescent dye from the ViaGram Red Bacterial Gram Stain and Viability Kit (Molecular Probes) were prepared according to the protocol provided by Molecular Probes. Light exposure, which causes the dye to fade, was minimized by using aluminum-foil-wrapped microfuge tubes to incubate the samples and to store the dye stock. Because of the pH sensitivity of the dye, all cell samples were resuspended in pH 7 BG-11 medium prior to mixing with SYTOX Green solution. Samples were added to slides evenly coated with 2% agarose in BG-11 before being viewed by bright-field and fluorescence microscopy using a Nikon X-Phot FX microscope. Bright-field microscopy was used to determine the total number of cells in a given field. Dead cells were visualized with SYTOX Green dye and fluorescence microscopy. Pictures were taken using SPOT Basic software.

Measurement of chlorophyll *a* fluorescence

The ratio of variable to maximum fluorescence of chlorophyll *a* (F_v/F_m) during pH stress was measured using a PAM fluorimeter (Valz) as a measure of the photochemical efficiency of photosystem II. To stress the cells, the pellet produced by centrifuging 20 ml of cells (OD_{750nm}=1.5), was resuspended in 10 ml of pH 1.8, 3.0 or 4.9 BG-11 medium. Two ml of this culture were placed in the chamber of the PAM fluorimeter, and the cell sample was incubated in the dark for 10 min by covering the chamber with a dark cloth. The sample was then exposed to a 20 s pulse of far red light (>700 nm), followed by a saturating pulse of white light. The F_v/F_m ratio (yield) determined by the fluorimeter using these methods was used as a measure of the photochemical efficiency of photosystem II.

Determination of internal pH using in vivo ^{31}P-NMR spectroscopy

Exponentially growing cells (with a final OD_{750nm} of 88–692)) were centrifuged and resuspended in 300–500 μl of medium plus 100 μl of D_2O. The suspension was placed in a 5-mm NMR tube containing a capillary filled with an aqueous solution of methylene diphosphonic acid (MDPA) which served as an external chemical-shift reference. The position of the MDPA reference line had been previously determined with respect to 85% H_3PO_4. Spectra were acquired at ambient probe temperature on a Bruker Avance 400 MHz NMR spectrometer operating at 161.98 MHz for ^{31}P. Spectra were run with a 6 μs pulse width, 0.4 s recycle delay, 16 K data points, 13 kHz sweep width, and between 100 and 1,600 scans. After a control spectrum was taken, the cells were centrifuged briefly and the pellet was resuspended in 10 ml of BG11 medium of various pH values, for 3 min, to produce the pH stress. The cells were centrifuged again, resuspended in 300–500 μl of the same medium and observed in the spectrometer. The change in the chemical shift of the inorganic phosphate (P_i) peak was used to determine internal pH based on a method used by Lawrence et al. (1997) in which the chemical shift of inorganic phosphate as a function of pH was determined at the ionic strength of cyanobacteria. Spectra were also taken of stressed cell supernatants to determine whether cell lysis had occurred.

Pigmentation studies

Cyanobacterial pigments were analyzed using a Perkin-Elmer Lambda Bio 40 UV/Vis spectrometer. Whole-cell samples were placed in quartz cuvettes, which were turned such that the frosted

sides were in the light path in order to offset the light scattering caused by whole cells (Shibata 1959).

Granule and water-soluble protein isolation and characterization

Two cell cultures (each 250 ml), one stressed at pH 3.7 for 3 days that contained granules, and the other of stationary-phase cells that contained cyanophycin, were broken and the cell contents processed using the isolation procedure for cyanophycin (Allen 1988). The cell cultures were centrifuged at 10,400×*g* for 10 min, and each of the cell pellets was resuspended in 5 ml of ddH_2O and broken using a French pressure cell at 0.08–0.1 MPa. The crude extracts were then centrifuged at 27,000×*g* for 10 min. The supernatants of each (water-soluble crude extract), which contained water-soluble proteins, were decanted and saved. The precipitates were washed with ddH_2O and Triton X-100 before being extracted for cyanophycin with 0.1 N HCl (Allen 1988).

