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Rapid Transient Growth at Low pH in the Cyanobacterium *Synechococcus* sp.

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The thermophilic cyanobacterium *Synechococcus* sp. strain Y-7c-s grows at its maximum rate at a high pH (pH 8 and above) and does not show sustained growth below pH 6.5. However, rapidly growing, exponential-phase cells from high-pH cultures continued to grow rapidly for several hours after transfer to pH 6.0 or 5.0. This transient growth represented increases in mass and protein, but cells failed to complete division. Viability loss commenced well before the cessation of growth, and cells at pH 5.0 showed no net DNA synthesis. When irradiated by visible light, cells at pH 6.0 and 5.0 maintained an internal pH of 6.9 to 7.1 (determined by ^{31}P nuclear magnetic resonance spectroscopy) and an extremely high ATP/(ATP + ADP) ratio even after growth had ceased. Cells exposed to a low pH did not show an increase in the spontaneous mutation rate, as measured by mutation to streptomycin resistance. However, cells already resistant to streptomycin were more resistant to viability loss at a low pH than the parental type. Cultures that could grow transiently at a low pH had higher rates of viability loss than nongrowing cultures in light or darkness. The retention of a high internal pH by cells exposed to a low pH suggested that a low pH acted initially on the cell membrane, possibly on solute transport.

Among microorganisms, cyanobacteria (blue-green algae) and other photosynthetic procaryotes are exceptional in that they lack the ability to grow at low pH values. A very limited number of cyanobacteria (3) and photosynthetic bacteria (22, 23) do grow at pH 4 to 5, but the growth of most cyanobacteria is optimal at high pH values, between pH 7.5 and pH 10.0 (6). In contrast, all other major groups of microorganisms have at least some representatives that grow at pH values well below 4.0 (17).

We found that the thermophilic cyanobacterium *Synechococcus* strain Y-7c-s shows sustained growth only at pH 6.5 and above, but that this organism maintains a near-neutral internal pH even when it is exposed (for 6 h or less) to pH 5 to 6 (13). This suggested that growth was not slowed by a decrease in the internal pH. However, these findings do not rule out the possibility of a gradual, growth-limiting loss of internal pH control. We also found a correlation between low rates of sustained growth and a low ATP/(ATP + ADP) ratio at low pH values, but we suggested that an inadequate energy supply is probably not what limits growth under these conditions.

Despite the lack of sustained growth at pH values below 6.5, exponential-phase *Synechococcus* cultures grew rapidly for several hours when they were resuspended in medium at pH 6.0 or 5.0. This rapid, transient growth at low pH provides a means for evaluating factors that may limit growth. In this work we investigated the phenomenon of transient growth at a low pH and tried to determine whether a gradual decline in the internal pH or the energy charge may lead to cessation of growth. We also examined the possibility of an increased mutation rate at low pH values as a factor which may account for the high loss of viability during transient growth.

MATERIALS AND METHODS

Cyanobacterial strain and culture conditions. *Synechococcus* strain Y-7c-s was grown as described in the accompanying paper (13). All inocula were from rapidly growing (specific growth rate, ca. 0.14 h^{-1}), exponential-phase cultures in D medium at pH 8.0 or above in bubbler vessels gassed with 1% CO_2 in air. Irradiation during growth experiments was provided by cool white fluorescent lamps (18 to 20 klx saturated growth; 1.0 klx = 10^3 lumens/m^2). Growth rates were expressed as the specific rate constants calculated in base *e* of natural logarithms.

Total and viable cell counts. Cells were counted with an American Optical Neubauer hemacytometer. Septate or double cells were cells which possessed a cross wall. In total (single) cell counts, septate cells were counted as two cells. When the count was expressed as the number of mechanical units, each septate cell was counted as one unit. The frequency of septate

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cells was determined by analyzing photomicrographs or by counting chamber observations. In some cases, the mechanical unit counts were determined by adjusting single-cell counts for the frequency of septate cells. The cell count values given below represent the means of quadruplicate counting chamber fillings. Viable cell counts were obtained from triplicate platings on DG_m agar at pH 8.2, as described in the accompanying paper (13). Error values represent standard errors of the mean ($S/\sqrt{n}$).

Intracellular pH. Phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy was used to determine the intracellular pH of *Synechococcus* in vivo. Spectra were obtained with a computer-controlled Varian XL-100 NMR spectrometer at a magnetic field strength of 23 kilogauss, as previously described (13a). Each spectrum represented 12,000 0.5-s pulses accumulated during 1.8 h from a concentrated cell suspension (ca. 50 mg [dry weight]per ml). A tungsten-halogen lamp provided an incident light intensity of ca. 100×10^4 ergs/cm^2 per s, and the effective light intensity under NMR conditions was at least as great as that under growth conditions at 4 klx (13a).

Peak locations (chemical shifts) were expressed as parts per million (ppm) of the field strength relative to 85% H_3PO_4 at 0 ppm. The scales shown with NMR spectra are approximate, and actual chemical shifts were obtained from data supplied by the computer. Internal pH values were determined from intracellular phosphorus compounds whose chemical shifts changed as a function of pH.

