Metallomics

RSC Publishing

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Cite this: *Metallomics*, 2013, **5**, 1595

Received 8th May 2013, Accepted 12th July 2013

DOI: 10.1039/c3mt00139c

www.rsc.org/metallomics

Novel surface associated polyphosphate bodies sequester uranium in the filamentous, marine cyanobacterium, *Anabaena torulosa*†

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A filamentous, heterocystous, nitrogen-fixing marine cyanobacterium, *Anabaena torulosa*, has been shown to harbour surface associated, acid soluble polyphosphate bodies. Uranium immobilization by such polyphosphate bodies, reported in cyanobacteria for the first time, demonstrates a novel uranium sequestration phenomenon.

Several studies have demonstrated the ability of cyanobacteria to accumulate heavy metals and radionuclides from their environment.[1–5] Cyanobacteria are known to bind metals extracellularly on their cell walls[1] or on extracellular polysaccharides (EPS).[5] However, cyanobacteria such as *Anabaena flos-aquae*,[3] *Anabaena cylindrica*,[4] and *Plectonema boryanum*,[6] also concentrate metals intracellularly in polyphosphate granules or bodies (PPBs). Inorganic polyphosphates (poly P) are linear phosphate polymers with chain lengths of two to a few hundred, and occur as prominent, ubiquitous structures found in bacteria, fungi, protozoa, plants and mammals.[7] There is strong evidence for the incorporation of heavy metals into the polyphosphate granules/bodies in several algae,[8] bacteria,[9] cyanobacteria,[10] diatoms[11] or in poly P containing vacuoles of the yeast *Saccharomyces cerevisae*.[12]

Our recent studies on the interactions of uranium with a filamentous, heterocystous, nitrogen-fixing marine cyanobacterium, *Anabaena torulosa*, revealed that this strain could sequester uranium in acid soluble polyphosphates, which could be extracted from the cells upon acidification with 1 N HCl at 100 °C.[13] Polyphosphate bodies are generally known to be localized intracellularly in cyanobacterial cells.[3,4,6] In the present study, data obtained using light, fluorescence and electron microscopy based imaging, coupled with Energy Dispersive X-ray (EDX) spectroscopy and spectrophotometric analyses, have revealed (a) the presence of novel surface associated polyphosphate bodies (SAPBs) in the filamentous cyanobacterium *A. torulosa*, and (b) the interaction of such SAPBs with uranium.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3mt00139c

Mid-exponential phase cells (at equivalent cell density, OD_{750} of 0.5) of the filamentous, heterocystous, nitrogen-fixing marine brackish water cyanobacterium, *A. torulosa*,[14] grown in a combined nitrogen free BG-11 medium, were used for the uranyl binding studies as described earlier.[13] *A. torulosa* was earlier isolated in our laboratory from the saline paddy fields of Trombay, Mumbai.[14] When challenged with 100 μM uranyl carbonate for 24 h at pH 7.8, under phosphate limited conditions, *A. torulosa* cells bound 65% (15.47 $\mu g\ mL^{-1}$) of the input U (23.8 $\mu g\ mL^{-1}$) (Fig. S1B, ESI†), resulting in a loading of 77.35 mg U g^{-1} dry wt. These U loaded cells, when imaged with a Carl Zeiss Axioscop 40 microscope, revealed distinct, dense, dark granules (Fig. 1D and the inset), as compared to the unchallenged cells (Fig. 1A). The uranium laden cells were harvested, washed with distilled water and treated with different concentrations of HCl (0.1–1 N HCl) at room temperature under agitation for 15 min, for the recovery of the cell bound uranium. Treatment with all the HCl concentrations at room temperature resulted in the loss of viability both in the control or U challenged cells, but did not lyse the cells. The HCl wash solutions and the uranium desorbed cell pellets were analysed for uranium content using the arsenazo III method.[15] Nearly 80% (12.37 $\mu g\ mL^{-1}$) of the bound uranium (15.47 $\mu g\ mL^{-1}$) (Fig. S1B, ESI†) could be released from the 24 h U challenged cells upon desorption with 0.1 N HCl.[13] These 24 h uranium challenged cells exhibited distinct 'hole' like structures upon acid desorption (Fig. 1E and inset), compared to the control unchallenged, acid desorbed cells which displayed somewhat distorted granular structures, but no holes (Fig. 1B and inset).