The water-soluble crude extract and the 0.1 N HCl extracts from the pH-stressed and stationary-phase cultures were analyzed using SDS-PAGE. The top portion of the putative granule layer was also prepared for SDS-PAGE by carefully resuspending it in 250 µl of ddH_2O without disturbing the bottom layer of the pellet, which was in contact with unbroken cells and cell membranes. Each of the cell samples were treated with 1×LDS sample buffer (Novex) and 1×sample reducing agent (Novex) before carrying out SDS-PAGE using a Novex X-CELL mini gel system with a 4–12% Bis-Tris gel under reducing conditions. The gel was stained with Gelcode R-250 stain (Pierce) and destained with ddH_2O.Cells were also placed on a slide coated with 1.5% agarose in BG-II and examined using a Leica TCS SP confocal microscope.

Results

Final culture OD_{750nm} and growth rate both decreased with increasingly acidic pH stress. Typical growth curves of control and acid-stressed cultures are shown in Fig. 1. Cell cultures stressed between pH 4.4 and 7.7 grew, following a lag of about 24 h, at rates from 30 to 100% of control cells. Cultures pH-stressed below pH 4.4, when inoculated to an OD_{750nm} of 0.4, did not grow and cells became blue.

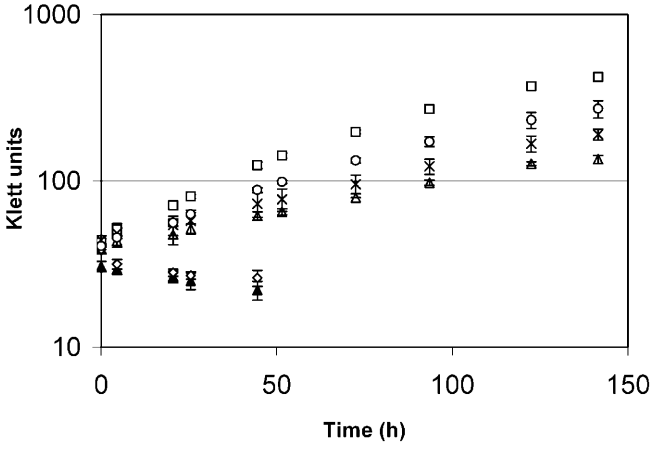

Fig. 1 Growth curve of *Synechocystis* sp. strain 6308 grown at different starting pHs under conditions described in Materials and methods. ❑ Controls, ❍ pH 7.7, × pH 5.5, Δ pH 4.4, ◊ pH 3.0, ▲ pH 1.7. *Bars* on each symbol indicate the variance between two samples at each pH

Since the generation time of *Synechocystis* sp. strain PCC 6308 was 30 h, as compared to much shorter generation times of the *Salmonella typhimurium* used in published work on ATR (Foster 1995), several different times for pre-stressing were used. All experiments using pre-stressed cells showed that pre-stressing at pH 5.1 did not enable cells to grow when stressed below pH 4.4, suggesting that no ATR is shown by *Synechocystis* sp. strain 6308.

Since this strain of *Synechocystis* does not grow well on plates, SYTOX Green fluorescent dye (Molecular Probes), which enters dead cells through compromised cell membranes, was used as a measure of viability. Red fluorescence of chlorophyll *a* showed that the majority of the cells in cultures stressed at pH 4.4 for 5 days were viable (Fig. 2C). Nearly all the cells that were stressed at pH 3.7 for 5 days stained green and therefore were not vi-