ATP, ADP, protein, and DNA content. ATP and ADP pool sizes were determined with a luciferin-luciferase assay, using the procedure described in the accompanying paper (13).

Protein was assayed by the Coomassie blue dye-binding method originally developed by Bradford (1). The reagent was obtained from Bio-Rad Laboratories, and values were expressed relative to bovine serum albumin.

Total DNA was assayed fluorometrically by using a modification of the methods of Kissane and Robbins (14) and Switzer and Summer (32). This method is based on the highly specific reaction of 3,5-diaminobenzoic acid with deoxyribose. Cells were extracted successively with 90% acetone, 0.6 N trichloroacetic acid at 0°C, 95% ethanol at 0°C, and 95% ethanol at 60°C to remove lipids. The final pellet was dried and reacted with 50 μl of 2 M 3,5-diaminobenzoic acid at 60°C for 30 min. After 5 ml of 1 N HCl was added, the relative fluorescence at 520 nm (excitation wavelength, 405 nm) was measured against a standard made from calf thymus DNA (Sigma Chemical Co.). A Hitachi model MPF-2A spectrofluorometer was used to make the measurements.

Streptomycin resistance. The frequency of mutation to streptomycin resistance was determined by spreading concentrated cell suspensions onto DG_m agar plates supplemented with 25 μg of streptomycin per ml. These plates were incubated at 45°C and 5 to 8 klx along with viable count plates.

RESULTS

Rapid, transient growth at low pH. When *Synechococcus* cells undergoing rapid, exponential growth at high pH values (8.0 or above) were resuspended at a low pH, rapid growth continued for several hours (Fig. 1). This transient growth represented increases in turbidity, dry weight, and protein and lasted 10 to 12 h at pH 6.0 and 5 to 6 h at pH 5.0. The initial rate (specific growth rate, 0.15 h^{-1}) equaled the maximum growth rate at a high pH. These data showed that a low pH at first had little or no effect on growth, but gradually caused complete growth inhibition. Cultures whose growth had stopped could not initiate a new burst of growth when they were transferred to fresh, low-pH medium.

Inhibition of cell division and loss of viability during transient growth. Despite initial increases in mass and protein, cells resuspended at pH 5.0 lost the ability to divide, and this loss rapidly became irreversible (Fig. 2). The optical density increased, but the total cell count remained unchanged. Moreover, after 2 h of exposure the viable cell count began to fall, and by 10 h it had decreased to about 3% of the original value. At 10 h, a pH shift to 7.93 (Fig. 2, arrow) caused an immediate and rapid increase in the viable cell count, whereas the absorbance and total cell

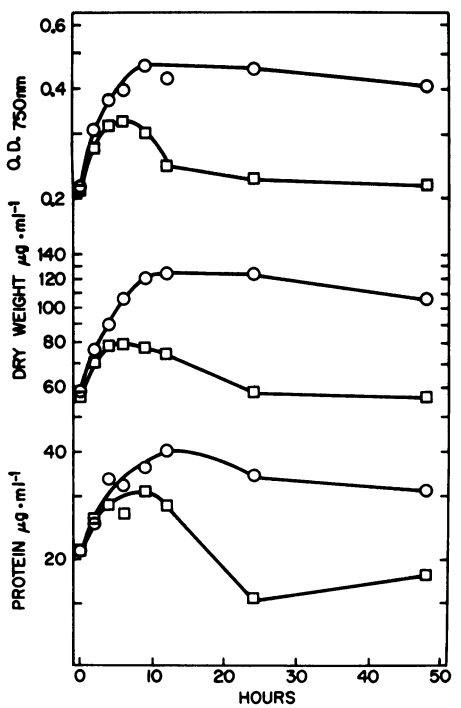

FIG. 1. Transient growth of *Synechococcus* in D-CMTC medium at pH 6.0 (○) or 5.0 (□) gassed with 1% CO_2 in air at a light intensity of 21 klx. The curves show the changes in optical density at 750 nm ($O.D._{750nm}$), dry weight, and protein.

