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A novel cyanobacterium exhibiting an elevated tolerance for iron

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Abstract

Studies directed at cyanobacteria inhabiting iron-depositing hot springs may provide insights into the role of both ancient and contemporary cyanobacteria mediated iron transformations. Here we phylogenetically, morphologically and physiologically characterize a novel cyanobacterium isolated from an iron-depositing hot spring. Phylogenetic analyses indicate that the bacterium is a representative of a new genus, exhibiting a maximum 95.2% homology to database sequences. The isolate is a unicellular cyanobacterium with bladder-like cells typically packed as duplexes, or in extracellular polymeric substance covered clumps and small chains without the ability to produce baeocystes. No growth without added combined nitrogen occurred. While requiring relatively large amounts of iron for growth (>40 μM), the isolate was shown to facilitate removal of iron from culture media. These results suggest that the isolate may be an important component of an iron-depositing microbial community. The name "Chroogloeocystis siderophila" for this cyanobacterium is proposed.

Keywords: Novel cyanobacterium; Iron tolerance; Iron depositing hot spring; Great Yellowstone area

1. Introduction

Precambrian cyanobacteria likely resided in aqueous environments characterized by very different physicochemical parameters than are typical today. Since ferrous iron of aqueous oxygenic environments is rapidly oxidized to ferric iron, which forms insoluble hydroxides at pH $\geqslant$ 7 [1,2], the biological availability of iron is severely reduced in most contemporary environments. In contrast, dissolved iron concentrations within Precambrian springs, rivers, lakes and oceans could have approached 100 mg kg^{-1}, levels significantly greater than all contemporary analogs excluding thermal springs and vents [3–5]. In these iron-enriched environments, iron detoxification mechanisms would have been necessary for cellular survival, while the iron-scavenging mechanisms exhibited by many contemporary marine and freshwater cyanobacteria [6–9] may have developed in response to more recent reductions in soluble iron availability.

Although the iron-dependent physiology of marine and freshwater cyanobacterial strains has been the focus of extensive study [9–11], very few studies dedicated to the physiology and diversity of cyanobacteria inhabiting iron-depositing hot springs have been conducted [12,13], despite the insights into contemporary, as well as ancient, microbial iron transformations such studies could potentially provide. One of the few studies that have been conducted [12] found that cyanobacterial members of iron depositing bacterial mat communities might increase the rate of iron oxidation in situ. Interestingly,

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these authors also found that ferrous iron concentrations up to 1 mM significantly stimulated light dependent consumption of bicarbonate [12], suggesting a specific role for elevated iron in photosynthesis of cyanobacteria isolated from iron depositing hot springs.

These works highlight the need for further studies addressing the diversity and ecophysiology of iron-resistant cyanobacteria and their interactions with iron-saturated environments. Elucidating the roles played by this unique group of organisms in iron transformations may require study of individual isolates. Our aim was therefore to obtain polyphasic information about single representatives of iron depositing hot spring cyanobacterial communities rather than to list simply their diversity.

Here we report the first isolation, taxonomic characterization and physiological properties of a Greater Yellowstone area iron-depositing hot spring cyanobacterium exhibiting elevated iron tolerance.

2. Materials and methods

2.1. Source of organism

Microbial mat samples were collected by scraping the side of a cement drainage culvert connected to La Duke Hot Spring (110°46′32″ W; 44°05′26″ N, Montana, USA) in July 2001. Although the site of collection is 5–8 cm above the main outflow stream (Fig. 1), it is continuously moistened by outflow water (60 °C; pH 6.85) containing 2.8 μM ferrous and 4.2 μM ferric iron. Analysis of water sampled directly from La Duke Hot Spring indicated substantially different ferrous and ferric iron concentrations (4.48 and 2.93 μM, respectively). Although further chemical analysis of outflow water was not conducted, Papke et al. [5] provide extensive physical and chemical information for water sampled directly from La Duke Hot Spring, and many other diverse and geographically dispersed hot springs.

Fig. 1. Site of cyanobacterial mat collection. Cyanobacterial mat materials were scraped from a permanently shaded area of the drainage culvert (white arrow), 5–8 cm above the outflow stream.