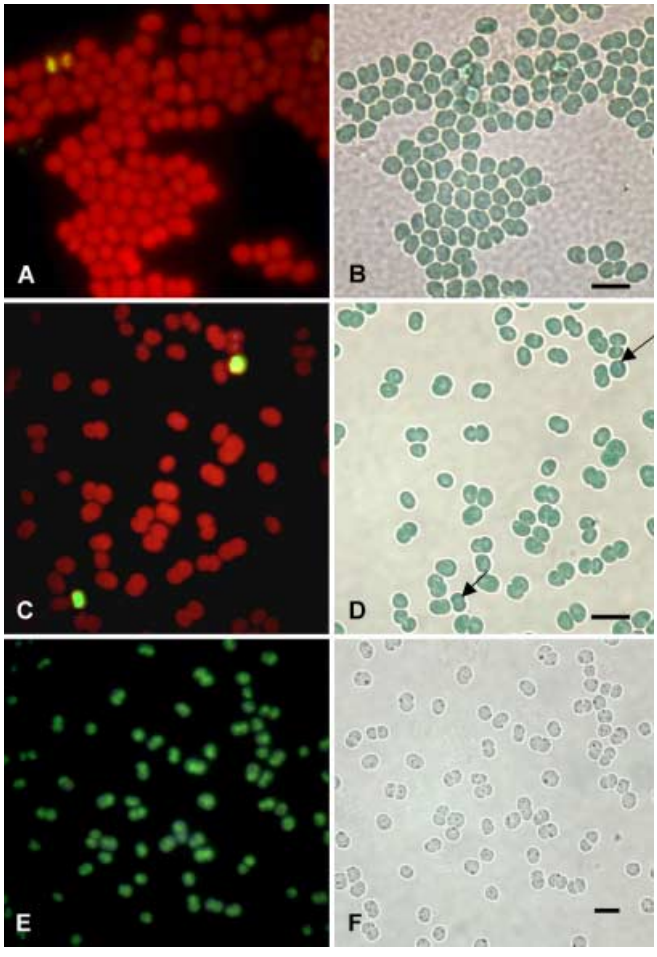

Fig. 2A–F Fluorescence and bright-field micrographs of pH-stressed *Synechocystis* sp. strain PCC 6308 after viability staining. Control cells and cells stressed at pH 4.4 and pH 3.7 were stained with SYTOX Green dye (Molecular Probes) and observed using a Nikon X-Phot FX fluorescence microscope. **A** Fluorescence micrographs of SYTOX-Green-stained control cells. **B** Bright-field micrographs of **A**. **C** Fluorescence micrographs of SYTOX-Green-stained cells stressed at pH 4.4 for 1 week. **D** Bright-field micrographs of **C**. *Arrows* indicate dead cells. **E** Fluorescence micrographs of SYTOX-Green-stained cells stressed at pH 3.7 for 5 days. **F** Bright-field micrographs of **E**

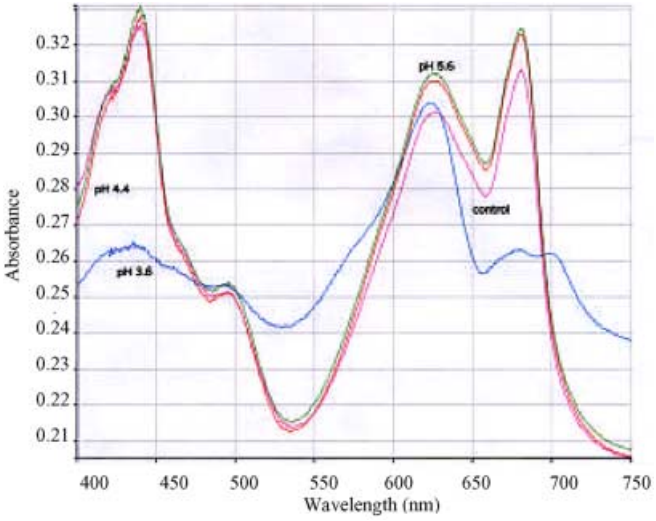

Fig. 3 Absorption spectra of pH-stressed *Synechocystis* sp. strain PCC 6308. The absorption spectra of cells 54 h after pH stress at pH 5.6, pH 4.4 or pH 3.6 were taken using a Perkin Elmer Lambda Bio 40 spectrophotometer