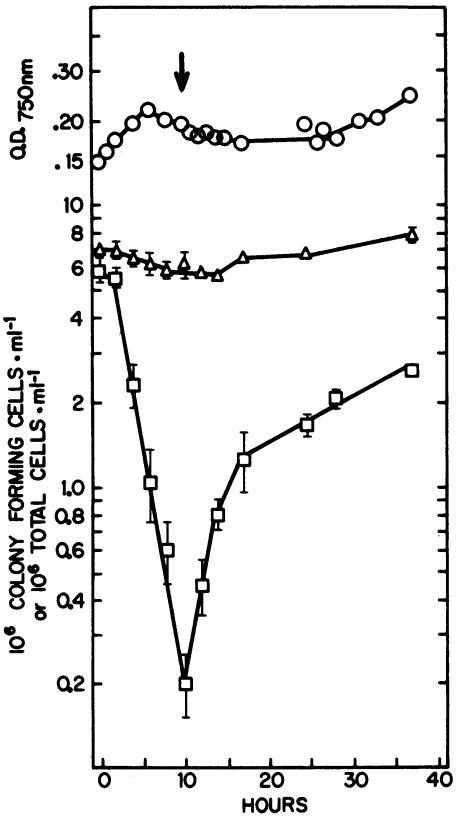

FIG. 2. Total and viable cell counts in a *Synechococcus* culture during transient growth at pH 5.0 in D-CMTC medium (1% CO_2 in air) at a light intensity of 20 klx. After 10 h (arrow), the pH of the medium was shifted from 5.03 to 7.93 by adding 2.1 ml 1.0 M Tricine (pH 12.45) to the remaining 210 ml of culture. Symbols: O, optical density at 750 nm ($O.D._{750nm}$); △, total cells per milliliter; □, viable cells per milliliter.

count increased only after long lag periods. These data are consistent with a low percentage of viable cells remaining after a 10-h exposure to pH 5.0.

After the shift-up to a high pH, the viable count increased at a rate (specific growth rate, 0.35 h^{-1}) that was approximately twice as high as the rate expected simply from the resumption of growth by the remaining viable cells. Perhaps the survivors of the low-pH exposure became multiseptate or reached a stage in the cell cycle where they could undergo a series of rapid divisions after the shift back to a high pH.

Cells resuspended at pH 6.0 also lost viability (but at a lower rate than cells at pH 5.0) and failed to complete cell division; however, they did form septa (Fig. 3). When septate (double) cells were counted as two single cells, the total cell count increased. However, this was accompanied by a large increase in the frequency of septate cells. Consequently, the number of mechanical units in the culture did not change, indicating that the cells had not completed division. In a control culture at pH 8.0, both the single-cell count and the mechanical-unit count increased exponentially, whereas the frequency of septate cells remained approximately constant at 0.20 (after a transient initial increase). At pH 5.0, neither the single-cell count (Fig. 2) nor the mechanical-unit count (data not shown) increased, and the septate frequency remained at 0.20 to 0.25. Hence, cells at pH 5.0 neither formed septa nor completed division.

Internal pH during transient growth at low pH. Figure 4 shows the ^{31}P-NMR spectra of cells harvested from a pH 6.0 culture and demonstrates that the spectra from intact cells, which retained internal pH control (Fig. 4, spectrum a), were readily distinguishable from the spectra of cells that lost internal pH control (spectrum b). Spectrum a (cells harvested after 1.5 h of growth at pH 6.0) showed an internal pH of 7.1 during

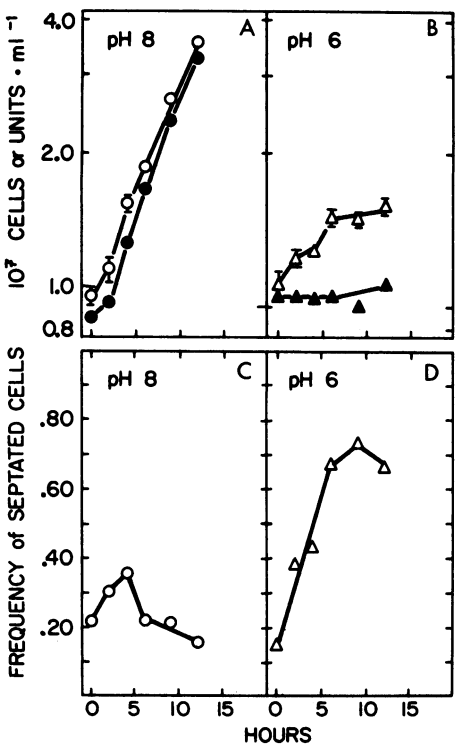

FIG. 3. Total cell number and frequency of septate cells during growth in D-CMTC medium (1% CO_2 in air; 19 klx) at pH 8.0 and 6.0. (A and B) Number of total (single) cells per milliliter at pH 8.0 (O) and pH 6.0 (△) and number of mechanical units per milliliter at pH 8.0 (●) and pH 6.0 (▲). (C and D) Frequency of septate cells in the cultures at pH 8.0 (O) and pH 6.0 (△). Mechanical-unit counts were calculated from single-cell counts and septate cell frequencies.