2.2. Media and cultivation

Immediately after collection, cyanobacterial mat samples were immersed in 50 ml flasks containing outflow water. Mat samples were used to inoculate three different liquid media: BG-11 [14], D [15] and DH (D medium supplemented with 1.2 g/L HEPES). Attempts to obtain enriched cultures of cyanobacterial mat inoculants were not successful until growth media BG-11, D and DH were supplemented with 40 μM of $FeCl_3 \cdot 6H_2O$. DH medium, unlike the other media tested, maintained a pH of 8.2 and supported greater growth. All subsequent growth experiments utilized DH media and were conducted at 50 °C, the approximate ambient temperature of the collection site, with illumination maintained at 30 $\mu E\ m^{-2}\ s^{-1}$.

Incubation of samples for two to three months resulted in enrichment of morphotypes resembling putative strain 5.2 s.c.1. Aliquots were streaked onto DH medium solidified with 1.5% of Sea Kem agarose (BioWhitaker Molecular Applications). The relative purity of Sea Kim agarose limits heterotrophic bacterial growth and therefore possible bio-inhibition of cyanobacterial growth (Rippka, R.R., personal communication). Single colonies were used to inoculate sterile microcentrifuge tubes containing 1 mL ironsupplemented DH medium and incubated for one month or more at 50 °C under illumination. The presence of single morphotypes was determined microscopically. Tentatively pure cultures were subcultured twice, resulting in the isolation of seven unialgal, nonaxenic strains. Each isolate was stored at −80 °C.

Cultures of 5.2 s.c.1 isolate were deposited at American Type Culture Collection (accession # ATCC BAA-845), Microbial Cultures Collection, Japan (accession # NIES-1031), and Culture Collection of Algae and Protozoa, Great Britain, (accession # 1419/1).

2.3. Determination of iron

Total iron contents were determined as described previously [16]. The ferric iron content was calculated by subtracting the ferrous iron content from the total iron content.

2.4. Determination of iron requirement and tolerance

Iron requirements and tolerance were estimated by measuring cell yield in DH media supplemented with a range of $FeCl_3 \cdot 6H_2O$ concentrations (0–1000 μM) using fluorometric chlorophyll determination as a surrogate for cell quantity [17].

2.5. Light microscopy

Standard light microscopy techniques were employed using a Nikon Microphot-SA microscope. Images were captured with the photocamera FX-35A and processed using Polaroid PolaColar Insight and Adobe Acrobat 7.0 software.

2.6. Transmission electron microscopy

Samples for TEM were collected by centrifugation (6670 × g, 10 min) of cyanobacterial cultures at room temperature. Pellets were washed twice with fresh DH medium and subsequently fixed with Karnovsky's fixative [18]. Cells were rinsed with 2% cacodylate buffer, post-fixed with 2% aqueous osmium tetroxide and dehydrated slowly in a graded ethanol series. After dehydration, cells were infiltrated with EMBed 812® resin (Electron Microscopy Sciences, Fort Washington, PA) and polymerized at 60 °C for 18 h. Blocks were sectioned (~70 nm) on an RMC MTXL Ultramicrotome with a diamond knife. Sections were captured on a 200 mesh Cu/Rh grid, stained with 2% uranyl acetate and Reynold's lead citrate, and subsequently examined using a Hitachi H-7100 TEM at 75 kV accelerating voltage. Images were captured with an AMT digital capture system on a 1 K by 1 K camera system.

2.7. Scanning electron microscopy

Samples for SEM were collected on 0.45 mm pore diameter filters (Millipore) and washed with distilled H_2O to remove soluble precipitate. Samples were then coated with Au–Pd, nominally 15 nm thick, using a Hummer VII coating device (Anatech Ltd). Sample mounts were visualized using a JEOL JSM-6100 SEM, with a LaB6 source.