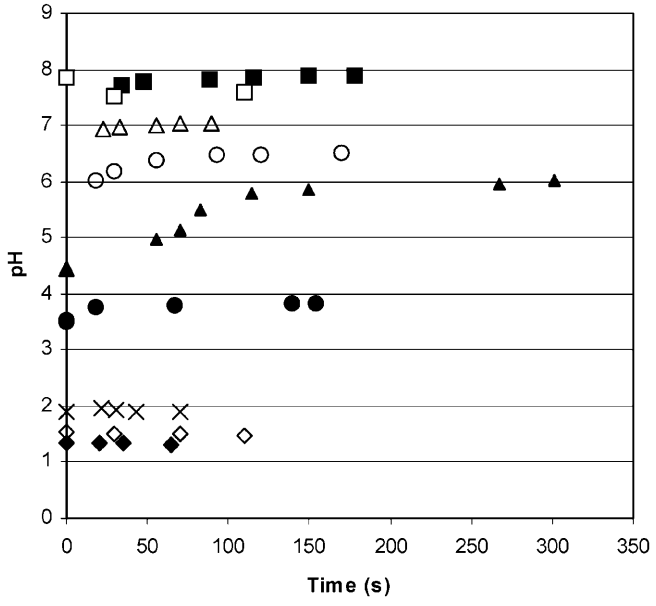

Fig. 4 External pH over time of *Synechocystis* sp. strain PCC 6308 during low pH stress. Cells were pH-stressed at pH 7.8 (control), pH 4.4, pH 1.5 and pH 3.5 at OD_{750nm} 0.54 and OD_{750nm} 5.4, and the pH of the cell suspensions were measured using a Corning pH meter. ■ OD_{750nm}=0.54 pH 7.8 (control), ❑ OD_{750nm}=5.4 pH 7.8 (control), ▲ pH 4.4 stressed OD_{750nm}=0.54, Δ pH 4.4 stressed OD_{750nm}=5.5, ● pH 3.5 stressed OD_{750nm}=0.54, ❍ pH 3.5 stressed OD_{750nm}=5.4, ◆ pH 1.5 stressed OD_{750nm}=0.54, ◊ pH 1.5 stressed OD_{750nm}=5.4, × pH 1.9 stressed OD_{750nm}=0.54

able according to this method (Fig. 2E). These cells were also blue after 3 days of pH stress due to the degradation of chlorophyll *a* without loss of phycocyanin pigments. The supernatants of the cultures, however, remained colorless. Figure 3 shows that cell cultures stressed at pH 3.6 had a significantly reduced amount of chlorophyll *a*, which absorbs at 420 and 680 nm, as compared to cells stressed at pH 4.4 and pH 5.6, which had similar absorption spectra to control cells. A new shoulder in absorbance at 700 nm was observed in cells at pH 3.6. Cells stressed below pH 3.5 turned yellow and lost their phycocyanin peak as well (data not shown).

Cells that survived low pH stress increased the pH of their growth medium (pH_o). Figure 4 shows that cell density at the time of pH stress influences the immediate post-stress pH of the growth medium. More concentrated cells increased the pH_o more, and at a higher rate, than less concentrated cells. Cell cultures stressed at pH 1 did not show any increase in supernatant pH regardless of cell concentration during stress. Control cells also did not significantly increase pH_o within the 10-min experiment time, regardless of cell concentration. Cell cultures stressed at pH 3.5 at high cell concentrations (OD_{750nm}= 5.4) increased the supernatant pH to 6 or above, 2 pH units more than cells that were at OD_{750nm}=0.54 at the time of pH 3.5 stress. In both the low and high concentrations of cells stressed at pH 4.4, the pH_o increased to 6 or above. All cultures that increased pH_o to 6 or above after pH stress were able to grow after a lag phase.

^{31}P-NMR spectroscopy was used to determine pH_i, to demonstrate the absence of polyphosphate granules in pH-stressed cells, and to show the absence of P_i in supernatants of stressed cells (data not shown). The P_i peak in cells that were resuspended in media at a non-tolerable pH (1.6) shifted upfield by 2.1 ppm, indicating that the pH_i had decreased below 6, the lowest value detectable by this method. P_i was abundant in pH-stressed cells, but neither it nor phycocyanin was present in the cell supernatant, suggesting that cells did not lyse (data not shown). Resuspension in pH 4.5 and pH 5.7 media caused no change in the chemical shift of the P_i peak, with the pH_i remaining at 8.0. Control cells, resuspended in medium of pH 11, displayed a peak of P_i at the same ppm as pH 8.0 phosphate buffer.

Chlorophyll *a* fluorescence was used to determine the photochemical efficiency of photosystem II during pH stress. Table 1 shows that 10 min after pH stress at pH 4.9, cells had an F_v/F_m value of 0.549. This value was similar to that of control cells, which had an average F_v/F_m of 0.546. Cells stressed at pH 3.0 had an F_v/F_m of 0.149, and those stressed at pH 1.8 had ratio values of 0 (F_v/F_m was not above normal noise levels).

