environmental microbiology

Environmental Microbiology (2012) **14**(3), 680–690 doi:10.1111/j.1462-2920.2011.02620.x

Nutrient-associated elongation and asymmetric division of the cyanobacterium *Synechococcus* PCC 7942

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Summary

While tightly regulated, bacterial cell morphology may change substantially in response to environmental cues. Here we describe such changes in the cyanobacterium *Synechococcus* sp. strain PCC7942. Once maintained in stationary phase, these rod-shaped organisms stop dividing and elongate up to 50-fold. Increase in cell length of a thymidine-auxotroph strain upon thymidine starvation implies that inhibition of DNA replication underlies cell elongation. Elongation occurs under conditions of limiting phosphorus but sufficient nitrogen levels. Once proliferative conditions are restored, elongated cells divide asymmetrically instead of exhibiting the typical fission characterized by mid-cell constriction. The progeny are of length characteristic of exponentially growing cells and are proficient of further proliferation. We propose that the ability to elongate under conditions of cytokinesis arrest together with the rapid induction of cell division upon nutrient repletion represents a beneficial cellular mechanism operating under specific nutritional conditions.

Introduction

Different bacteria are characterized by unique size and shape, reflecting tightly controlled developmental programmes (Koch, 1996; Young, 2006). Upon variations in environmental conditions, however, cells may deviate from their ongoing developmental schemes and change their size and morphology (Golden and Yoon, 2003; Cabeen and Jacobs-Wagner, 2005; Justice *et al*., 2008). A prominent example of such divergences is cell elongation (Wortinger *et al*., 1998; Ogawa *et al*., 2001; Rosenberger and Finlay, 2002; Miller *et al*., 2004; Pernthaler, 2005; Chauhan *et al*., 2006; Justice *et al*., 2006; Piao *et al*., 2006).

Elongated cells, sometimes referred to as filamentous cells, are obtained when cell growth continues without subsequent cell division. Recent observations suggest that these morphologies may serve adaptive roles [reviewed in (Justice *et al*., 2008)]. For instance, it has been shown that filamentous cells are resistant to protist predation in a number of marine environments (Hahn and Hofle, 2001; Corno and Jurgens, 2006). An additional example of advantageous cell elongation is illustrated by uropathogenic *Escherichia coli*, which invade the superficial epithelial cells of the bladder. Some of the cells in the intracellular bacterial community exhibit dramatic elongation. Upon release into the luminal space of the bladder, bacillary members of the population undergo phagocytosis by neutrophils whereas the filamentous form evades this process (Justice *et al*., 2006). Both of the above mentioned examples, avoidance of protist predation or escape from phagocytosis, manifest an advantage emerging from the morphology of the elongated cell. Another case of an adaptive morphological change is exhibited by *Caulobacter crescentus*. Under phosphorus limitation, this bacterium develops long prostheca (stalk), a cell extension that serves for the uptake of phosphate. It was suggested that the stalk represents an effective fitness strategy for low-phosphorus environments by maximizing contact with diffusing nutrients while imposing minimal increase in cell volume (Wagner *et al*., 2006).

In this study we report on morphological changes of the cyanobacterium *Synechococcus* sp. strain PCC7942 (hereafter *Synechococcus*). While maintained at stationary phase (hereafter referred to as aging) these cells elongate up to 50-fold. Cell elongation occurs under conditions where phosphorus shortage is combined with relative excess of nitrogen and is likely associated with inhibition of DNA replication. We also find that, once proliferative conditions are restored, the cells resume cytokinesis and divide into normal-sized progenies, but division occurs atypically in an asymmetric fashion. It is proposed

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that the aforementioned changes in cell morphology and division mode represent a cellular response to phosphorus limitation under a background of sufficient nitrogen, which becomes beneficial upon nutrient repletion.

Results

Cells in aged Synechococcus *cultures exhibit substantial elongation but maintain cell diameter and the concentric arrangement of the photosynthetic membranes*

The unicellular cyanobacterium *Synechococcus* is rod-shaped, typically 2–5 μm in length and ~ 1 μm in diameter. When allowed to age in the standard cyanobacterial growth medium, BG11, a majority of the cell population exhibited a gradual but substantial increase in cell length with time (Fig. 1A). Median cell length increased about fourfold (Fig. 1B); however, particular cells elongated up to 50-fold, reaching up to ~ 100 μm (Fig. 1A and C). Cell elongation was reproducibly observed, but for unknown reasons, maximal cell length varied between experiments, ranging from ~ 25 to *c.* 100 μm. Aside from the elongated cells, aged cultures contained short cells; 10–15% of the cells were of length characteristic of exponential growth (Fig. 1A).

Electron microscopy was used to compare the cellular morphology of exponentially growing and aged *Synechococcus* cells (Fig. 2). Importantly, there was no difference in cell diameter between exponentially growing and aged cells [1.02 μm ± 0.11 ($n = 28$) and 1.02 μm ± 0.17 ($n = 40$), respectively]. Notably, the organization of the photosynthetic (thylakoid) membranes in concentric shells as typical of exponentially growing cells of this strain (Nevo *et al.*, 2007) was maintained in aged cells. Additionally, glycogen granules accumulated in between the thylakoid membranes and in the internal space of aged cells (Fig. 2).