2.8. Molecular methods

DNA was extracted from unialgal cultures using a bead-beating method described previously [19]. PCR-mediated amplification of genomic DNA was performed using cyanobacteria 16S rRNA gene-specific primers cy781f (A) and cy781f (B), and their reverse compliments, or CYA106F [20] in conjunction with the "universal" bacterial 23S rRNA gene or 16S rRNA gene reverse primers L23cyR [21] or 1492R [22] respectively. Fifty picomoles of each primer, 25 nmol of each deoxynucleoside triphosphate, 10 μl of 10 × PCR buffer, 0.5 U of SuperTaq DNA polymerase (Promega) and 10 ng of template DNA were combined in H_2O to a volume of 50 μl in 0.2 ml tubes. In reactions employing cy781f (A) and cy781f (B), equimolar amounts each were used. Thirty incubation cycles, each consisting of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C were performed using a GeneAmp PCR System 2700 (Applied Biosystems) thermocycler. Products of these reactions were included in sequencing reactions utilizing Big Dye terminator mix (Applied Biosystems, Foster City, CA), using the manufacturers recommended protocol. Sequence determination was performed using a Prism 310 genetic analyzer (Applied Biosystems).

2.9. Phylogenetic analyses

To determine potential phylogenetic affiliations of isolate 5.2 s.c.1, the ARB software package, developed by W. Ludwig and O. Strunk (http://www.mikkro.biologie.tumuenchen.de), was used to align the partial 16S rRNA gene of isolate 5.2 s.c.1 with reference 16S rDNA sequences in the latest version of ARB database (ssujun02). Initial phylogenetic analyses incorporated 16S rDNA sequences identified by BLAST (http://www.ncbi.nlm.nih.gov) as having homology to isolate 5.2 s.c.1, and all cyanobacterial sequences in the ARB database ($n = 599$) as the outgroup configuration. Phylogenetic trees were then generated in ARB using neighbor joining with Olsen correction and the Quick add sequence insertion tool using parsimony.

From these initial analyses, 42 cyanobacterial 16S rDNA reference sequences were selected to both include near full length sequences having relatively high homology to isolate 5.2 s.c.1 and to be representative of the known diversity of the cyanobacteria (database accession numbers of sequences used are represented in Fig. 2).

Phylogenetic trees using this dataset were constructed on the basis of nucleotides 221–1425 (*E. coli* numbering). Ambiguous positions of 16S rDNA alignments were excluded from similarity calculations by applying a maximum-frequency filter available in the current ARB database (ssujun02). A distance matrix using this alignment was constructed in ARB using the Jukes–Cantor model.

Five methods were employed to generate tree topologies using the software package PAUP* [23]. They include three evolutionary distance (ED) methods (neighbor joining with Kimura two-parameter correction and the general time reversible (GTR) model (empirically determined base frequencies and empirically determined γ-distribution models of site-to-site rate variation), the log determinant transformation [24], maximum parsimony (MP) (default settings, heuristic search) and maximum likelihood (ML) in conjunction with the GTR substitution model [25] (estimated shape and proportion of invariable sites, empirically determined base frequencies; heuristic search).

The robustness of ED and MP tree topologies was determined by bootstrap resampling (1000 repetitions). Branches were considered resolved by ML if branch lengths were positive at $p < 0.01$ level of significance by a likelihood ratio test (PAUP*, ZeroLenTest).