Unique to cells that are stressed for 2 days at pH 3.5–4.0 is the presence of multiple, small intracellular

Table 1 Ratios of the variable over maximum fluorescence (F_v/F_m) of chlorophyll a during pH stress of *Synechocystis* sp. strain PCC 6308

pH stress	F_v/F_m
control (no pH stress)	0.546±0.003 (*n*=5)
pH 4.9	0.549±0.006 (*n*=3)
pH 3.0	0.149±0.019 (*n*=3)
pH 1.8	0 (*n*=3)

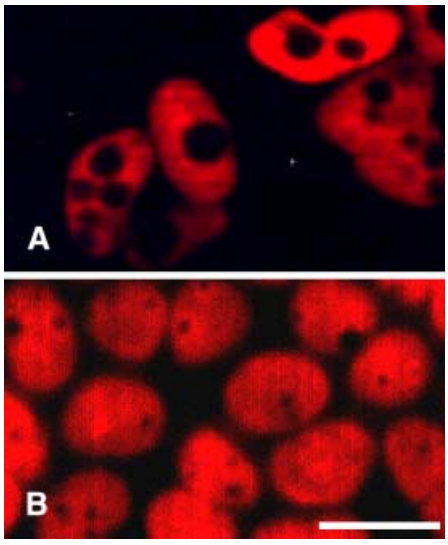

Fig. 5A, B Confocal micrographs of *Synechocystis* sp. strain 6308. Cells were examined using a Leica TCS SP confocal microscope. **A** light-limited stationary-phase cells, with cyanophycin granules. **B** pH-stressed at pH 3.7 for 3 days. The samples were prepared by placing cells on a slide coated with 1.5% agarose in BG11 medium. *Marker* 5 μm

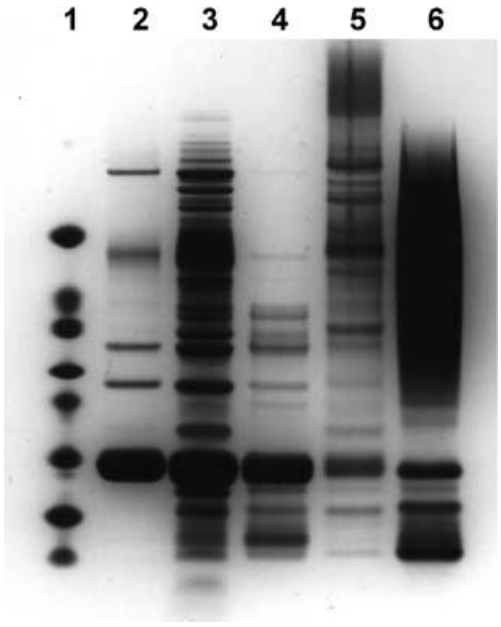

Fig. 6 SDS-PAGE of stationary-phase and pH-stressed *Synechocystis* sp. strain PCC 6308 crude extracts. Stationary-phase cells were grown for 4 weeks under normal laboratory conditions, and pH-stressed cells were grown for 3 days in pH 3.7 BG 11 medium. Cells were French-pressed and centrifuged to separate water-soluble proteins from insoluble cellular components and unbroken cells. *Lanes 1* Low molecular mass standards (Sigma): 66, 45, 36, 29, 24, 20, 14.2, 6.5 kDa; *2* crude extract (water-soluble proteins) from pH-stressed cells, 14.3 μg protein; *3* crude extract (water-soluble proteins) from stationary-phase cells, 20.8 μg protein; *4* 0.1 N HCl cyanophycin extract from pH-stressed cells, 6.6 μg protein; *5* ddH_2O suspension of granule layer from pH-stressed cells 8.0 μg protein; *6* 0.1 N HCl cyanophycin extract from stationary-phase cells, 30.5 μg protein

granules located near the cell periphery. These granules are different in appearance, both by bright-field microscopy (Fig. 2F) and by confocal microscopy (Fig. 5), from cyanophycin granules, which are large and typically distort the cell shape (Fig. 5). Cell fractionation, granule isolation and SDS-PAGE of pH-stressed cells with granules, as well as of stationary-phase cells containing cyanophycin, showed that the granules differed biochemically from cyanophycin. The water-soluble crude extract of the pH-stressed cells was more blue in color than the extract from the stationary-phase cells, which was dark green.

