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Metal-induced phosphate extracellular nanoparticulate formation in *Ochrobactrum tritici* 5bvl1

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ABSTRACT

Hexavalent chromium (Cr(VI)) is a toxic environmental contaminant which detoxification consists in reduction to Cr(III). In this work, the Cr(VI)-resistant and reducing *Ochrobactrum tritici* 5bvl1 produced phosphate nanoparticles upon exposure to Cr(VI) and Fe(III), effectively removing chromium from solution. Under Cr(VI) stress, higher siderophore production by strain 5bvl1 was observed. Cr(VI) toxicity was decreased in presence of Fe(III), increasing the growth and Cr(VI)-reduction rates in cell cultures, lowering the amount of morphologically compromised cells and promoting chromium immobilization as insoluble extracellular phosphate complexes. The formation of phosphate nanoparticles increased with Cr(VI) and Fe(III) concentrations and was also stimulated by Ni(II). Under these experimental conditions, nanoparticle formation occurred together with enhanced inorganic phosphate consumption by cells and increased polyphosphate kinase (PPK) activity. NMR analysis of the particles showed the presence of both polyphosphate and phosphonate together with orthophosphate, and FT-IR supported these results, also showing evidences of Cr(III) coordination. This work demonstrated that *O. tritici* 5bvl1 possesses protection mechanisms against chromium toxicity other than the presence of the Cr(VI) pump and SOD related enzymes previously described. Future assessment of the molecular regulation of production of these nanoparticles will open new perspectives for remediation of metal contaminated environments.

1. Introduction

Hexavalent chromium (Cr(VI)) is a carcinogenic environmental contaminant [1]. Remediation of contaminated soils and waters is achieved by reducing Cr(VI) to the less toxic and less soluble trivalent chromium (Cr(III)) using microorganisms [2].

Several Cr(VI)-resistant bacteria species have been isolated in recent years. Cr(VI)-resistance is a consequence of the summed effects of various strategies, which include Cr(VI) reduction to Cr(III) aerobically or anaerobically [3–6], repair of damaged DNA, proteins and lipids, free-radical scavenging enzymes and the efflux of Cr(VI) from the cytoplasm [7], or downregulation of the sulfate transport system, responsible for chromate uptake [8]. Among the most resistant microorganisms are the strains *Brevibacterium* sp. CrT-13 [9], *Ochrobactrum intermedium* SDCr-5 [10] and *Ochrobactrum tritici* 5bvl1 [11].

The efflux of chromate is performed by the membrane-potential dependent ChrA membrane transporter [12], a protein coded by the *chrA* gene, present in *Pseudomonas aeruginosa* (plasmid pUM505) [13], in *Cupriavidus metallidurans* (plasmid pMOL28) [14] and in *O. tritici* 5bvl1 (transposon Tn*OtChr*) [15]. In this last microorganism, transposon Tn*OtChr* contains the operon *chrBACF*, which also codes a regulatory protein, ChrB, responsible for the induction of the *chrA* gene and a superoxide dismutase (SOD), ChrC [15].

Metal sequestration and precipitation occurs in several bacteria, but the contribution of this phenomenon to cell survival under metal stress is not fully understood. Microbiological metal precipitation in extracellular phosphate complexes was previously reported as important in uranium or chromium bioremediation strategy [2,16]. Recently, after Cr(VI) reduction by bacterial consortia, Cr(III) was found coordinated octahedrically to phosphate in the biofilm [17]. In contrast, phosphate solubilization by plant growth-promoting bacteria under metal stress leads to a higher heavy metal bioavailability which results in uptake by plants [18]. In recent reports, polyphosphates and phosphonates were found in abundance in marine sediments, and suggested to originate from benthic microorganisms, in response to redox potential changes [19]. Until

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today however, there is a lack of evidences linking polyphosphates to the metal-phosphate extracellular aggregates produced by bacteria.

O. tritici strain 5bvl1 was used in this work as a model. This strain was isolated from a Cr(VI)-contaminated wastewater treatment plant [20], and is one of the most Cr(VI)-resistant microorganisms known [11], as opposed to *O. tritici* SCII24^T [21]. Strain 5bvl1 possesses capacity to reduce Cr(VI) and was shown in the current work to form insoluble extracellular nanoparticles in presence of this metal, provided Fe(III) was also available. Consequently, the goals of this work include the study of the chemical structure of the nanoparticles, and of its metal dependence. To achieve these goals, siderophore production was followed in presence of Cr(VI), nanoparticles were characterized by SEM-EDS, FT-IR and ^{31}P NMR, and total phosphate present in nanoparticles and growth medium was quantified and correlated to the concentrations of metals present in solution. Polyphosphate kinase (PPK) activity of cell extracts was tested on cells exposed to metals. Physiological assays were also performed in order to determine if the presence of iron and nanoparticle formation improved the Cr(VI) resistance and reduction abilities of the model strain.

2. Experimental

2.1. Bacteria strains

O. tritici strain 5bvl1 was isolated from activated sludge in a chromium-contaminated area [20]. The type strain *O. tritici* SCII24^T was obtained from LMG Culture Collection (Gent, Belgium) and is Cr(VI)-sensitive. The strains were maintained at −80 °C in Nutrient Broth (Difco) containing 15% (w/v) glycerol.