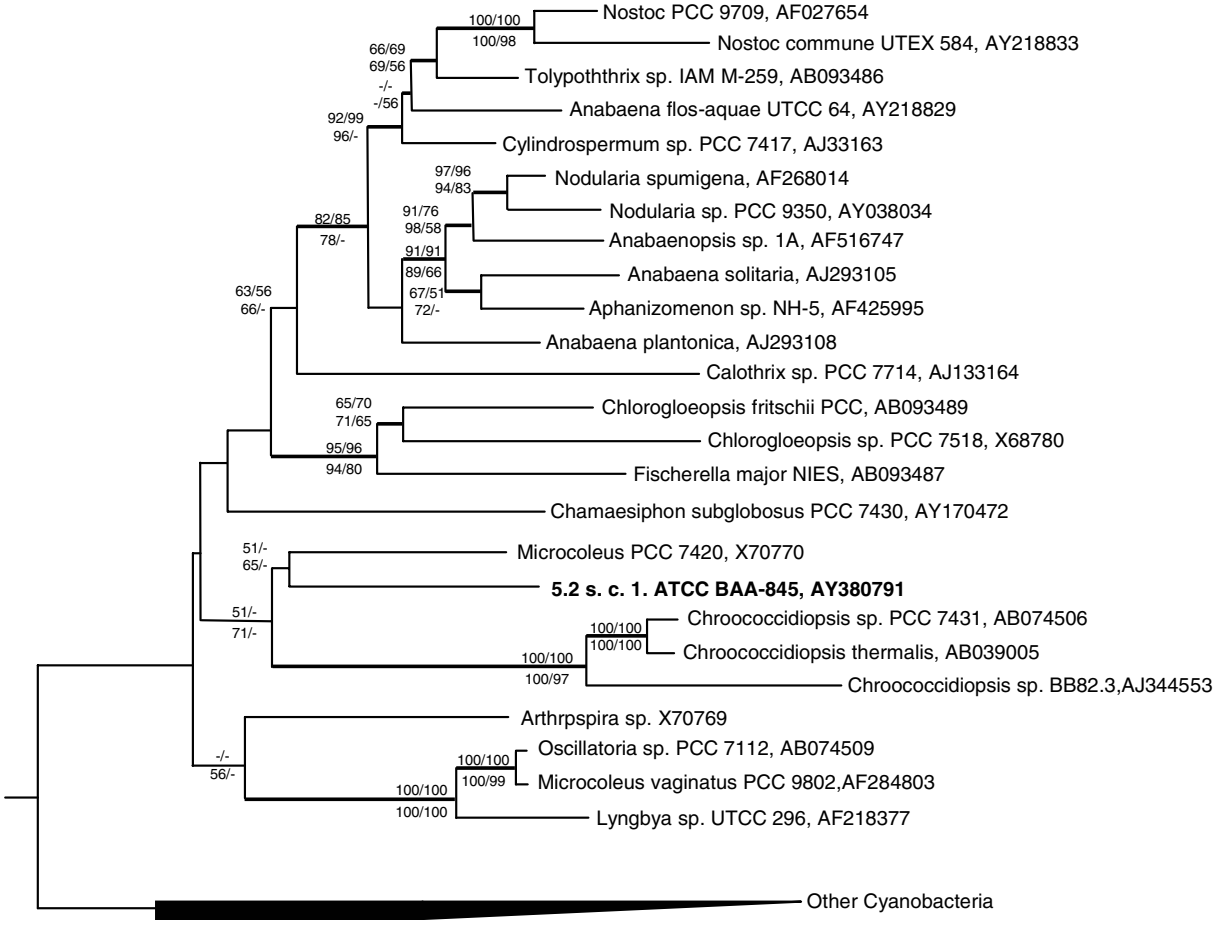

Fig. 2. Modified phylogenetic dendrogram generated using neighbor joining (Olsen correction) in ARB. 16S rDNA sequences from *E. coli* and *B. subtilus* comprised the outgroup. Values associated with internal nodes indicate bootstrap support from 1000 repetitions calculated using evolutionary distance [Kimura two-parameter model (upper left), LogDet transformation (upper right) and the GTR model (lower left)] and maximum-parsimony (lower right). Thick lines mark internal nodes resolved in maximum-likelihood trees (GTR model, ZeroLenTest) and at least two ED or MP analyses (bootstrap support >50%).

3. Results and discussion

3.1. Phylogenetic analyses

Comparison with database sequences using Blast N indicated that the 16S rRNA gene of isolate 5.2 s.c.1 (the GenBank accession number for the 16S rRNA gene sequence reported in this study is AY380791) exhibits the highest homology to that of *Microcoleus chthonoplastes* PCC 7420 (93%). A distance matrix based on the Jukes–Cantor model using our 42 cyanobacteria dataset also indicated that the sequence of *M. chthonoplastes* PCC 7420 exhibits the least distance from 5.2 s.c.1 (4.8%) and is on the borderline of accepted values for genus classification [26,27]. Even though initial phylogenetic analyses performed using ARB suggested a relationship between isolate 5.2 s.c.1 and *M. chthonoplastes* PCC 7420, more rigorous analyses failed to support this relationship. Although the node connecting isolate 5.2 s.c.1 and *M. chthonoplastes* PCC 7420 was resolved by ML using the GTR model, bootstrap support for placement of isolate 5.2 s.c.1 within any established cyanobacterial group was lacking for all analyses (Fig. 2). Lack of stable support for phylogenetic placement within the cyanobacterial tree indicates that 5.2 s.c.1 represents what has been referred to as a cyanobacterial "loner" sequence, having no close relatives, for which no taxonomic inferences can be made except for its isolated position [28].