The water-insoluble cell pellets of each of the cells also differed. The cell pellet from pH-stressed cells contained two layers, an olive-green top layer, which was hypothesized to be pH-stress granules, above a dark green layer of unbroken cells and cell membranes. The layer containing the putative pH-stress granules was not present in stationary-phase crude extracts, was distinct in color and location from the layer of unbroken cells and cell membranes, did not contain whole cells when examined under the light microscope, and was not soluble in 0.1 N HCl or ddH_2O. The pellet from stationary-phase cells contained three layers: an orange carotenoid layer above a white cyanophycin layer and a dark green bottom layer of unbroken cells and cell membranes.

SDS-PAGE of the unbroken cell pellets from both pH 3.7-stressed and pH 10 stationary-phase cells showed that the two cultures contained the same protein profile (data not shown). However, the proteins are located in different places within the two types of cells. Figure 6 shows that the polymer cyanophycin, which ranged in size from 30 to 120 kDa, was extracted from stationary-phase cells (lane 6) but not from pH-stressed cells (lane 4). The water-soluble crude extract from pH-stressed cells (lane 2) contained many fewer proteins than the same extract from stationary-phase cells (lane 3). Lane 5, the putative pH-stress granules suspended in ddH_2O, shows that the granule layer contained a large number of proteins, many of which were not present in the water-soluble crude extracts of the pH-stressed cells (Fig. 6, lane 1, and Table 2). Proteins that were not present in the water-soluble crude extract from pH-stressed cells, but present in the putative granule layer as well as in stationary-phase cell extracts, are proteins of molecular mass 97, 85, 53, 37, 22, 12, 10, 7.9 kDa (Table 2). The majority of cellular proteins in stationary-phase cells were in the water-soluble crude extract (lane 3), not the cyanophycin pellet (lane 6). In cells exposed to pH stress, there were comparatively fewer proteins in the crude extract fraction (lane 2), and the majority of cell proteins were shown to be in the granule fraction (lane 5).

Discussion

The ability of the cyanobacterium *Synechocystis* sp. strain PCC 6308 to tolerate pH stress as low as pH 4.4, in media

Table 2 Molecular masses (kDa) of protein bands present in SDS-PAGE (Fig. 6) of water-soluble crude extracts from pH-stressed cells and from stationary-phase cells, 0.1 N HCl-extracted "cyanophycin" pellet from pH-stressed cells and ddH_2O suspension of the granule layer from the pellet of pH-stressed cells

Crude extract stationary-phase cells	Crude extract pH-stressed cells	0.1 N HCl extract pH-stressed cells	ddH_2O granule layer pH-stressed cells
157			
138			
132			
126			
116			
106	106		106
97			97
85			85
78			
63	63	63	63
53		53	53
42		42	
40		40	
37		37	37
34	34	34	
26	26	26	
23		23	22
18–20	18–20	18–20	18–20
12		12	12
10			10
9.5		9.5	
7.9			7.9

nearly 10,000 times more acidic than its optimal pH, is remarkable considering the physiological changes caused by an increase in external proton concentration. Changes in the proton concentration of a cell's environment (pH_o) can affect the dissociation rate of CO_2, the electrical charge of the cell-wall surface, ion transport systems and membrane potentials. Acidic pH_o can interfere with the function of outer-surface cell components such as pili, chemoreceptors, cell walls, exopolysaccharide, periplasmic proteins and flagella. Changes in external pH, such that pH_i homeostasis is lost, can cause disruption of the plasma membrane, protein denaturation and loss of enzyme function, as well as damage to macromolecules or ionization of nutrient molecules, which affects the availability of these compounds to the cell (Park et al. 1996). The dissociation of protein functional groups is directly affected by pH stress, and changes in pH_o and pH_i can interfere with enzyme activity.