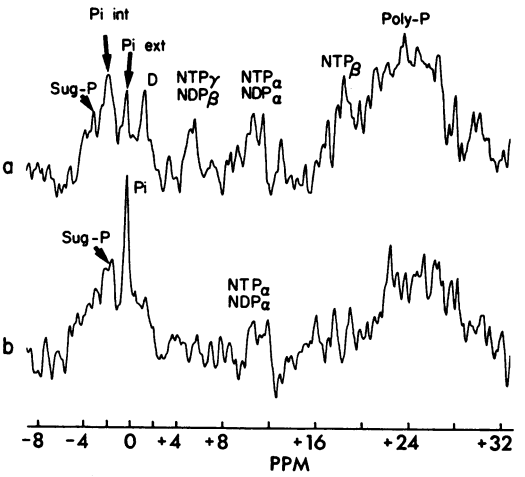

FIG. 4. Phosphorus-NMR spectra obtained from irradiated *Synechococcus* suspensions in a Krebs Ringer buffer containing 0.1 M 2-(*N*-morpholino)ethanesulfonic acid. Spectrum a represents cells that had grown in D medium containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.0 (1% CO_2 in air) and a light intensity of 23 klx for 1.5 h before harvest and NMR analysis. Spectrum b represents cells that had been grown at pH 6.0 for 2 h under identical culture conditions and then grown for an additional 1 h in the presence of 10 μM CCCP before harvest and NMR analysis. The external pH values during NMR analysis were 5.95 to 5.91 and 5.89 to 5.77 for suspensions a and b, respectively. P_i at a concentration of 0.9 mM was added to each suspension as an external marker. Peak identities: Sug-P, sugar phosphate; Pi int, internal P_i; Pi ext, external P_i; D, unidentified; NTP_γ NDP_β, γ-phosphate nucleotide triphosphates or β-phosphate nucleotide diphosphates; NTP_α NDP_α, α-phosphate nucleotide tri- or diphosphates; NTP_β, β-phosphate nucleotide triphosphates; Poly-P, polyphosphates.

data accumulation at an external pH of 5.95 to 5.91. This internal pH value was based on the chemical shifts of internal sugar phosphate, P_i, and γ-phosphate nucleotide triphosphate (or β-phosphate nucleotide diphosphate) at −3.20, −2.00, resonances and +5.21 to +5.56 ppm, respectively (13a).

Spectrum b was obtained from cells that were treated with 10 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) before harvest and NMR analysis. The uncoupler CCCP makes membranes permeable to protons (12), and in the absence of an electrical charge gradient across the cell membrane, the intracellular pH becomes equal to the external pH. Spectrum b shows a single, intense P_i peak at −0.34 ppm, which corresponded to the intra- and extracellular P_i at the pH of the medium.

A control experiment (data not shown) demonstrated that the phosphorus compounds corresponding to the peaks labeled sugar phosphate, P_i internal, and γ-phosphate nucleotide triphosphates (or β-phosphate nucleotide diphosphates) were acid extractable and that the chemical shifts of the acid-extracted compounds changed with changes in pH. Other controls showed that these peaks belonged to intracellular compounds (data not shown). Hence, the chemical shifts of these compounds were appropriate indicators of internal pH.

Table 1 summarizes internal pH maintenance during transient growth at low pH values. These data were obtained by analyzing spectra such as those shown in Fig. 4. Cells harvested during transient growth at pH 6.0 and 5.0 showed internal pH values of 6.9 to 7.1. The cessation of transient growth always preceded any decline in the cytoplasmic pH.

ATP/(ATP + ADP) ratio during transient growth at low pH. Figure 5 shows the results of one experiment at pH 5.0. The ATP/(ATP + ADP) ratio at zero time in darkness was 0.86. With the onset of illumination at zero time, the ATP/(ATP + ADP) ratio increased to an extremely high value, 0.98. Gradually the ratio decreased, but it was still 0.83 12 h after the cessation of growth (18 h after the onset of illumination). Control experiments showed that this ratio was not artifactually high because of an underestimation of ADP (data not shown). Viability decreased sharply, again before cessation of growth and long before any drop in the energy level (Fig. 5).

Other control experiments showed that virtually no ATP could be detected in a culture at pH 5.0 2 h after the addition of 10 μM CCCP and 5 μM valinomycin (a potassium ionophore). Similarly, within 5 min of the addition of 10 μM CCCP to a culture growing at pH 6.0 (2-h exposure), growth stopped, and the ATP/(ATP + ADP) ratio decreased from 0.91 to 0.26. These data and the data in Fig. 5 support the conclusion that transient growth at a low pH did not cease because of a limited energy supply.

Factors affecting loss of viability at low pH. Figure 6 shows the effects of four different culture conditions on transient growth at pH 6.0. Cells resuspended in an irradiated bubbler vessel (1% CO_2 in air) showed normal, transient growth (Fig. 6A). Cells from the same inoculum did not grow in a darkened bubbler vessel (1% CO_2 in air), in a darkened nonshaken flask, or in an irradiated nonshaken flask. However, the cells in the irradiated bubbler vessel suffered by far the highest rate of viability loss, whereas those in the darkened nonshaken flask had the lowest rate. The latter culture retained 60% of its initial viability after 24 h at pH 6.0.