These results indicate that isolate 5.2 s.c.1 diverges independently from any other genus-level cyanobacterial group and, therefore, merits genus status.

3.2. Physiological observations, morphology and ultrastructure

The isolate is a unicellular cyanobacterium with cells typically packed as duplexes, or in clumps or small chains (Figs. 3 and 4), covered with extracellular polymeric substance (EPS). Cells exhibit oval shape (average

longitudinal diameter of 4 ± 0.77 μm, and average transverse diameter of 3.3 ± 0.64 μm; $n = 10$). Micrographs (Figs. 3–5) indicate a bladder-like shape, similar to that of *Synechocystis* PCC 6803 cells [29]. Therefore, the root "cystis" (from Greek "*kystis*" – bladder or bag) was incorporated into generic name of isolate 5.2 s.c.1.

Light, TEM and SEM micrographs indicate that isolate 5.2 s.c.1 reproduces by binary fission in either one or two successive planes at right angles (Figs. 3–5). Although the morphology of 5.2 s.c.1 cells is reminiscent of the *Synechocystis* genus, a salient difference is that isolate 5.2 s.c.1 has a EPS cover, visible on SEM and TEM micrographs as a 0.3–0.4 m skin-like envelope (Fig. 5(a)–(c)). In accordance with this observation, the root "Chroo" (from Greek *Chroo* – skin) was incorporated into the generic name of isolate 5.2 s.c.1. The

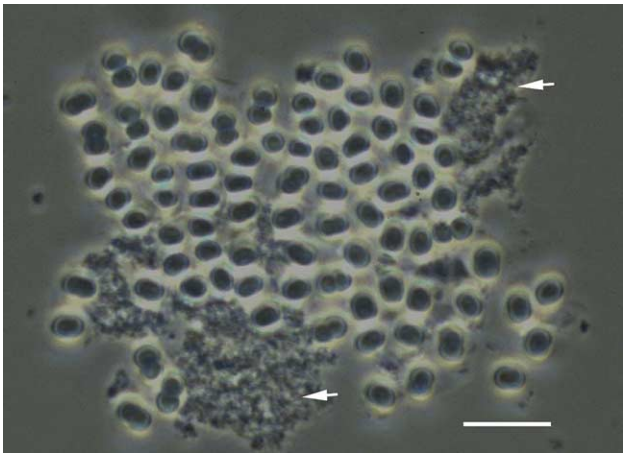

Fig. 3. Light micrograph of the 5.2 s.c.1 isolate during reproduction in liquid medium DH supplemented with 40 μM Fe^{3+}. Phase contrast. Bar = 5 μm. Thin arrows indicate mineral precipitates on cell surfaces.

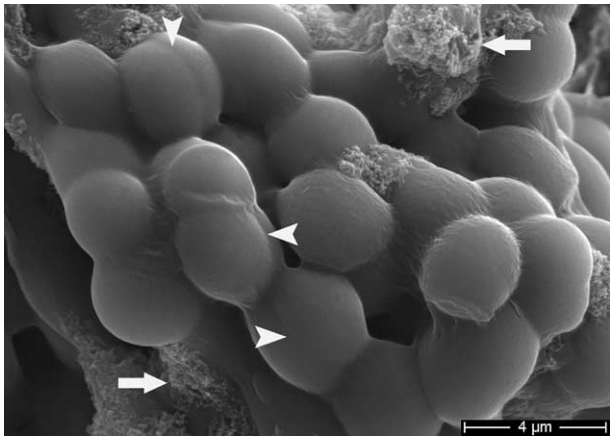

Fig. 4. Scanning electron micrograph of the isolate 5.2 s.c.1 cells showing binary fission of cells during reproduction (arrow heads), and precipitates (bold arrows). No baeocytes were observed. Regarding to energy dispersive X-ray spectroscopy (EDS) the crystal contained (in %): C – 0.01; Pd – 0.91; Ca – 3.15; O – 63.36; Fe – 24.87; Mg – 0.71; Au – 1.29; P – 5.15; Cl – 0.53.