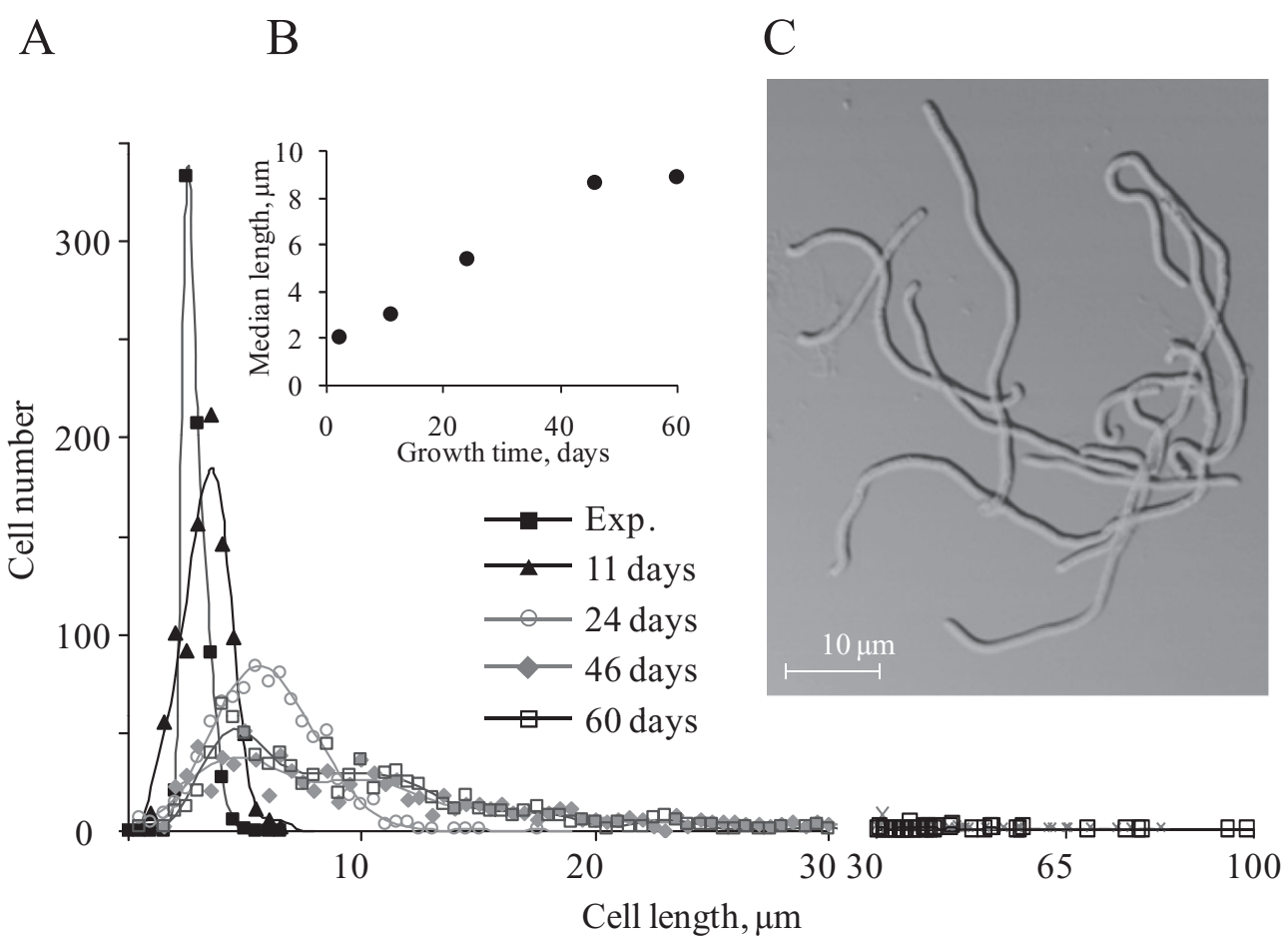

Fig. 1. Cell elongation upon culture aging.
A. Distribution of cell length at various times following inoculation. Data points represent the number of cells falling within bins of 0.5 μm; trend lines are shown for clarity. Exp. – exponential growth phase (2-day-old cultures).
B. Median cell length as a function of time.
C. An image of elongated (8-week-old) cells. Scale bar: 10 μm.

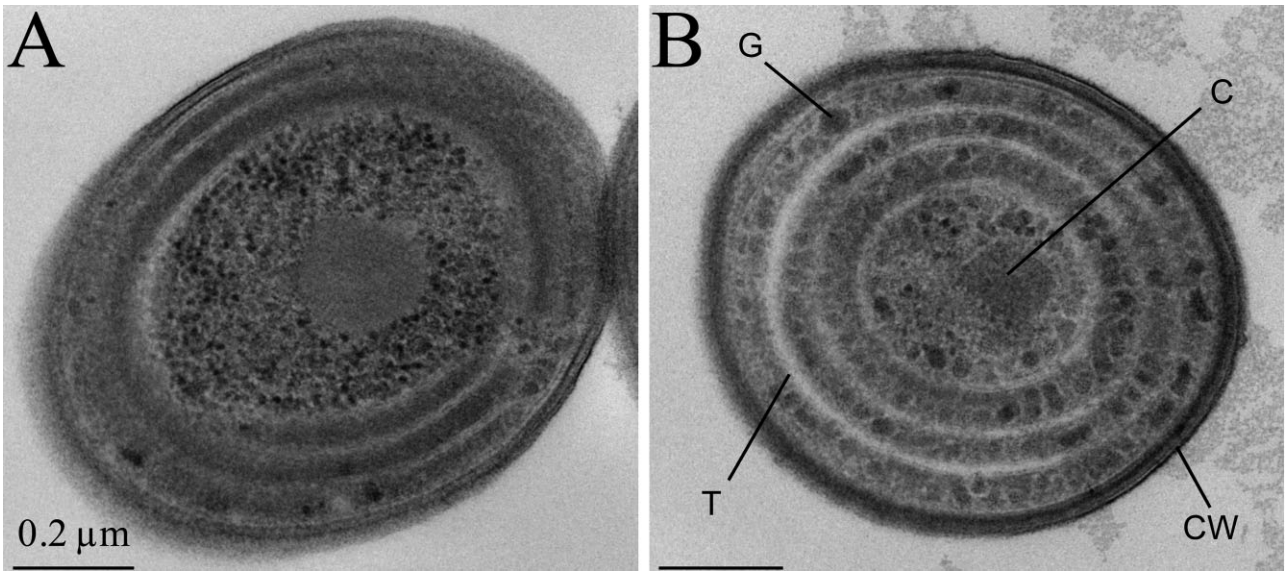

Fig. 2. Electron micrographs of (A) exponentially growing cells and (B) cells from an 8-week-old culture. Images were obtained from high pressure-frozen, freeze-substituted cell samples. The numerous granules (G) observed in aged cells are glycogen granules, as confirmed by carbohydrate staining (not shown). CW – cell wall; C – carboxysome; T – thylakoid membrane. Scale bars: 0.2 μm.