Irradiation of the darkened bubbler culture after 24 h at pH 6.0 still resulted in a burst of

TABLE 1. Internal pH maintenance in *Synechococcus*[a]

Conditions before harvest	Specific growth rate (h^{-1})		pH during NMR assay[b]		
	Initial	Before harvest	External (electrode)	Internal (cytoplasmic)	ΔpH (internal pH minus external pH)
Unbuffered D medium, pH 8 or above	0.09	0.09	8.4–7.9	7.3	−1.1 to +0.6
Exposed to pH 5.0 for:					
1.5 h	0.10	0.10	5.0–5.2	7.1	+2.0
2 h	0.08	0.05	5.1–5.3	6.7–6.8	+1.6
5 h	0.08	0.04	5.0–5.1	7.0	+2.0
10 h	0.10	0.02	5.0	6.9	+1.9
11 h	0.11	0	5.0	7.1	+2.1
12 h	0.10	0.01	5.0	5.0	0
Exposed to pH 6.0, for:					
1.5 h	0.08	0.08	6.0–5.9	7.1	+1.1
14 h	0.08	0	5.9–5.8	7.0	+1.2
pH 6.0, 10 μM CCCP added 1 h before harvest	0.09[c]	0	6.0–5.8	5.7	−0.2

[a] These data were from ^{31}P-NMR experiments, including those shown in Fig. 4.

[b] The external pH values represent electrode measurements taken immediately before and after the completion of each NMR assay. External pH values obtained from NMR spectra were in close agreement with the electrode-measured values. The ΔpH values represent the pH of the cytoplasm minus the pH of the medium.

[c] This specific growth rate (0.09 h^{-1}) represents the growth during a 2-h exposure to pH 6.0 immediately before the addition of CCCP.

transient growth. The level of this growth was approximately 70% of the level shown by the bubbler culture at zero time. The irradiated nonshaken flask culture showed little growth when it was transferred after 24 h to an irradiated bubbler flask.

These data showed that the conditions which permitted growth at a low pH resulted in the highest rate of viability loss. These data suggested several possible interactions of pH, light, and growth, which are discussed below.

Net DNA synthesis at low pH. The inhibition of cell division or the loss of viability at low pH or both might reflect an effect (direct or indirect) of low pH on DNA synthesis. We tested this hypothesis by performing the experiment shown in Fig. 7. Cells from a high-pH inoculum were resuspended at pH 8.0, 6.0, and 5.0, and these cells grew as shown in Fig. 7A. The amount of DNA increased at pH 8.0 and 6.0, but after a slight increase during the first 2 h, no net DNA accumulation occurred at pH 5.0 (Fig. 7B).

Does exposure to low pH increase the frequency of spontaneous mutation? Figure 7 shows that low pH values affected DNA synthesis. Without causing a decrease in the internal pH, exposure to a low external pH might alter the intracellular (chemical) environment sufficiently to cause a loss of fidelity in DNA replication. This could result in an increased mutation rate and could account for the loss of viability observed at low pH values. To test this hypothesis, we studied the frequency of spontaneous mutation to streptomycin resistance in *Synechococcus* at pH 8.0 and 5.0 (Table 2). The viable cell count increased exponentially at pH 8.0 and, after a transient increase, decreased rapidly at pH 5.0. At pH 8.0, the frequency of streptomycin resistance remained approximately 1.0 per 10^8 viable cells throughout the experiment. The initial frequency at pH 5.0 was the same, but after 13 h of exposure the frequency of streptomycin resistance had risen eightfold. These data suggested an increase in the spontaneous mutation rate during exposure to pH 5.0. However, when expressed on a total cell basis, the streptomycin resistance frequencies at pH 5.0 differed only slightly from those at pH 8 (Table 2).

Are streptomycin-resistant cells more resistant to low pH? The data described above suggested the following two alternatives: either the mutation frequency to streptomycin resistance increased during exposure to low pH, or cells that were already resistant to streptomycin were, for some reason, also more resistant to low pH. This was resolved by the experiment shown in Table 3. A spontaneous, streptomycin-resistant strain was obtained at pH 8. This strain (strain SR-3) had never been exposed to low pH, and at high pH its growth rate equaled that of the parental strain. When resuspended at pH 5.0, strain SR-3 exhibited growth kinetics that were