The integrity of the HCl washed cells was confirmed with a fluorescence microscope (Carl Zeiss Axioscop 40) fitted with a charge-coupled device (CCD) Axiocam MRc (Zeiss) camera. Phycocyanin is a highly soluble cyanobacterial phycobilisome, easily released upon cell damage or lysis.[16] The integrity of the HCl washed cells was carefully assessed with fluorescence microscopy by exciting the cells with green light (λ: 520 nm), which is absorbed by phycocyanin, and visualizing the red chlorophyll *a* fluorescence (λ: 680 nm) arising as a consequence

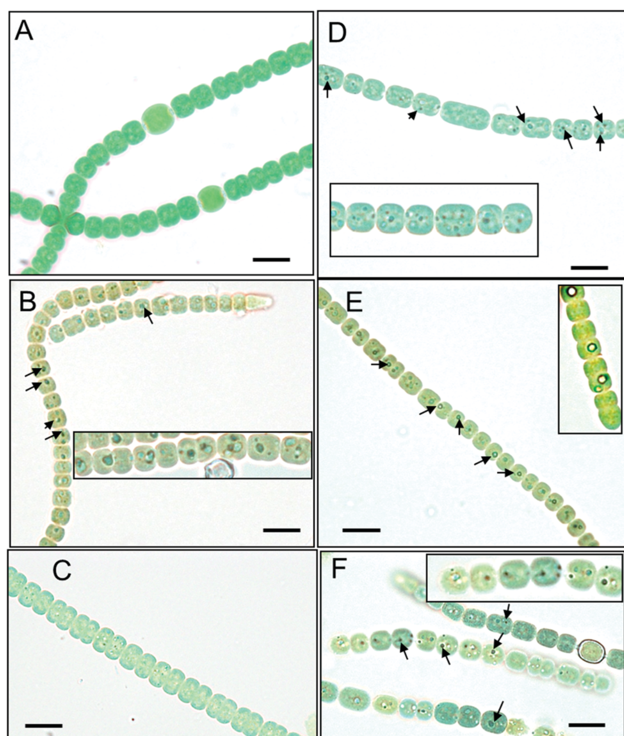

Fig. 1 Light microscopy of the uranium exposed *A. torulosa* cells (magnification: 1500×, bars indicate 5 μm) grown in a combined nitrogen free BG 11 medium (pH 7.2). The mid-exponential phase cells were incubated (A) under control conditions, and subsequently washed with either (B) 0.1 N HCl, or (C) 10 mM EDTA, pH 5.0. Alternatively, the cells were exposed to (D) 100 μM uranyl carbonate, $[UO_2(CO_3)_2]^{2-}$, at pH 7.8 for 24 h resulting in a loading of 77.35 mg U g^{-1} dry wt, and subsequently washed with (E) 0.1 N HCl, or (F) 10 mM EDTA, pH 5.0. The cells were observed with bright field microscopy using a Carl Zeiss Axioscop 40 microscope, with oil immersion objectives. The corresponding insets show an enlarged view (magnification: 4000×) of morphological alterations caused by the uranium loading (D), and HCl (B & E) or EDTA (F) washing of cells.

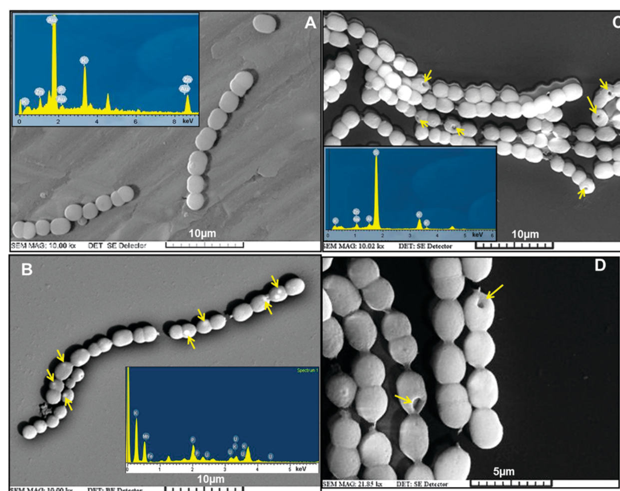

Fig. 2 SEM-EDX examination of *A. torulosa* cells exposed to uranium. The mid-exponential phase cells were incubated (A) under control conditions, (B) challenged with 100 μM uranyl carbonate for 24 h or (C and D) subsequently washed with 0.1 N HCl. The resulting samples were examined by a scanning electron microscope (SEM) provided with Energy Dispersive X-ray (EDX). The insets in figures (A)–(C) display the EDX spectra of the corresponding *A. torulosa* images. Arrows indicate the high contrast spots in the uranium loaded *A. torulosa* cells (B), or the 'hole' like structures formed after desorption by HCl (C and D).