Thymidine starvation of an auxotroph strain triggers cell elongation

Cytokinesis is commonly linked to DNA replication (Pritchar and Zaritsky, 1970; Slater and Schaechter, 1974; Huisman *et al*., 1984; Schapiro *et al*., 2003; Yoshida *et al*., 2005). It is therefore conceivable that the observed elongation of the cells in aged cultures stems from inhibition of DNA replication and arrest of cell division, while accumulation of biomass and cell growth continues. To examine this possibility we constructed a *Synechococcus* strain that exhibits auxotrophy to thymidine.

Thymidine/thymine auxotrophic cells (collectively referred to as thymidine-auxotrophs) are obtained by impairment of the *de novo* pathway for pyrimidine synthesis. Thymidine starvation of such strains inhibits DNA replication due to lack of precursors for DNA synthesis [reviewed in (Zaritsky *et al*., 2006)]. Once external thymidine is supplied it may be taken up and used for DNA synthesis, following intracellular phosphorylation. Biochemical analyses (Restaino and Frampton, 1975) as well as a bioinformatic study we performed (not shown) have indicated that thymidine kinase (TK), the enzyme that catalyses thymidine phosphorylation, is absent from *Synechococcus*. Therefore, aside from impairment of the *de novo* pathway by inactivation of thymidylate synthase (TS) we inserted the TK gene from *E. coli* (Fig. 3A, see details in *Experimental procedures*) to obtain thymidine-auxotrophy in *Synechococcus*. PCR analysis confirmed replacement of the native TS gene with the inactivated one in all the chromosomes of a particular transformant (Fig. 3B). This transformant demonstrated thymidine-auxotrophy since once diluted into thymidine-free medium it ceased to divide (Fig. 3C), and eventually underwent pigment bleaching (not shown).

Upon depletion of thymidine in the medium, the thymidine-auxotrophic cells exhibited rapid elongation (Fig. 3D). Notably, the relative change in the distribution of cell length following 2 days of thymidine depletion (Fig. 3D) is comparable with that exhibited by wild-type cells after 24 days in standard BG11 medium (Fig. 1A). This observation supports the suggestion that cell elongation in aged *Synechococcus* cultures results from inhibition of DNA replication, and consequently, the inability to undergo cell division.

To compare the amount of cellular DNA in exponentially growing and elongated cells, we employed Vybrant green staining and flow cytometry (Fig. S1). The analysis revealed slightly lower DNA content in aged as compared with exponentially growing cultures. Cyanobacteria possess several copies of the chromosome [up to eight chromosomes (Binder and Chisholm, 1990; 1995)]. It therefore could be that in some of the cells, cytokinesis occurred at the expense of reducing the chromosome copy number. In most of the cells in aged culture, however, DNA content is similar to that of exponentially growing cells, an observation supporting the hypothesis that cells that do not replicate their chromosome-set do not undergo division.

Elongation occurs under phosphorus limitation and nitrogen sufficiency

The conventional BG11 growth medium (Stanier *et al*., 1971), which was used for this study, contains a large

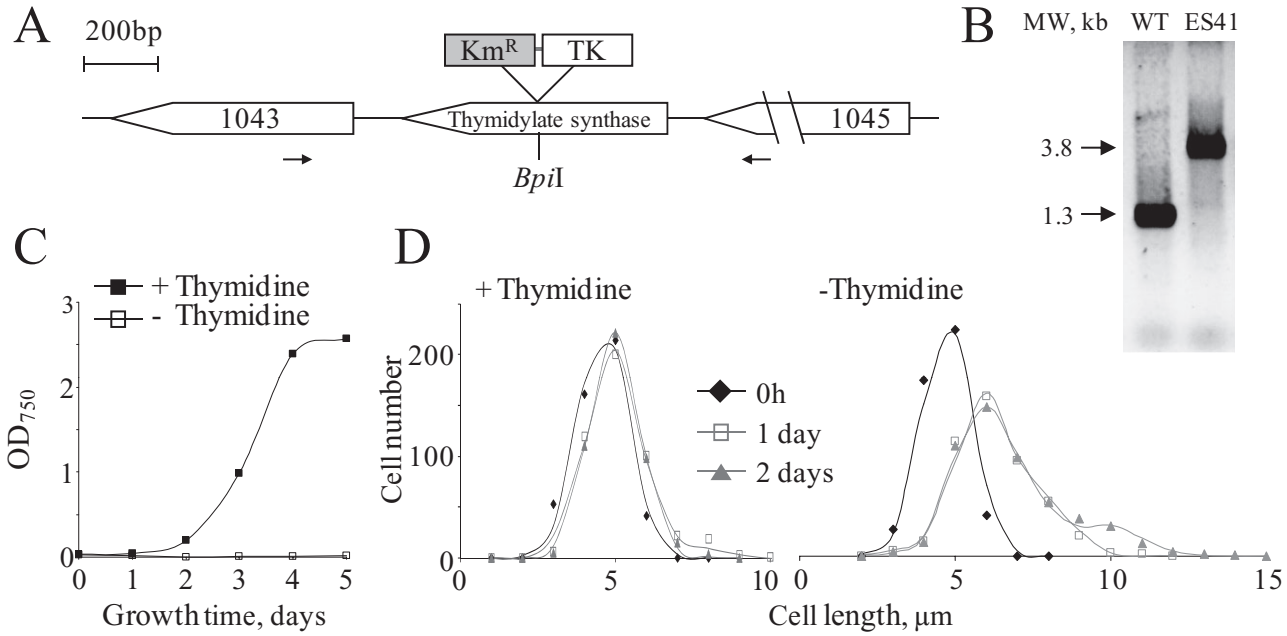

Fig. 3. Thymidine starvation of a thymidine-auxotrophic strain triggers cell elongation.
A. Physical map of the genomic region of *Synechococcus* thymidylate synthase. BpiI indicates the restriction site used for insertion of a kanamycin resistance (Km^R) cassette and thymidine kinase (TK) from *E. coli*. Arrows indicate primers used for PCR. ORFs 1043 and 1045 encode a hypothetical protein and an amidase enhancer-like protein respectively.
B. PCR analysis using the primers shown in (A) of wild-type (WT) and the thymidine-auxotroph strain (ES41). This analysis indicates inactivation of thymidylate synthase as well as the absence of a wild-type copy of the gene encoding this enzyme. MW – molecular weight markers.
C. Growth curves of strain ES41 in medium containing 750 μM thymidine or in the absence of thymidine.
D. Distribution of cell length of the thymidine-auxotroph strain (ES41) as affected by thymidine in the medium. Cells were grown in the presence of 750 μM thymidine, washed with growth medium devoid of thymidine and re-inoculated into medium containing 750 μM thymidine, or lacking thymidine. Distribution of cell length is shown at the time of re-inoculation and following 1 or 2 days in the indicated media. Data points indicate the number of cells falling within bins of 1 μm.

excess of nitrogen and, comparatively, a low level of phosphorus. As suggested above (Fig. 3), inhibition of DNA replication resulted in cell elongation. Because phosphorus is required for synthesis of DNA, we postulated that cell elongation is caused by phosphorus limitation encountered by *Synechococcus* cells in a depleted culture. Cell elongation under these conditions might occur due to the autotrophic metabolism that provides the required carbon skeletons and the presence of sufficient levels of nitrogen, a major macronutrient.