Several conclusions can be drawn about how *Synechocystis* sp. strain 6308 responds to pH stress. At low cell concentrations, stress at pH 1–2 is immediately lethal; cells rapidly change color to yellow-green, they show a rapid decrease in OD_{750nm}, they do not maintain a transmembrane pH gradient, their F_v/F_m is zero, and they do not form granules. Cell cultures stressed at pH 3–4 at an OD_{750nm} of 0.4 are not killed immediately, but turn blue after 2–3 days, do not grow, and form granules at the cell periphery. Stress at pH 4.4 and above is tolerated by cells at OD_{750nm} of, or greater than, 0.4. These cells return to growth, after a lag of about 24 h, at rates similar to control cells and neither change their pigmentation nor form granules. They maintain a transmembrane pH gradient.

Cyanobacteria normally increase their pH_o due to photosynthesis (Baas-Becking et al. 1960). Unstressed *Synechocystis* sp. strain 6308 cells increase their pH_o by 1–2 units over several days, but cells stressed at a tolerable pH increase their pH_o, from 4 and above to pH 6 and above, within 5 min (Fig. 4). These latter cells then continue to increase their pH_o, but at a lower rate, similar to that of control cell cultures. Cells placed in the dark do not grow and do not increase the pH_o (data not shown). This suggests that cells must photosynthesize to increase external pH. FTIR spectroscopy of lyophilized spent medium showed the absence of any new covalently bonded compounds in pH-stressed cell supernatants as compared to control cells (data not shown). Preliminary experiments suggest that ammonia may be excreted into the medium (Shea, K., Wellesley College, unpublished data) by pH-stressed cells; this could be responsible for the increase in pH_o. Ammonia would have been lost during lyophilization of the spent medium.

Cell density could be an important factor in pH stress tolerance. It has been shown that cell density is important for the initiation of growth in cultures of pH-stressed *L. lactis* (Augustin et al. 2000), an observation consistent with data for this cyanobacterium. As shown by Fig. 4, increase in pH_o following pH shock is cell concentration-dependent; more concentrated cell cultures raise their pH_o at a faster rate than lower concentration cell cultures, further suggesting that cells are excreting something to increase the pH_o. An increase in pH_o to pH 6 or above is a good predictor of the ability of cyanobacteria to grow after pH stress. Why pH 6 is important is not known, although it has been found that below pH 6 *Anabaena* sp. strain PCC 7120 cells stop evolving oxygen (Giraldez-Ruiz et al. 1997). If the acid stress response of one bacterium involved the neutralization of its local environment, this response would be intensified in a denser culture.

The increase in pH_o observed in these experiments is not due to cell lysis. First, the medium did not turn blue from the release of phycobiliproteins, which are water-soluble pigments released upon cell lysis. Second, in vivo NMR spectroscopy showed that very dense suspensions of pH-stressed cells increase the amount of intracellular P_i over time, but P_i is not detected in the cell supernatant. Other microorganisms have also been found to respond to pH stress by increasing pH_o. When engulfed by the host macrophage, the pathogenic yeast *Histoplasm capsularis* survives by neutralizing the acidic environment of the phagosome compartment within the macrophage through an unknown process (Eisenberg et al. 1993). Low pH also induces the production of amino acid decarboxylases in *E. coli*, which have been found to neutralize the external environment by decarboxylating amino acids such as lysine and arginine (Gale and Epps 1942; Meng and Bennett 1992). The basic amine compounds that are secreted neu-

tralize the medium, although this buffering effect is insignificant unless there is a high concentration of organisms (Park et al. 1996).

It is unclear how cells that can tolerate pH stress maintain pH homeostasis inside while neutralizing the external medium. The ^{31}P-NMR results suggest that the cells in media above pH 4.4 maintain a pH gradient across the cell membrane. Dilworth and Glenn (1999) showed that if pH_i homeostasis was disturbed, even small shifts at alkaline pH resulted in slowed growth. Zilberstein et al. (1982) suggested that the process of cell division in *E. coli* is pH sensitive, and that pH_i could serve a central role in the regulation of metabolism and cell growth. Changes in pH_i of pH-stressed *Synechocystis* sp. strain 6308 could potentially be involved in the growth lag of pH-stressed cells.