2.2. Growth conditions and Cr(VI) quantification in resistance assays

The strain was cultured in buffered mineral medium (MMH) [12] using double-distilled water (ddH_2O) and incubated at 30 °C. Growth was determined by optical density (O.D.) at 600 nm. The glass apparatus was previously washed with concentrated HNO_3 to remove trace amounts of iron and washed thoroughly with ddH_2O. The assays performed with iron contained 100 μM $FeCl_3$ while iron-deprived assays contained less than 2 μM Fe. Chromium was used either as sodium dichromate ($Na_2Cr_2O_7$) or sodium chromate (Na_2CrO_4), ranging from 0 to 4 mM. Cr(VI) concentration in culture was followed using the diphenylcarbazide method [22]. Total chromium present in cell pellets was quantified using the same method after re-oxidation of Cr(III) with $KMnO_4$ [22]. All assays were performed in triplicate and are expressed as averages, with standard deviation.

2.3. Siderophores

Strain 5bvl1 was tested positive for siderophore production in Chrome Azurol S (CAS) agar medium incubated at 30 °C. The strain was then tested for the presence of soluble siderophores in MMH and for the presence of membrane-bound siderophores. The assays consisted of inoculated MMH with 2 mM Cr(VI) as chromate or dichromate, and with or without $FeCl_3$. Soluble siderophores were tested with CAS, Arnow's assay, for catechol-type siderophores, and Atkin's assays, for hydroxamate-type siderophores, as described by Clark [23]. Membrane-bound ochrobactin-like siderophores were extracted with ethanol from cells recovered from the culture (2 ml) [24]. The extract was concentrated by evaporation, applied on CAS agar medium, and plates were incubated at room temperature for 48 h before digitalization, densitometry analysis and normalization.

2.4. Scanning electron microscopy with X-ray microanalysis (SEM-EDS)

Strain 5bvl1 was grown for 72 h in MMH with 2 mM Cr(VI) as chromate or dichromate, and either under iron deficiency, or with 100 μM Fe(III). Cell samples were prepared and solidified in blocks of Spurr resin (TAAB) as previously described [12]. Samples were thin sectioned, applied to a copper grid and analysed by a Jeol JSM 6301F scanning electron microscope coupled with EDS (Oxford INCA 350).

2.5. Nanoparticle metal-dependent formation

Cells suspensions were obtained from cultures that reached stationary phase after 72 h, at 30 °C, in MMH under iron deficiency and supplemented with 0.25 mM Na_2CrO_4. Cells were concentrated, washed and resuspended in MMH medium. Nanoparticle metal-dependence was tested on cell suspensions in 20 ml MMH medium with an initial O.D. of 2.0.

2.5.1. Effect of iron

To evaluate the iron nanoparticle formation dependence, assays were performed with cell suspensions containing 2 mM Na_2CrO_4 and $FeCl_3$ concentrations of 0, 50, 100, 150, 200, 250 and 300 μM. Assays were incubated at 30 °C during 96 h and sampled (2 ml) at set time intervals. Cr(VI) was quantified from samples as described in Section 2.2. Reduction rates were compared by one-way ANOVA analysis followed by Dunett's multiple comparison post-test. Cells were resuspended in 1 ml ddH_2O and subjected to sucrose gradient centrifugation (4 °C, 3220 × *g*, 30 min), with the following 2 ml sucrose phases, from bottom to top: 70%, 45%, 30%. The pellet formed was further purified after resuspension in 500 μl ddH_2O and centrifugation against a 1:1 85% sucrose phase (16,100 × *g*, 15 min). The 45% sucrose phase contained most cells, while the 85% sucrose phase contained a pellet of purified metal-rich aggregates. Total intracellular phosphate and phosphate from aggregates were quantified using an ascorbic acid method [25] after 2 washes with ddH_2O (3220 × *g*, 15 min), resuspension in 1 ml ddH_2O and HCl digestion at 120 °C, during 45 min. All assays were performed in triplicate and averages of three values were plotted with standard deviations. Graphs and statistical analysis were performed using GraphPad Prism v5.0 for windows, GraphPad software, San Diego, California, USA, www.graphpad.com.

2.5.2. Effect of chromium

To evaluate the chromium nanoparticle formation dependence, assays were performed with cell suspensions containing 300 μM $FeCl_3$ and Na_2CrO_4 concentrations ranging from 0 to 5 mM. Free phosphate ions present in the suspension and phosphate from the nanoparticles were quantified as described in Section 2.5.1. Cr(VI) was quantified for the *O. tritici* strain 5bvl1 assays. The different responses were compared by one-way ANOVA analysis followed by Turkey's multiple comparison and linear trend post-tests.

2.5.3. Effect of other metals and paraquat

All samples were processed and analysed as in Section 2.5.1. Assays containing an initial concentration of 1 mM of: paraquat, $NaAsO_2$ (As(III)), $HAsNa_2O_4$ (As(V)) or $NiCl_2$ (Ni(II)) were compared with 1 mM Cr(VI).