To directly test the role of phosphorus, we added phosphorus to the growth medium, while keeping the nitrogen concentration constant, to fix the nitrate to phosphate ratio (N : P) at 15:1 [instead of N : P of 100:1 (Fig. 4A), as in typical growth medium, see *Experimental procedures* for details]. In this high phosphorus medium, cell elongation was not observed (Fig. 4B). Furthermore, we demonstrate that nitrogen excess is also a prerequisite for cell elongation; a second modified medium containing the standard phosphate concentration but reduced nitrate, at an N : P ratio of 15:1, similarly did not support cell elongation (Fig. 4C). Growth medium in which both N and P concentrations were reduced by 10-fold, while maintaining an N : P ratio of 100:1, did not allow cell elongation (Fig. 4D). Therefore, a particular N : P ratio *per se* is not sufficient for triggering cell elongation. Rather, the combination of phosphorus limitation and sufficient nitrogen supply results in elongation of the majority of the *Synechococcus* cells in the culture.

Importantly, experiments involving manipulations of nutrient level were performed on cells grown in a standard growth medium to mid-exponential phase and concentrated to $OD_{750} \approx 4$ to apply the cell density of aged culture. Thus, thought nutrient status is clearly a crucial parameter, it may be speculated that by itself, it is insufficient, and density dependent mechanisms also come into play.

Elongated cells divide asymmetrically once provided with fresh growth medium

To determine if the elongated cells could resume cell division, we followed their length distribution following nutrient repletion. Aged *Synechococcus* cells were size-fractionated by filtration and the elongated cells were inoculated into fresh liquid growth medium. As shown in

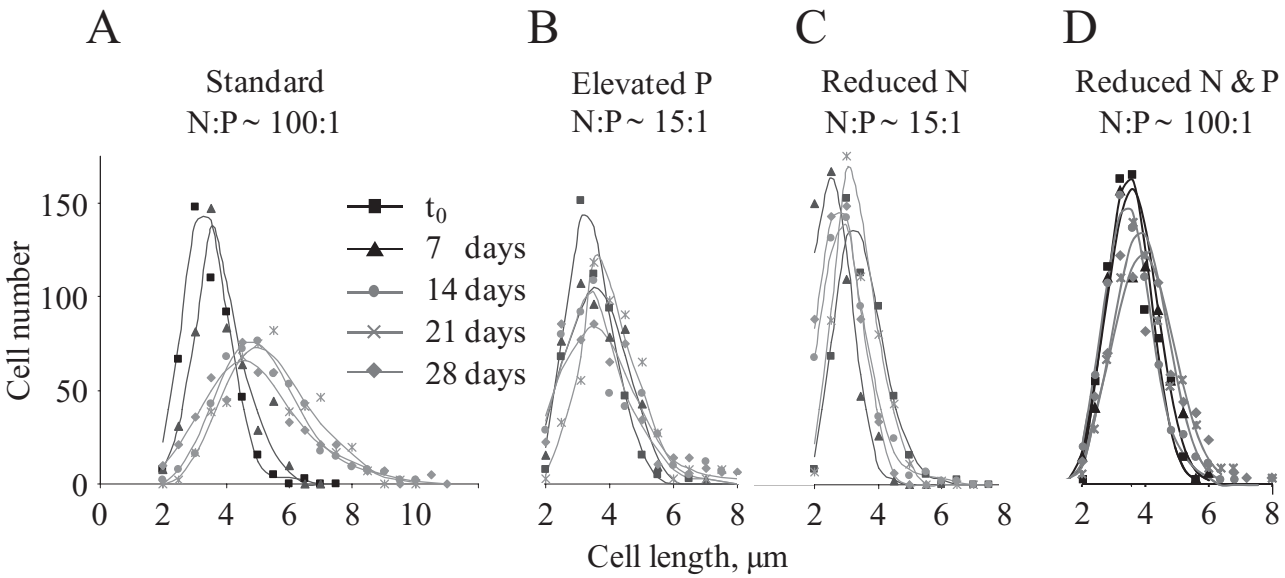

Fig. 4. Effect of the nutritional state on cell morphology. Distributions of cell length at various time points following inoculation into a standard growth medium (A) or into media modified as follows: (B) elevated phosphate (P) (C) reduced nitrate (N) and (D) reduced nitrate and phosphate. Before inoculation, the cells were grown in a standard growth medium to mid-exponential phase and concentrated to $OD_{750} \approx 4$. Analyses of cell length in these sets of experiments were performed for 4 weeks, as cultures subjected to low nitrogen level collapsed when maintained longer under these conditions. Data points represent the number of cells falling within bins of 0.5 μm.

Fig. 5A, cell length was reduced as a function of time, and within 2 days, the culture was characterized by the typical length distribution of exponentially growing cells.

Exponentially growing *Synechococcus* cells reproduce by symmetric division. In this process, often termed 'binary fission', constriction occurs in mid-cell, yielding equal size daughter cells. Microscopic examination of elongated cells following inoculation into nutrient sufficient liquid medium revealed, however, that they do not use this canonical mode, but rather divide in an asymmetric fashion (not shown). To follow consecutive divisions of elongated cells, we performed live cell imaging following inoculation onto solid fresh growth medium (Figs 5B and S2). The images revealed initial cell elongation, which was followed, in most cases, by asymmetric division (Fig. 5B, t_0 and 20 h).