A reduced viable cell population could also explain why cells stressed at pH values lower than optimal have longer lag periods. Survival of the culture after pH stress is most likely not due to adaptation because cultures stressed at pH 4.4 and above consistently returned to growth, suggesting that there is always a population of cells in a culture able to deal effectively with pH stress. Studies of cultures stressed at pH 4.4, using SYTOX Green viability dye, showed that the majority of a cell population stressed in this way does not have compromised membranes and is most likely viable (Fig. 1C). Nearly all cells stressed at pH 3.5 were shown to have compromised membranes (Fig. 1E), and observations from plating experiments further support the hypothesis that the cells in these cultures are not viable (data not shown). In addition, these cells did not increase in OD_{750nm} after stress, turned blue, and formed granules. The data in Table 1 suggest that photosystem II may have been damaged or down-regulated during pH stress. Cells (OD_{750nm} of 3.0) stressed at pH 3.0 had significantly lower F_v/F_m values than control cells. In contrast, cells at the same concentration stressed at the tolerable pH of 4.9 had F_v/F_m values similar to control cells. Maximum values for F_v/F_m for nutrient-replete phytoplankton are approximately 0.65 when all photosystem II reaction centers are photochemically competent (Falkowski et al. 1994).

Adaptive ATR is an important part of the pH stress response in a variety of bacteria such as *S. typhimurium* (Foster 1992), *E. coli* (Slonczewski 1992) and *Mycobacteria* sp. (O'Brien et al. 1996), although some bacteria, such as *Shigella flexineri* (Hengge-Aronis 1993), that are able to tolerate acid stress do not possess this response. Pre-adaptation is thought to induce the production of protective proteins, which, when synthesized, protect the cell from future pH stress (Villarreal et al. 2000). *Synechocystis* sp. strain PCC 6308 does not appear to have an ATR. Pre-exposure at a sub-lethal pH for 1 h, 24 h, or 1 week did not enable cultures to then survive lower pH challenges.

Cells stressed at a tolerable pH did not exhibit changes in pigmentation (Fig. 3). Cells stressed at pH 3.6 turned blue after 2–3 days as a result of the degradation of chlorophyll *a*, which absorbs at 420 and 680 nm. A new absorption peak was seen at 700 nm in these cells compared to control cells and cells stressed at a tolerable pH (Fig. 3), which suggests that properties of the pigment may also have been changed by the stress. The new shoulder in absorbance at 700 nm observed in cells at pH 3.6 is not pheophytin, which absorbs at only 1 nm above chlorophyll *a* (data not shown). These non-viable cells, representative of cells stressed between 3.5 and 4.4, also formed small, dark granules at the periphery of cells (Figs. 2F and 5B). ^{31}P-NMR spectroscopy showed no polyphosphate to be present, and the SDS-PAGE analysis (Fig. 6) indicated that cyanophycin was not present. Differential centrifugation and cyanophycin extraction methods followed by SDS-PAGE on various cellular fractions from these cells suggested that the granules are aggregates of denatured cellular proteins. Since all cells with these granules also had compromised membranes (Fig. 2E), it is likely that cellular proteins formed aggregates due to the loss of internal pH homeostasis.

Cyanobacteria have been found in almost all ecosystems studied (Tandeau de Marsac et al. 1993) and in natural environments of varying acidity (Brock 1970; Almer et al. 1973; Steinberg et al. 1998). *Synechocystis* sp. strain PCC 6308, originally isolated from a Wisconsin lake in 1963 and has been growing in laboratory cultures since, has been shown in the current study to have a lower pH limit for growth of pH 4.4. It is not known whether the ability of this bacterium to survive significant pH stress is intrinsic to the organism, or if this strain acquired acid tolerance mechanisms once it became cultured in the laboratory. Although it is not certain whether these bacteria respond similarly in nature, some aspects of the pH stress response elucidated in the laboratory could be similar to those used in nature to survive pH stress. The cell concentration-dependent pH_o increase would not typically be a plausible survival strategy in nature, since cells would not be present at the high densities required to change the pH of their environment. An exception could be cyanobacterial mats, which are composed of very high densities of cyanobacteria and could potentially be very resistant to acid stress. Such mats have been found to exhibit light-dependent increases in external pH (Pierson et al. 1999); to date, *Synechocystis* sp. strain 6308 has not been found to exist in nature in mats. Whether cyanobacteria behave similarly in response to acid stress in natural environments remains to be determined, but our studies show that cyanobacteria, like other bacteria in which pH stress has been studied, are able to respond to and survive encounters with acidic pH stress in a predictable way.

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