of the resonant energy transfer from phycocyanin to chlorophyll *a*. The U loaded cells remained intact after 0.1–1 N HCl treatment, as visualized by red fluorescence (Fig. S2B, E and H, ESI†). HCl (0.1–1 N) treatment of the U loaded cells indicated the removal of distinct 'acid soluble' structures from the intact cells, possibly resulting in the formation of the observed 'hole' like structures (Fig. 1E and Fig. S2A, D and G, ESI†). Absence of such 'holes' in the U unchallenged cells (Fig. 1B) implied that uranium binding played some role in the creation of these easy to detect structures on the cell surface and their loss upon HCl washing. It has been shown earlier that uranium increases the membrane permeability of cells due to its toxicity.[9]

EDTA washing was used to release the weakly/surface bound uranium and for distinction of the adsorbed and absorbed metal.[17] Washing the 24 h U challenged *A. torulosa* cells with 10 mM EDTA (pH 5.0) could release ~26% (4.02 μg mL^{-1}) of the bound uranium[13] (Fig. S1B, ESI†). The EDTA treated control and 24 h U challenged cells (Fig. 1C and F, respectively) appeared to be similar to the EDTA untreated cells (Fig. 1A and D). The U treated cells subjected to EDTA treatment showed distinct, dark granular structures in the cells, but no 'holes'.

A. torulosa cells challenged with uranium for 24 h, or challenged with uranium for 24 h followed by treatment with 0.1 N HCl, were subjected to scanning electron microscopy and compared with the control unchallenged cells. The cells were washed, fixed with 3% glutaraldehyde, serially dehydrated in ethanol and sputter coated with gold using standard methods. The resulting samples were examined by a TESCAN Vega 40 T scanning electron microscope (SEM) provided with an Energy Dispersive X-ray (EDX) facility (INCA Energy 250, Oxford Instruments). The images were scanned at an accelerating voltage of 10 keV. The backscattered electron SEM image of the uranium loaded cells revealed high contrast spots (Fig. 2B), as compared to the control unexposed cells (Fig. 2A). A high concentration of a heavy element with high atomic number, like uranium, causes more back scattered electrons to bounce back, resulting in bright or high contrast areas. Visualization of such discrete, high contrast spherical granular structures on the cell surface of *A. torulosa* after uranium exposure (Fig. 2B), suggested the concentration of uranium in these surface granular bodies. Previously, electron microscopic observations of uranium exposed *Arthrobacter illicis*,[9] *Acidithiobacillus ferrooxidans*,[18] and *Sphingomonas* sp.[19] cells revealed the accumulation of uranium within intracellular granules, corresponding to polyphosphate bodies. The EDX spectra from such uranium bearing polyphosphate granules showed the presence of uranium and phosphorus. The EDX spectra for the images of the cells collected between 0 to 20 keV indicated the presence of both uranium and phosphorus in the U loaded *A. torulosa* cells (Fig. 2B, inset), as compared to the unchallenged cells (Fig. 2A, inset). The co-occurrence of U and P in the EDX spectra of U laden *A. torulosa* cells, exhibiting discrete high

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contrast spots, suggested the possible association of U with the polyphosphate bodies.

SEM examination of the U loaded *A. torulosa* cells washed with 0.1 N HCl revealed distinct 'crater' like structures (Fig. 2C and D) and simultaneous loss of both the uranium and phosphorus peaks in the respective EDX spectrum (Fig. 2C, inset). These results indicated the occurrence of distinct surface bodies in *A. torulosa*, harbouring uranium and phosphorus, and their physical removal upon HCl treatment.