To quantitatively represent the position of cell constriction, we measured the length of each of the two daughter cells and derived the ratio between the shorter and longer cell. About 95% of the dividing cells in an exponentially growing cell culture were characterized by a ratio of 0.9–1 and none of the cells showed significant asymmetric division (defined as a ratio of 0.6 or below, Fig. 6A). Contrary, most of the long cells divided asymmetrically with only about 5% of the daughter cell pairs exhibiting ratios greater than 0.9 (Fig. 6A). About 80% of the short cells produced by the asymmetric division were of length typical of cells in exponentially growing culture (Fig. 6B). Approximately 2% of the elongated cells exhibited two asymmetric constriction sites per elongated cell upon nutrient restoration (not shown).

Elongated cells are competent proliferative units

As shown, elongated cells are capable of cytokinesis once proliferative conditions are restored. The elongated morphology, however, may be associated with a physiological state that does not allow a rapid switch to a proliferative programme. Furthermore, cells that are not capable of further proliferation may be produced. Therefore, we examined if an elongated cell represents a proficient proliferative unit.

The live cell imaging experiments, which represent proliferation on solid growth medium, revealed that elongated cells could divide and, importantly, that their progenies are capable of further proliferation (for example, the cell indicated by the arrow in Fig. 5B at 20 h yielded two daughter cells by 46 h). They also indicated that elongated cells produce a higher number of progenies compared with short aged cells (compare Fig. 5B and C). To examine if these properties are also manifested in liquid culture, we compared the bulk growth characteristics of elongated versus aged short cells. To compare cultures representing an equal number of potentially proliferating units of either elongated or short aged cells, an aged *Synechococcus* culture was size-fractionated and an equal number of cells from the shortest and longest fractions were inoculated separately into fresh growth medium. As shown

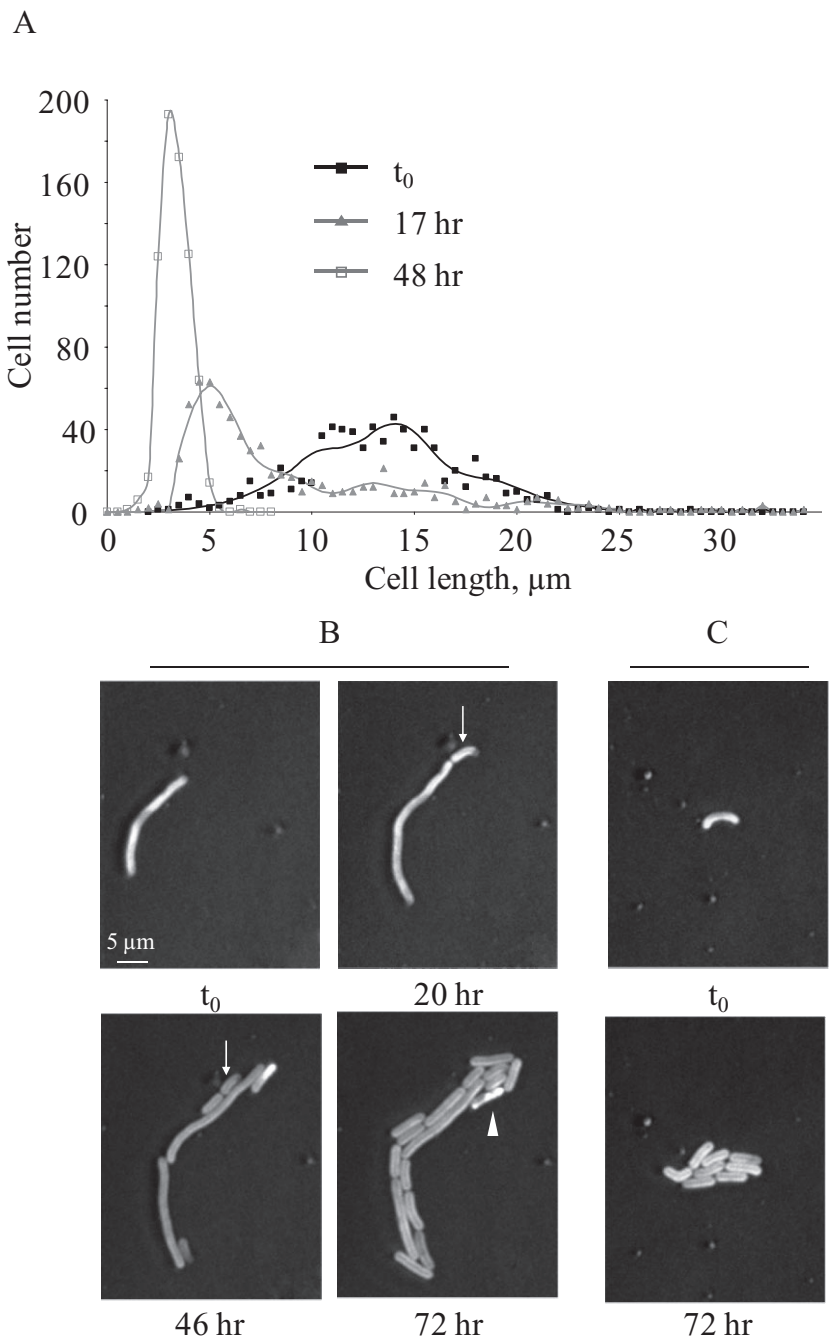

Fig. 5. Elongated *Synechococcus* cells resume proliferation and acquire standard cell length once provided with fresh growth media.
A. Distribution of cell length of elongated *Synechococcus* cells obtained following size-fractionation, and of the same culture after 17 and 48 h in fresh medium. ROC curve analysis was used to evaluate the size-fractionation of the cells. Area under the curve: 0.981 (95% confidence interval: 0.967–0.996). The elongated-cell-enriched fraction used in the experiment contained 95% 'long cells', defined as cells longer than 6 μm. [This length was chosen as the cut-off as cells ≥ 6 μm were rarely observed in exponentially growing cultures (see Figs 1, 3 and 7)]. Data points represent the number of cells falling within bins of 0.5 μm.
B and C. Fluorescence images, obtained from live cell recording (Fig. S2), of elongated (B) and short (C) aged cells following nutrient repletion. The selected frames show cells at the time of inoculation (t_0), and following growth on a solid fresh medium for the indicated times. Arrows point at the first short cell produced by asymmetric division and the daughter cells produced form this progeny (B, 20 and 46 h, respectively). Arrowhead indicates a daughter cell that did not undergo further division.