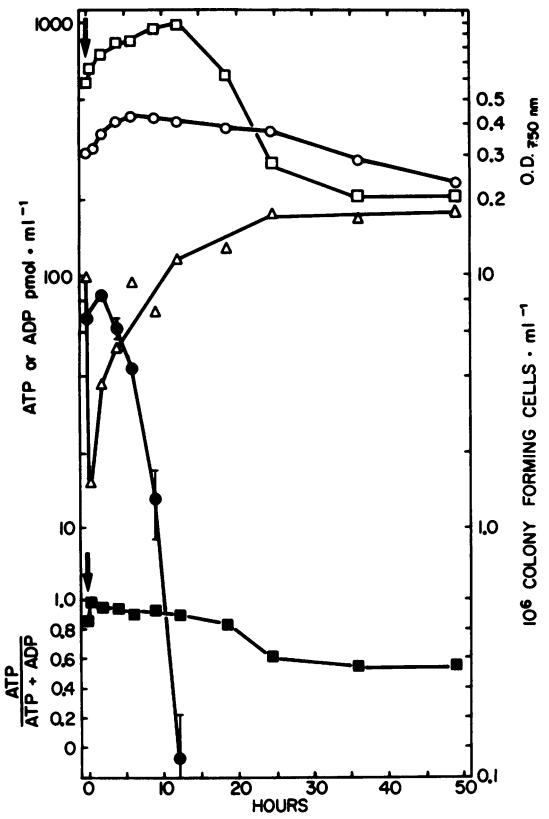

FIG. 5. ATP and ADP in *Synechococcus* during transient growth at pH 5.0 in D-CMTC medium (1% CO_2 in air) at a light intensity of 20 klx. Each value for ATP concentration (□), ADP concentration (△), or ATP/(ATP + ADP) ratio (■) represents the mean of a triplicate determination. Symbols: ○, optical density at 750 nm ($O.D._{750nm}$); ●, viable cell count. The samples at zero time were taken in darkness; then the culture was irradiated (arrow), and the next samples for ATP and ADP determinations were taken 20 min later.

similar to the growth kinetics of the parent (data not shown). However, the kinetics of viability loss differed markedly from those of wild-type *Synechococcus*. The viability of strain SR-3 declined sharply during the first 2 h, but then continued to decline only gradually. After 12 h of exposure, strain SR-3 still retained 40 to 45% of its initial viability, compared with 3% for the wild type. These data showed that there was a lower viability loss at low pH in streptomycin-resistant cells, and this accounted for the apparent increase in streptomycin resistance during exposure to pH 5.0 (Table 2).

DISCUSSION

In this paper we show that the transfer of *Synechococcus* from high pH to low pH results in rapid, transient growth, but is accompanied by a loss of viability, a loss of cell division, and (at pH 5.0) inhibition of net DNA synthesis. The maintenance of a high internal pH, even after the cessation of transient growth (Table 1), strongly suggests that the inhibition of growth at low pH does not result from an acidification of the cytoplasm.

A recent study with *Coccochloris peniocystis*

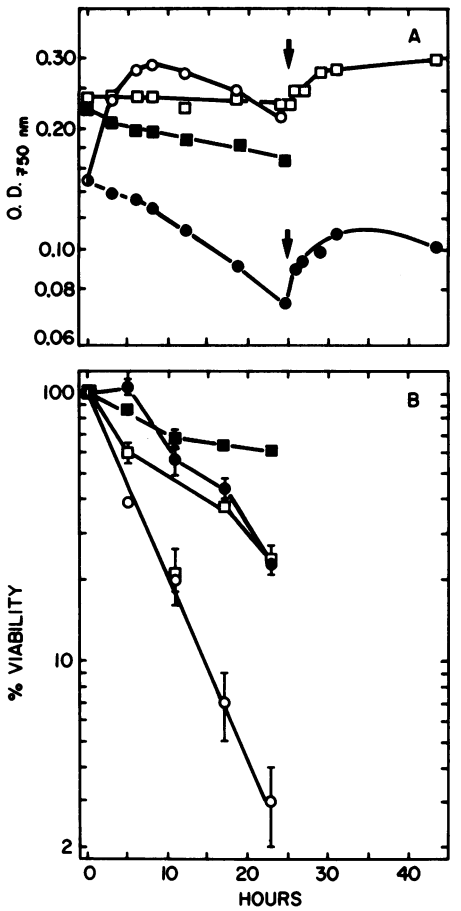

FIG. 6. Growth and viability of *Synechococcus* exposed to pH 6.0 in light and darkness under two different culture conditions. An exponential-phase inoculum from a high-pH culture was suspended in D medium containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.0 under the following conditions: in bubbler vessels (1% CO_2 in air) at a light intensity of 13 klx (○) or in darkness (●); in nonshaken 125-ml Bellco flasks at a light intensity of 13 klx (□) or in darkness (■). (A) Optical density at 750 nm ($O.D._{750nm}$). (B) Percent viability relative to zero-time viable cell counts as a function of exposure time. After 24.5 h (arrows) the darkened bubbler culture was exposed to light (13 klx), and the irradiated flask culture was maintained at the same light intensity but transferred to a bubbler vessel and gassed with 1% CO_2 in air.

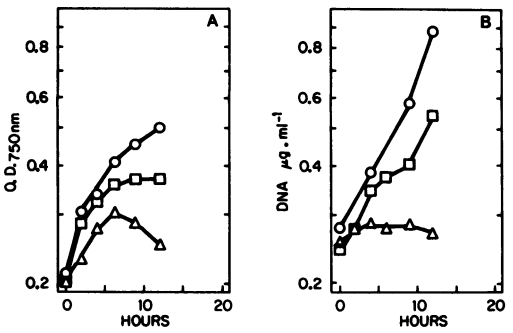

FIG. 7. Net DNA synthesis in *Synechococcus* cells suspended at pH 8.0 (○), pH 6.0 (□), and pH 5.0 (△) in D-CMTC medium (1% CO_2 in air) at a light intensity of 20 klx. (A) Optical density at 750 nm ($O.D._{750nm}$). (B) Concentration of DNA as a function of time.