Polyphosphates in prokaryotes are known to be either acid soluble or acid insoluble.[7] The staining of the polyphosphate bodies or granules with the basic dye toluidine blue shows a characteristic red colour (γ-metachromasia).[7,20] The acid lability characteristics of phosphoanhydride bonds in polyphosphates are well established and the latter have been found to be completely hydrolyzed to phosphate (Pi) units at 100 °C in 1 N HCl.[7,21] Distinct, dark red spheres (metachromasy), characteristic of polyphosphate bodies (PPBs),[7,20] appeared in the control and U challenged *A. torulosa* cells upon staining with toluidine blue (0.05% at pH 1) (Fig. 3A and D). The metachromasy (resulting in the characteristic red colour) at pH 1.0 is known to be specific for polyphosphate bodies.[20] The total cellular polyphosphates of the control and 24 h U challenged cells (equivalent to OD_{750} of 0.5) were precipitated and purified from the cells by an ice cold TCA method.[22] The purified PPBs were hydrolyzed with 1 N HCl at 100 °C for 15 min and analyzed for their inorganic phosphate (Pi) content.[22] The total Pi contents for the control or 24 h U challenged cells were found to be 2.21 or 2.68 μg mL^{-1}, respectively (Fig. S1A and B, ESI†).

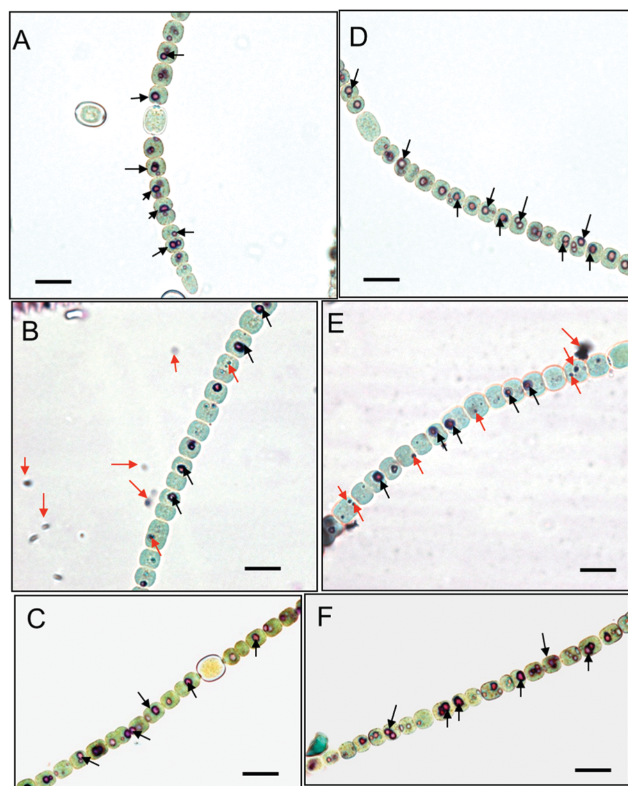

Fig. 3 Polyphosphate bodies (PPBs) in uranium challenged *A. torulosa* cells (magnification: 1500×, bars indicate 5 μm). The polyphosphate bodies were stained and visualized with bright field microscopy using a Carl Zeiss Axioscop 40 microscope, with oil immersion objectives. The microphotographs show the cells incubated under (A) control conditions and subsequently washed with either (B) 0.1 N HCl or (C) 10 mM EDTA, pH 5.0, or the cells (D) exposed to 100 μM uranium for 24 h and subsequently washed with (E) 0.1 N HCl or (F) 10 mM EDTA, pH 5.0. The black arrows show the stained PPBs, while the red arrows indicate the partially solubilized PPBs, desorbed with 0.1 N HCl.

When the cells exposed to U for 24 h were washed with 0.1 N HCl and subsequently stained with toluidine blue, both intact red spheres (black arrows) surrounded by dark deposits, as well as dark spots located on the cells or extracellularly released (red arrows), could be clearly visualized (Fig. 3B and E). Measurable amounts of phosphates (Pi) were detected in the 0.1 N HCl wash solution of both the control (0.44 μg mL^{-1}) and U-loaded (0.54 μg mL^{-1}) cells, indicating that this treatment resulted in partial hydrolysis/solubilization (~20%) of the total cellular polyphosphates of the cells, *i.e.* 2.21 or 2.68 μg mL^{-1}, respectively (Fig. S1A and B, ESI†). The dark spots (red arrows) displayed on the acid desorbed, stained control and U loaded cells (Fig. 3B and E), appear to have resulted from the partial extraction or detachment of surface associated PPBs. The cell morphology and the profile of the polyphosphate bodies in the 24 h U challenged cells remained unaltered (no cell lysis) upon treatment with 0.1–1 N concentration of HCl (Fig. S2A, C, D, F, G and I, ESI†). There was no further increase in the amount of uranium or phosphate released from the U loaded cells following treatment with higher concentrations of HCl (0.5–1 N).