EPS envelope of single 5.2 s.c.1 cells may reach ~1 μm, suggesting its importance to adhesion of 5.2 s.c.1 cells to surfaces. Thus, the Greek root "*Gloe*" (sticky) was incorporated into the generic name of this isolate – "Chroogloeocystis".

This finding, as well as its pronounced capacity to form biofilms on culture flask walls (data not shown), suggests that isolate 5.2 s.c.1 is a sessile organism. It is interesting to note that our three years fieldwork in Great Yellowstone thermal waters did not reveal any planktonic cyanobacterial populations. The cultivation of 36 primary cyanobacterial samples, isolated in different hot springs, in vitro suggested that all primary isolates of thermophilic cyanobacteria have an enhanced affinity to a substratum. Cyanobacteria from thermal springs may therefore be ideal candidates for biofilm-based photo-bioreactors.

Electron microscopy of 5.2 s.c.1 sections revealed that thylakoids were arranged concentrically along the inside of cytoplasmic membranes (Fig. 5(c)). Polyhedral bodies (carboxysomes) and lipid inclusions are apparent in Fig. 5(c). Unlike filamentous cyanobacteria of Section III [30], no junctional pores were observed between dividing cells (Fig. 5 (c)).

No growth without added combined nitrogen occurred, suggesting that 5.2 s.c.1 isolate is unable to fix atmospheric nitrogen, thus providing a physiological distinction between isolate 5.2 s.c.1 and the genus *Nostocales* [14].

Isolate 5.2 s.c.1 does not exhibit extensive vegetative binary fission following generation of baeocytes, a trait characteristic of the genus *Chroococcidiopsis* [31]. Additionally, isolate 5.2.s.c.1 is quite morphologically distinct from filamentous *Microcoleus* [32].

These results indicate that isolate 5.2 s.c.1 is morphologically and physiologically, as well as phylogenetically, distinct from any other cyanobacterial genus.

3.3. The interaction between 5.2 s.c.1 isolate and iron

Although cell division was suppressed in culture media supplemented with $FeCl_3 \cdot 6H_2O$ concentrations less than 40 μM and greater than 1000 μM, iron concentrations between 40 and 400 μM stimulated growth throughout 18-day incubations (Fig. 6). These results strongly suggest that exponential growth of 5.2 s.c.1 requires elevated concentrations of soluble iron (>40 μM). Several authors have observed elevated iron requirements for optimal cyanobacterial growth [33,34]. Thus, one might speculate that the iron concentrations of widely used media (see for a reference [35]) are not sufficient for polyphasic studies of the diversity and physiology of cyanobacteria isolated from iron-saturated biotopes.

Elevated iron requirements suggests that 5.2 s.c.1 may employ a siderophore independent mechanism of