(Fig. 7A), progenies of elongated cells accumulated faster compared with those of short aged cells. These data strongly suggest that elongated cells represent an efficient reproductive unit and that the biomass accumulated during elongation is effectively converted into progeny cells capable of further proliferation.

To further examine this notion, *Synechococcus* cells possessing different antibiotic resistance cassettes were used to enable selection of a particular subpopulation. Similar to the wild-type strain, the antibiotic resistant strains exhibited cell elongation upon aging. The kanamycin- and spectinomycin-resistant cells (Km^R and Spc^R, respectively) were aged in separate cultures and were then size-fractionated. Elongated Km^R cells were inoculated into fresh medium together with an equal number of short aged Spc^R cells. Assessment of the strain composition of the mixed culture was performed by plating aliquots of the liquid cell suspension on selective solid growth media containing either Km or Spc. Figure 7B, which represents the strains' composition fol-

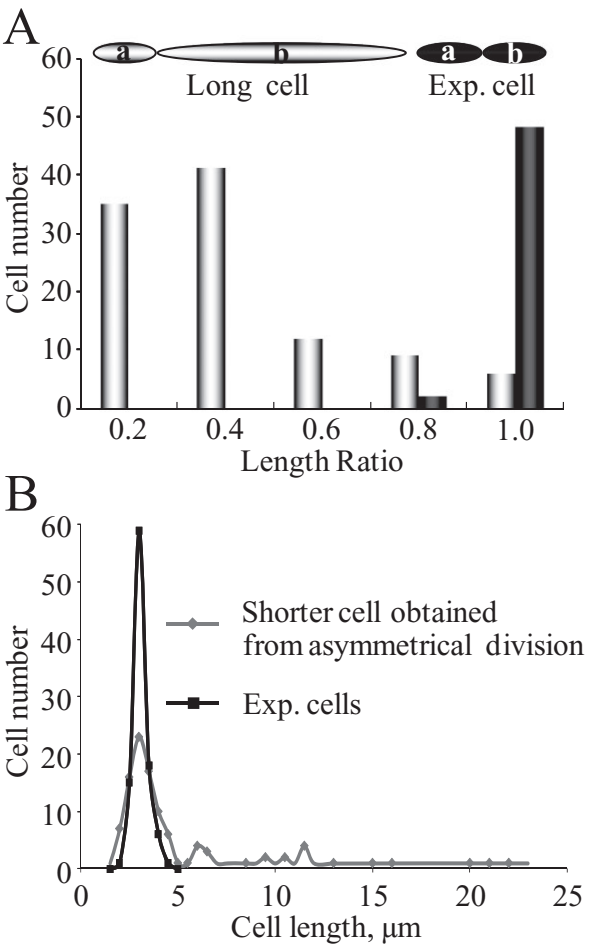

Fig. 6. Elongated *Synechococcus* cells exhibit asymmetric division once inoculated into fresh growth media.
A. Site of constriction, expressed as the length ratio (a/b in the diagrams) between daughter cells, in long cells (grey bars) and exponentially growing cells (Exp; – black bars). Bars represent the number of cells falling within bins of 0.2.
B. Length distributions of the short cells obtained from asymmetric division of aged elongated cells and of cells in exponentially growing culture.

lowing 10 days of growth in a mixed cultures, demonstrates that the number of progenies of the aged elongated cells was 1.6 times higher compared with the number of progenies of aged short cells. The reciprocal experiment in which aged Spc^R elongated cells were inoculated with short Km^R cells resulted in similar offspring enrichment of elongated cells (not shown).

Approximating the initial biomass of the elongated and the short aged cells from the length distributions, we found a 1.7-fold increase in elongated cells biomass. Because the number of progenies of these cells was 1.6 higher compared with the number of progenies of aged short cells, we conclude that the efficiency of biomass conversion into progeny cells was similar between the two populations.

Discussion

Our study describes nutrient-associated changes in *Synechococcus* sp. PCC7942, which include arrest of cytokinesis and cell elongation, as well as asymmetric cell division once proliferative conditions are resumed. In spite of the substantial increase in cell length, the characteristic arrangement of the photosynthetic membranes in concentric shells is fully maintained in the elongated cells (Fig. 2). The preservation of thylakoid membrane structure in the nutrient-depleted cells likely contributes to their ability to maintain growth, as well as to their capacity to readily resume proliferation upon nutrient repletion.

The propensity of *Synechococcus* to elongate is actually reflected in the species name, as this cyanobacterium is also termed *Synechococcus elongatus*. The elongation phenomenon, however, was not addressed in details and the conditions inducing it were generally not investigated. Elongated *Synechococcus* cells i.e. 10–15 μm in length, were observed under particular conditions of relatively low temperature and elevated CO_2 concentration (Burns *et al.*, 2005). The cell elongation we observed, however, is not restricted to conditions of CO_2 enrichment, as substantial cell elongation also occurred in aging cultures bubbled with air (not shown).

Our microscopic examinations indicate that cell elongation is not accompanied by a change in the thickness of the cells; both elongated and exponentially growing cells are characterized by a cell diameter of ~ 1 μm (Fig. 2). Changes in object dimensions may have a strong impact on the surface area (S) to volume (V) ratio. For certain geometries, such changes may impose S/V ratios that would slow down metabolism by limiting exchange of materials between the cells and their surroundings (Young, 2006). In an elongated cell, the contribution of the 'domes' at the cell ends is relatively small, and S/V may be approximated by the ratio 4/d (where d is the cell diameter). Given that cell elongation is not accompanied by changes in cell diameter, S/V remains essentially constant in *Synechococcus*. Contrary, a spherical cell (with an S/V of 6/d), which grows by expanding its diameter, would experience limitations born of a decrease in its S/V ratio. Accordingly, *Synechocystis* sp. PCC6803, a spherical cyanobacterium, does not exhibit an increase in cell size in response to culture aging (not shown).