(equivalent to *Synechococcus* PCC 6307 [26]) showed a loss of in vitro ribulose-1,5-bisphosphate (RUBP) carboxylase activity at pH 6.5, a loss of photosynthetic activity (O_2 evolution) in intact cells at external pH values below 5.25, and a decrease in the internal pH to 6.6 in cells exposed to pH 5.25 (4). Coleman and Colman (4) suggested that inactivation of RUBP carboxylase (the principal CO_2-fixing enzyme in procaryotes and eucaryotes) by a lower internal pH results in the inhibition of photosynthesis at low pH values. This appears not to be the case in *Synechococcus* strain Y-7c-s. First, strain Y-7c-s cells continued to evolve O_2 at a high rate when they were exposed to pH 5.0 (data not shown). Second, transient growth at pH 5.0 (mass and protein accumulation) implied that there was RUBP carboxylase activity (*Synechococcus*, an obligate autotroph, has no alternative pathway for carbon assimilation). Since cells at pH 5.0 maintained an internal pH of 6.9 to 7.1 (Table 1), it appears unlikely that the immediate cause of growth stoppage was a loss of RUBP carboxylase activity which resulted from a drop in the internal pH.

Nonetheless, the absence of diverse strains of cyanobacteria from environments with extremely low pH's ultimately may be explained by an inability to keep cytoplasmic pH values above 6.5 to 7.0. Under the conditions described in the accompanying paper (13), the internal pH of *Synechococcus* did decrease to about 6.5 during exposure to pH 4.8. In addition to the suggested loss of RUBP carboxylase activity at pH 6.5 (4), an internal pH below 7.0 may lead to the activation of glucose-6-phosphate dehydrogenase (27), a key regulatory enzyme of cyanobacterial light and dark metabolism (30). The activation of this enzyme in a photosynthetic organism exposed to light might result in a futile, short-circuited cycle of CO_2 fixation.

The data in Fig. 5 strongly suggest that growth at low pH's is not limited by an inability to sustain the energy supply. Cells at pH 5.0 retained an ATP/(ATP + ADP) ratio of 0.83 12 h after the cessation of transient growth. This ratio is equal to that found during exponential growth at pH 8 (13). In the accompanying paper we report a correlation between lower ATP/(ATP + ADP) ratios and lowered sustained growth rates at intermediate pH values, but these results were obtained with slowly growing inocula (13). After prolonged (24 h or more) exposures to low pH, the ATP/(ATP + ADP) ratios in cells from rapidly growing inocula also decreased to values of 0.60 or below (Fig. 5).

A high internal pH in cells during growth-limiting and lethal exposures to low external pH values suggests that low pH acts initially on the cell membrane. A defect in solute transport caused by changing intracellular concentrations of nutrients or cofactors might lower the fidelity of DNA, RNA, or protein synthesis and result in the loss of viability shown in Fig. 2.

TABLE 2. Streptomycin resistance frequency of *Synechococcus* strain Y-7c-s under growth conditions at pH 8 and 5[a]

Time of exposure (h)	10^6 viable cells per ml		No. of streptomycin-resistant cells per 10^8 viable cells		No. of streptomycin-resistant cells per 10^8 cells	
	pH 8	pH 5	pH 8	pH 5	pH 8	pH 5
0	20	17.0	0.9	0.9	1.2	1.0
4		23.0		0.5		0.6
7	36		0.8		1.2	
9		7.1		3.5		1.3
13	61	1.6	1.0	8.1	1.8	0.7

[a] *Synechococcus* cells from an exponential inoculum (unbuffered D medium) that had reached pH 9.5 were resuspended at pH 8.0 and 5.0 in D-CMTC medium (1% CO_2 in air) at a light intensity of 20 klx. At intervals, samples were taken from the cultures for viable and streptomycin-resistant cell counts, as described in the text. All values represent the means of triplicate platings. Total cells were estimated on the basis of the following relationship: 1 unit of optical density at 750 nm was equivalent to 3.3×10^7 cells per ml.

TABLE 3. Resistance to low pH of wild-type and streptomycin-resistant *Synechococcus* strain Y-7c-s[a]

Time of exposure to pH 5 (h)	Fraction of zero-time colony-forming cells	
	Wild type	Streptomycin resistant
0	1.00	1.00
2	1.12	0.58
4	0.90	0.50
6	0.62	0.58
9	0.19	0.54
10		0.40
12	0.03	0.44
18	0.005	
25	0	0.00002

[a] A *Synechococcus* clone (strain Y-7c-s, SR-3) resistant to 25 μg of streptomycin per ml was obtained at pH 8. An inoculum of this streptomycin-resistant strain was harvested from mid-exponential phase (in unbuffered D medium that had reached pH 10.45) and then suspended in D-CMTC medium at pH 5.0 under otherwise identical conditions (1% CO_2 in air; light intensity, 20 klx). At intervals, samples were taken from the culture and plated in triplicate onto DG_m agar at pH 8.2. The results are expressed as fractions of zero-time viability as a function of exposure. Viability at pH 5.0 in a similarly treated wild-type culture is shown for comparison.