The polyphosphate bodies in EDTA treated control and uranium challenged cells appeared as distinct red granular spheres, which could not be removed by EDTA (Fig. 3C and F). In earlier reports, EDTA treatment of the cyanobacterium *Synechocystis* sp. PCC 6308 had revealed the occurrence of two intracellular pools of polyphosphate. One of these pools was found to be solubilized with 10 mM EDTA, resulting in the detection of inorganic phosphate (Pi) in the supernatant of the EDTA treated *Synechocystis* cells.[16] In contrast, no inorganic phosphate was detected in the EDTA washes of the control and U challenged *Anabaena* cells (Fig. S1A and B, ESI†). The microscopic observations were therefore consistent with the inability of EDTA to solubilize the polyphosphates in *A. torulosa* (Fig. 3C and F).

Incubating the uranium loaded cells in 1 N HCl at 100 °C for 15 min resulted in bleaching, extensive cell lysis and complete extraction of the total cell bound uranium (15.47 μg mL^{-1}) or the total polyphosphate (2.68 μg mL^{-1})(Fig. S1B, ESI†). Occasional intact filaments were observed after this treatment (Fig. S2J, ESI†), which completely lacked red fluorescence (Fig. S2K, ESI†) or stainable PPBs (Fig. S2L, ESI†).

The sequestration of heavy metals by poly P in bacteria is well known. Although the polyphosphate bodies are largely known to be intracellular inclusion bodies, the occurrence of surface associated polyphosphates has been reported in microorganisms such as *Chlorella fusca*,[8] *Acinetobacter*[23] and *Saccharomyces cerevisae*,[24,25] where they have also been implicated in uranium chelation on the surface of the cells.[25] A similar study in

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Chlorella fusca found that a part of the cellular polyphosphates were located outside the cytoplasmic membrane and tightly bound to the cell wall or cytoplasmic membrane, and were not susceptible to hydrolysis by a strong acid, such as perchloric acid, at room temperature in contrast to intra-cytoplasmic polyphosphates.[8]

As far as the authors are aware, there is no report suggesting the occurrence of surface associated polyphosphate bodies in cyanobacteria. The surface PPBs susceptible to hydrolysis by hydrochloric acid (0.1–1 N) at room temperature comprised of only ~20% of the total polyphosphate content of the *A. torulosa* cells. The light microscopic (Fig. 1E) or SEM images of the HCl treated 24 h U challenged cells (Fig. 2C and D) also show occasional 'hole' or 'crater' like structures, although the polyphosphates seem to be uniformly distributed throughout the cells (Fig. 3D). We have earlier seen that 0.1 N HCl led to the desorption of 80% of the total cell bound uranium from intact cells of *A. torulosa*. This proportion of desorbed U is extracellular and is likely to be distributed between the extracellular polysaccharides[13] and the surface PPBs. The probability of uranium precipitation *via* constitutive phosphatase activity or polyphosphate degradation is unlikely. The phosphatase activity, estimated by the liberation of *p*-nitrophenol from disodium *p*-nitrophenyl phosphate (*p*NPP),[26] was found to be negligible, ranging from 0.006–0.008 μg mL^{-1} in the control or U treated *Anabaena* cells, respectively. Also, our studies did not show any measurable degradation of the total polyphosphate content of *A. torulosa* cells exposed to uranium under phosphate limiting conditions over 24 h, and it remained almost consistent (2.68 μg mL^{-1}) with the total polyphosphate content of the cells prior to U exposure, *i.e.* 2.72 μg mL^{-1}.

Conclusions

The present investigation provides high resolution physical evidence for the occurrence of surface associated polyphosphate bodies (SAPBs) in the marine, filamentous, heterocystous microbe *A. torulosa*, reported in cyanobacteria for the first time, and the involvement of such bodies in the sequestration of uranium. This is evident from the co-localization of uranium with the surface associated polyphosphate bodies (SAPBs) and detachment or extraction of such bodies resulting in large craters on the cell surface accompanied by loss of U and Pi upon HCl based desorption. Extraction of polyphosphate bodies, along with uranium sequestered therein, by dilute HCl without causing cell lysis, further substantiates their surface association and acid solubility.

Acknowledgements

The authors thank Dr Shovit Bhattacharya, Technical Physics Division, BARC, for technical help with the SEM-EDX analyses of cyanobacterial samples.

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