Experiments on thymidine-auxotrophic cells (Fig. 3) imply that elongation of *Synechococcus* cells is associated with inhibition of DNA replication. In agreement, under relative shortage of phosphorus, which likely limits the availability of phosphorylated deoxyribonucleotides – the precursors for DNA synthesis, increase in cell length was observed (Fig. 4A), whereas addition of phosphorus precluded cell elongation (Fig. 4B). Taken together, it is conceivable that phosphorous shortage encountered by

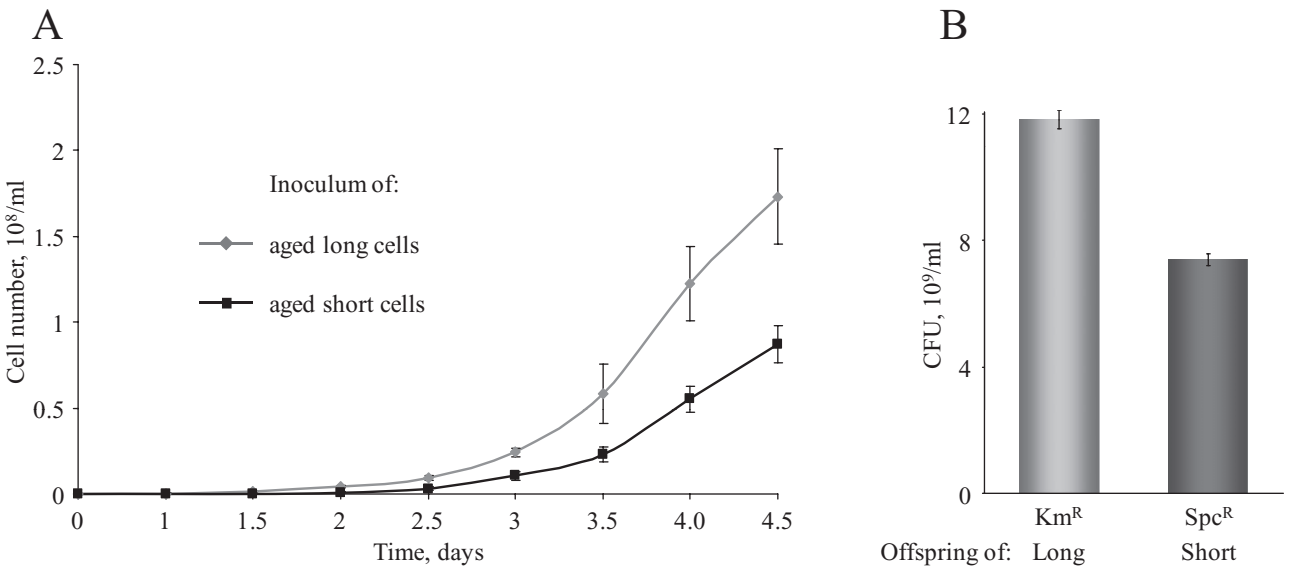

Fig. 7. The biomass accumulated in elongated cells is effectively converted to proliferation proficient daughter cells.
A. Cell number of elongated and short aged cells (obtained by filtration, see *Experimental procedures*) as a function of time, following inoculation into fresh growth media. ROC curve analysis was used to evaluate the size-fractionation of the cells. Area under the curve: 0.957 (95% confidence interval: 0.934–0.979). The elongated cell-enriched fraction contained 90% long cells and the short cell-enriched fraction of aged cells contained 7% long cells.
B. Colony forming units (CFU) obtained following plating on selective media of a mixed culture containing the progeny of kanamycin resistant (Km^R) and spectinomycin resistant (Spc^R) cells. Strains possessing different antibiotic resistance genes were aged in separate cultures and were size-fractionated; equal numbers of Km^R-elongated cells and Spc^R-short aged cells were then inoculated into a single tube containing fresh medium. ROC curve analysis of the Km^R culture revealed area under the curve: 0.928 (95% confidence interval: 0.901–0.955). Analysis of Spc^R culture indicated area under the curve: 0.98 (95% confidence interval: 0.967–0.992).

aging cultures inhibits DNA replication and, consequently, cytokinesis is precluded. (Evidently, an adequate amount of phosphorus is allocated to support the apparent cell elongation, e.g. to produce RNA precursors and phospholipids). Importantly, sufficient amount of nitrogen is essential to support cell elongation (Fig. 4C). Thus, in an autotroph like *Synechococcus*, the combination of phosphorus limitation, which prevents cytokinesis, and sufficiently high nitrogen levels (and likely additional nutrients), which enable cell growth, is a prerequisite for cell elongation.

The live cell imaging experiments, which allowed following consecutive cell divisions, indicated that progeny cells produced upon nutrient repletion are capable of further proliferation. Thus, these experiments clearly demonstrate that adequate chromosome replication and segregation occurs upon asymmetrical division.

Elongated *Synechococcus* cells divide asymmetrically upon nutrient repletion (Figs 5B and 6A). In most cases, the shorter cell produced is of a normal size, namely it is characterized by length typical of exponentially growing cells (Fig. 6B). This suggests that the site of constriction in the elongated cell is not random and plausibly, a mechanism favouring the formation of 'normal-sized' cells directs their positioning within the cell. Previous studies highlighted several features of cyanobacterial cytokinesis. Interestingly, *Synechococcus* possesses homologues of the Min system, which is involved in the division machinery of Gram-negative bacteria, as well as homologues to proteins typical of cytokinesis in Gram-positive bacteria and in addition some unique components (Kuhn *et al*., 2000; Koksharova and Wolk, 2002; Mazouni *et al*., 2004; Miyagishima *et al*., 2005; Sakr *et al*., 2006; Klint *et al*., 2007; Koksharova *et al*., 2007; Marbouty *et al*., 2009a,b,c,d; Dong *et al*., 2010). The detailed mechanism underlying the position of constriction in cyanobacterial symmetric fission as well as determination of the sites of asymmetric division of elongated cells, as observed in this study, are yet to be elucidated.

Bacterial cell elongation, followed by asymmetric division, has been observed under several different conditions. For example, thymine starvation of thymine auxotrophs of *E. coli* induces cell elongation (Zaritsky *et al*., 2006). Additionally, DNA damage and induction of the SOS response leads to elongation (Mukherjee *et al*., 1998), followed by uneven division once proliferation resumes (Maguin *et al*., 1986); [reviewed in (Justice *et al*. 2008)]. The predatory bacterium *Bdellovibrio* also exhibits morphological flexibility. In this case, elongation occurring within the cytoplasm of the bacterial host cell is followed by asymmetric division once the host cytoplasm is consumed (Angert, 2005). Thus, it is likely that

nutrient starvation triggers uneven division in *Bdellovibrio*, in contrast to *Synechococcus*, which resumes cytokinesis and exhibits asymmetric division upon nutrient supply.