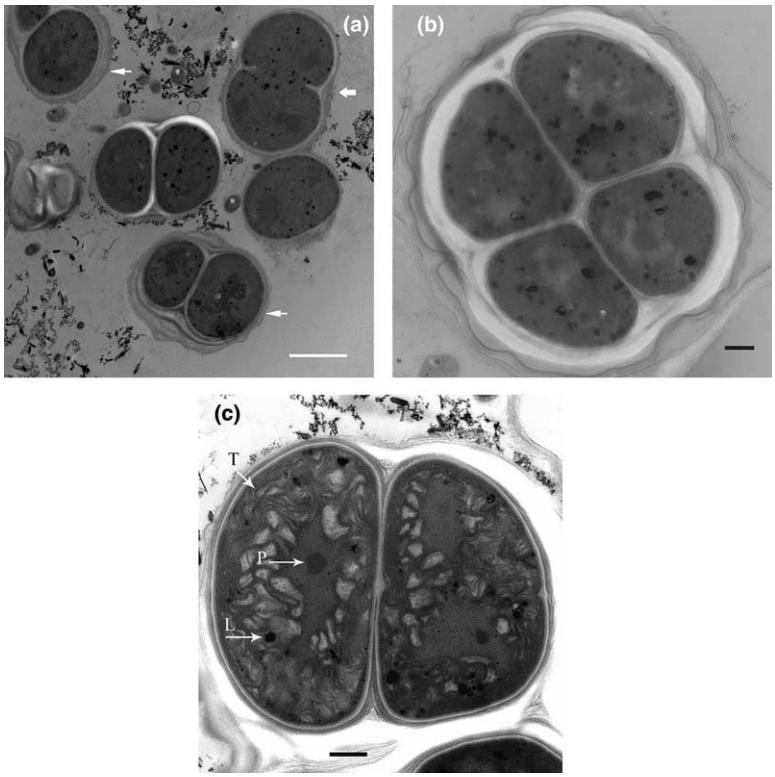

Fig. 5. Transmission electron micrograph of the isolate 5.2 s.c.1 cell cross-sections. (a) Dividing cells of "C. siderophila" in transverse direction (bold arrow). Note symmetrical cell division. Multilayer EPS (thin arrows). Bar = 2 μm. (b) "*C. siderophila*" cells dividing in two plains. Bar = 500 nm. (c) Inclusions in *C. siderophila* cells: T – concentric arrangement of thylakoids; L – lipid inclusions; P – polyhedral bodies (carboxysomes). Bar = 500 nm.

iron uptake, as previously postulated [36], rather than siderophore-based iron uptake [1], such as is found in the cyanobacterium *Synechocystis* sp. PCC 6803 [8], which requires only 1–2 μM ferrous iron for maximal growth [7]. On the other hand it is clear that elucidation of cyanobacterial iron tolerance mechanism(s) will require further research.

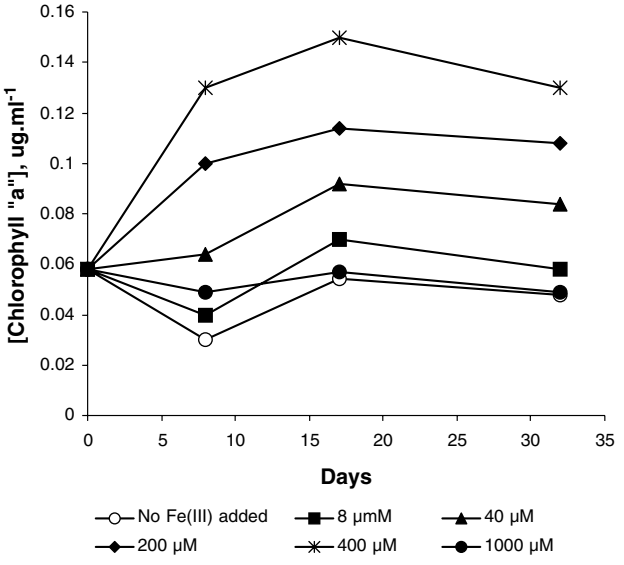

Fig. 6. Influence of $FeCl_3 \cdot 6H_2O$ concentration on the isolate 5.2 s.c.1 growth as indicated by fluorometric chlorophyll analysis. Initial medium pH was 8.2 and the figure shows arithmetic increases in chlorophyll.

Two weeks after inoculation, DH media supplemented with 400 μM $FeCl_3 \cdot 6H_2O$ exhibited a nearly threefold decrease in total soluble iron concentration compared to uninoculated media, indicating that 5.2 s.c.1 facilitates the removal of soluble iron from growth medium (Table 1). This result suggests that 5.2 s.c.1 cells may constitute a repository for environmental iron. While the EPS layer of cells incubated in the presence of 400 μM iron exhibited orange–brown coloration (Fig. 7), this coloration was absent in cells

Table 1
The removal of soluble iron from incubation media during 5.2 s.c.1 growth

	Fresh medium	Uninoculated medium	Inoculated medium
Repetition	3	3	3
Fe^{3+} concentration (ppm)	5.42	5.42	1.73
SD	±0.5	±0.5	±0.2

Iron determination was conducted after two weeks incubation (DH medium supplemented with 400 μM $FeCl_3 \cdot 6H_2O$, 50 °C, pH 8.2, illumination 30 μE m^{-2} s^{-1}.