Exposure to pH 5.0 did inhibit net DNA synthesis (Fig. 7). However, we were not able to show an increased frequency of mutation to streptomycin resistance, because streptomycin-resistant cells had a greater resistance to viability loss at low pH (Table 3). Another problem with streptomycin resistance as a marker is that it may take several generations of growth before the genetic acquisition of resistance can be expressed phenotypically (31). Hence, low pH may have caused mutations to streptomycin resistance which we were unable to detect because we subjected cells to the drug immediately after they were removed from low-pH cultures. Increased mutation rates at low pH values remain possible, and this question may be answered more easily by using other genetic markers (11).

Streptomycin binds to ribosomes and inhibits protein synthesis (28). Two possible explanations for the increased tolerance to low pH of streptomycin-resistant cells are the following: (i) cells with altered ribosomes may escape the lethal effects of both streptomycin and low pH; and (ii) streptomycin-resistant cells may not accumulate streptomycin (thus, they may have a lesion in transport which also confers increased tolerance to low pH) (16). The characterization of streptomycin-resistant, low-pH-tolerant mutants should help clarify the possibly interesting relationship between streptomycin resistance and low pH tolerance.

Figure 6 shows that the cells which were able to grow transiently at low pH had the highest rate of viability loss. This suggests that the accumulation of some compound (perhaps RNA or protein) may lead to killing at low pH. At least under specific conditions, certain bacteria lose viability if they cannot curtail biosyntheses (29). However, irradiated but nongrowing *Synechococcus* cells also lost viability, at a higher rate than the equivalent darkened cells, and showed little growth when they were transferred to growth-permissive conditions (Fig. 6). Our data do not distinguish between the involvement of growth and photodynamic effects (13) as possible causes of killing at low pH.

Low pH may block cell division and septation (Fig. 3) by acting externally on the cell membrane or cell wall, or these phenomena may reflect an indirect, internal effect of low external pH. Specific protein synthesis events appear to be necessary for septation in *Bacillus subtilis* (20) and also for cell division in *B. subtilis* (20), *Escherichia coli* (5, 33), and *Synechococcus* AN (19). Hence, exposure to a low pH might alter the intracellular environment and cause the synthesis of defective septation- and division-specific proteins, or such exposure might alter the activities of these proteins. At present however, we have no evidence to support this hypothesis.

We have suggested that exposure to low pH may affect solute transport. All bacteria appear to establish electrochemical gradients across their cell membranes, generally by translocating protons from the cytoplasm to the outside (7, 10). Such gradients may be used for ATP generation, motility, or active transport of solutes. A requirement for growth at either high or low pH appears to be the retention of a cytoplasmic pH near neutrality (8, 15). In alkalophiles, excessive alkalinization of the cytoplasm appears to be prevented by the activity of cation-proton antiporters (2, 24). Such antiporters allow cells at high pH to regulate their internal pH and yet conserve the energy of the transmembrane electrochemical gradient ($\Delta\mu H$) by exchanging a gradient of protons (with chemical [ΔpH] and electrical charge [$\Delta\Psi$] components) for a gradient of K^+, Na^+, or Ca^{2+}.

However, antiporter activity may prevent growth at lower pH values. Wild-type *Bacillus alcalophilus* grows at pH 8.5 to 11.5, but a mutant that lacks the activity of an Na^+-H^+ antiporter grows at pH 5.0 to 9.0 (16). The wild type may not grow at neutral and low pH values because of a limited capacity of the cytoplasm to buffer protons entering via the Na^+-H^+ antiporter.

Cyanobacterial cell membranes appear to be

analogous to the cell membranes of *E. coli* in that the ΔpH and ΔΨ components of the ΔμH gradient can be exchanged (21, 25), suggesting the presence of a cation-proton antiporter. Growth at pH 10 and above (data not shown) and the retention of a neutral cytoplasm in cells exposed to high pH (Table 1) suggest that there is antiporter activity in *Synechococcus*. Cells at low pH may face the following dilemma: the active accumulation of certain solutes requires an electrical charge gradient (ΔΨ) of a specific ion, whereas the accumulation of other solutes requires ΔpH and the accumulation of still others may be driven by either component of ΔμH (9, 18). If *Synechococcus* requires a specific cation gradient for the accumulation of an essential solute, this gradient and internal pH regulation can be accomplished by a cation-proton antiport in cells exposed to high pH values. However, at low external pH values the establishment of a steep cation gradient may lead to excessive acidification of the cytoplasm. Alternatively, curtailed antiporter activity at low pH may result in the failure to accumulate an essential nutrient. Either effect could halt growth. Our data show no evidence for an early acidification of the cytoplasm in cells exposed to low pH (Table 1). This suggests a possible absence of antiporter activity and an inability to accumulate certain solutes.

The data reported here suggest that viability loss and cessation of growth at low pH result neither from a lowered internal pH nor from a low energy charge. We propose that a defect in solute transport at low pH may be the immediate cause of growth loss and viability loss. Therefore, it should be possible to isolate transport mutants which have somewhat altered pH limits for growth.

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