The conditions leading to cell elongation, i.e. phosphorus limitation with an adequate supply of nitrogen, may be encountered by native populations of cyanobacteria. Phosphorus restricts microbial production in freshwater and oceanic environments (Hudson *et al*., 2000; Wu *et al*., 2000). Soluble nitrogen, however, may be provided to the system by microorganisms (including certain cyanobacteria) capable of N_2 fixation (Capone *et al*., 1997; Berman-Frank *et al*., 2001). High rates of N_2 fixation were recently reported to occur in the North Pacific Ocean. Thus, reduced nitrogen could be supplied to the upper water column at a rate comparable with that of the equatorial upwelling system, without any supply of PO_4^{3-} (Montoya *et al*., 2004). Additionally, short-lived transport events connect deep stocks of nitrate to nutrient-poor surface waters (Johnson *et al*., 2010). It may therefore be suggested that the morphological flexibility described in this study represents a beneficial strategy of rod shape photoautotrophs to environmental conditions involving phosphorous limitation under a background of sufficient levels of reduced nitrogen.

Experimental procedures

Growth conditions and strains

Synechococcus cells and all strains resulting from molecular manipulations of the parental strain were grown at 30°C in BG11 growth medium (Stanier *et al*., 1971). Cultures were illuminated with incandescent light (30 μmol photons m^{-2} s^{-1}) and bubbled with 2% CO_2 in air. In particular experiments (as specified in *Results*) cultures were bubbled with air. To examine the effect of nutrient status on cell morphology the N : P ratio was set at 15:1 (instead of 100:1 in classical BG11) by either increasing the phosphate concentration while keeping the nitrate concentration unchanged or by or lowering the concentration of the latter while keeping the phosphate concentration fixed. To maintain the ionic strength of the medium NaCl was added as required. For experiments involving nutrient manipulations the cells were grown in a standard growth medium to mid-exponential phase, and before inoculation were concentrated to $OD_{750} \approx 4$ to yield cell density typical of aged culture.

Synechococcus cells possessing either Km^R or Spc^R resistance genes were obtained by insertion of the relevant cassettes into a genomic neutral site referred to earlier (Sendersky *et al*., 2005).

Preparation of thymidine-auxotroph Synechococcus *strain*

Thymidine-auxotroph *Synechococcus* cells were obtained by inactivation of thymidylate synthase (TS) and insertion of thymidine kinase (TK) of *E. coli* as follows: DNA fragment of 1334 bp containing *Synechococcus* TS gene was obtained by PCR on genomic DNA using the primers 5′ TCGAACCAGAGCAGGATGG 3′ and 5′ CACCCGAGAACAGCACCG 3′. This fragment was cloned into pGEM-T Easy (Promega) and a cassette encoding for Km^R was cloned into the BpiI site located within the coding region of the TS gene. The TK gene of *E. coli* K-12 was obtained by PCR on genomic DNA using the primers 5′ ATGGTAGGATCCAGTTGTGTTTACGAGAATTCC 3′ and 5′ ATGGTAGGATCCTCTTAAGTGAACGGCATTGC 3′. This 1238 bp fragment was cloned into the BamHI site adjacent to the Km^R cassette. *Synechococcus* was transformed with the resulting construct. Selection was performed in the presence of 50 μgr ml^{-1} Km and 250, 500 or 750 μM thymidine. PCR was used to screen about 400 transformants to identify a clone that underwent complete segregation and possessed only the inactivated TS gene (see *Results*, Fig. 3).

Length fractionation and microscopy

Fractionation of cells in aged cultures according to cell length was performed by filtration using nitrocellulose filters (Schleicher & Schuell). A fraction highly enriched with elongated cells was collected as the 'filter cake' on filters with 12 μm pores and resuspended in fresh BG11 growth medium. Filtrate obtained by passing the cell suspension through a 5 μm pore membrane contained the aged short cells. These cells were washed and resuspended in fresh BG11. For assessment of cell length, 200–800 cells were visualized using Axio Imager.Z1 (Zeiss) with a 1.3-NAx100 (EC Plan-NeoFluar) objective, and analysed by the image analysis software, ImageJ. The results were subjected to statistical analysis by Receiver Operating Characteristic (ROC) curve to evaluate the quality of separation (see Figure legends). For inoculation at a defined cell density and for growth analysis, cells were counted by Fluorescent Activated Cell Sorter (FACS). Electron microscopy was performed as described in Nevo *et al*. (2007), on high-pressure frozen, freeze-substituted cell samples. Live cell imaging was carried out using an IX70-based (Olympus) DeltaVision deconvolution microscope system (Applied Precision), with a 0.9-NA × 40 (UplanApo) objective, enhanced further by 1.6-fold auxiliary magnification. Ten regions of interest (103 × 103 μm) were visited in a computer-controlled manner at 2 h intervals for 72 h; the focus was adjusted automatically for the fluorescence signal. The temperature was 30 ± 0.2°C. Both DIC and fluorescence images were recorded. For the latter, Cy5 filters (CWL/BP: 640/20 and 685/40 nm, for excitation and emission, respectively) were used.

Acknowledgments

We would like to thank Prof Arieh Zaritsky for critically reading the manuscript, Prof David Steinberg and Dr Yury Kamenir for advising on statistical analysis and Uriel Karo for assistance with flow cytometry. This study was supported by the German Israel Foundation (Grant No. I-729–27.9/2002 and by the Israel Science Foundation (Grant No. 683/03–17.1).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DNA content in exponentially growing and aged (7-week-old) cells. Visualization of DNA was obtained by staining with the membrane permeable fluorescent stain Vybrant DyeCycle Green (Molecular Probes) at a final concentration of 10 μM. DNA-stained cells were analysed by flow cytometry.
Fig. S2. A representative live cell recording of an aged elongated *Synechococcus* cell following inoculation onto fresh growth medium. Cell suspension (10–20 μl) was spotted into a 14 mm glass-bottom Petri dish and a layer of 1.5% agarose dissolved in BG11 was placed over the drop. For more details, see *Experimental procedures* in the text.

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