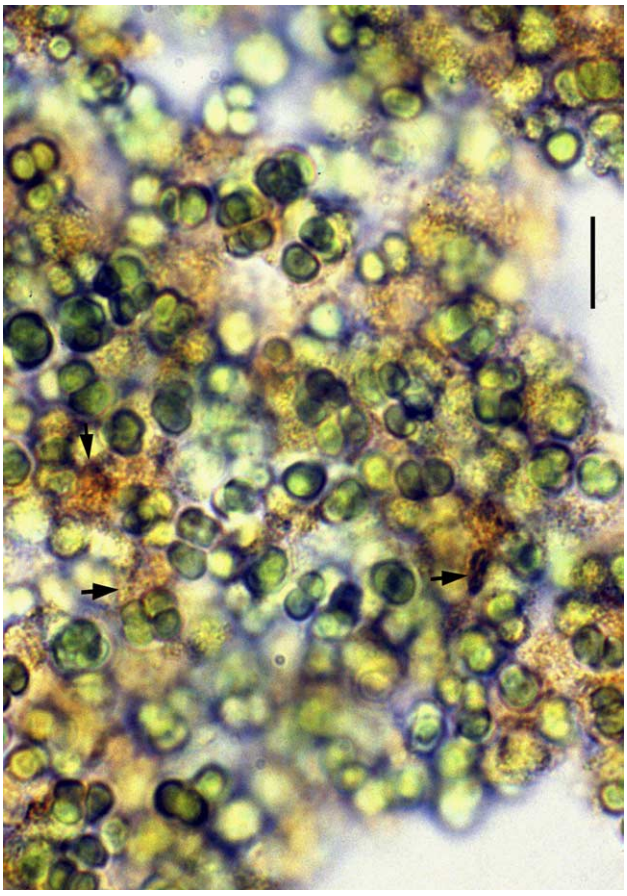

Fig. 7. Light micrograph of the isolate 5.2 s.c.1 grown in liquid medium DH supplemented with 400 μM Fe^{3+}, indicating iron-containing precipitates on cell surfaces (black arrows). Bar = 5 μm.

grown in the presence of only 40 μM iron (data not shown). Additionally, crystals observed in light and SEM micrographs (Figs. 3, 4 and 7) were confirmed to contain iron using energy dispersive X-ray spectroscopy (see legend to Fig. 4), further suggesting that the EPS layer of 5.2 s.c.1 is a repository for iron. A similar conclusion was made for marine cyanobacterial mats [37].

Extracellular accumulation of iron by iron-resistant cyanobacteria may potentially serve two functions: to decrease the chemical potential of active (accessible for cells) iron [2] and to produce a pool of reserve iron for times of low iron availability. The latter is in agreement with the finding that colloidal iron resulting from *Synechococcus* decomposition was also accumulated by *Synechococcus*, whereas colloidal Fe from diatom *Thalassiosira pseudonana* decomposition was unavailable to them [38].

Although our data provide little insight into the iron-resistance mechanisms utilized by 5.2 s.c.1, elevated iron dependence and iron tolerance are suggestive of iron resistance mechanisms attributed to ancient cyanobacteria. Therefore, the isolate may potentially provide a model for study of Fe^{2+}-dependent photosynthesis, as suggested previously [13,39].

Enhanced iron requirements, in addition to the ability to absorb iron from the medium, suggested the species epithet for this isolate "siderophila" (From Greek *sideros* – iron; *phila* – loving; *siderophila* – iron loving). The name "Chroogloeocystis siderophila" is proposed for this novel cyanobacterial isolate.

Phylogenetic analyses, morphological characteristics and physiological features suggest that "C. siderophila" belongs to a novel genus and represents a novel species of this genus. "C. siderophila" was isolated from, and is physiologically well adapted to, an environment likely similar to that expected for Precambian environments. Therefore, study of this isolate may provide insights into ancient iron-dependent prokaryotic physiology and environmental iron transformations, as well as cyanobacterial evolution and phylogenetic relationships.

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