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# Microbial Reduction of Hexavalent Chromium as a Mechanism of Detoxification and Possible Bioremediation Applications

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## 1. Introduction

### 1.1. Characteristics of chromium

Chromium (Cr) is a naturally occurring element with atomic number 24 and atomic mass of 51.996 amu. The element belongs to the group of transition metals and in the oxidation state elementary presents an electronic configuration (Ar) 4d<sup>5</sup>s<sup>1</sup>. Chromium is naturally present in the environment, it is widespread in rocks, animal, plants and soil, and is the seventh most abundant element on Earth's crust, at concentrations ranging from 100 to 300  $\mu\text{g g}^{-1}$ . In nature, Cr is found in the form of its compounds, and the most important chromium ore is chromite, (Fe, Mn)Cr<sub>2</sub>O<sub>4</sub> [1,2].

Chromium exists in different oxidation states, the most stable and common forms are the trivalent [Cr(III)] and the hexavalent [Cr(VI)] species, which display quite different chemical properties [1]. Cr(III) in the form of oxides, hydroxides or sulfates, exists mostly bound to organic matter in soil and aquatic environments. Cr(VI) is usually associated with oxygen as chromate (CrO<sub>4</sub><sup>2-</sup>) or dichromate (Cr<sub>2</sub>O<sub>7</sub><sup>2-</sup>) ions [1]. Cr(VI) is a strong oxidizing agent and in the presence of organic matter is reduced to Cr(III); this transformation is faster in acid environments such as acidic soils [1]. However, high levels of Cr(VI) may overcome the reducing capacity of the environment and thus persist in this form [3].

Chromium represents an essential micronutrient for living organisms, considering that Cr(III) is an essential trace element known for its particular role in the maintenance of normal carbohydrate metabolism in mammals and yeasts [4]. Moreover, it has also been suggested

that Cr(III) is involved in the tertiary structure of proteins and in the conformation of cell RNA and DNA [5,6].

## 1.2. Toxicity of chromium

Cr(VI) exposure in humans can induce allergies, irritations, eczema, ulceration, nasal and skin irritations, perforation of eardrum, respiratory track disorders and lung carcinoma [7,8,9]. Moreover, Cr(VI) evidences the capability to accumulate in the placenta, damaging fetal development [10]. Cr(VI) pollution in the environment alters the structure of soil microbial communities [11], reducing microbial growth and related enzymatic activities, with a consequent persistence of organic matter in soils and accumulation of Cr(VI) [12].

The toxic action of Cr(VI) is due to its capability to easily penetrate cellular membranes, and cell membrane damages caused by oxidative stress induced by Cr(VI) have been extensively reported, both in eukaryotic and prokaryotic cells, with effects such as loss of membrane integrity or inhibition of the electron transport chain [13,14]. Moreover, Cr(VI) enters cells using the sulfate transport system of the membrane in cells of organisms that are able to use sulfate [15,16,17,18,19,20].

Once Cr(VI) entered into cells, spontaneous reactions occur with the intracellular reductants as ascorbate and glutathione, generating the short-lived intermediates Cr(V) and/or Cr(IV), free radicals and the end-product Cr(III) [21,22,23]. In the cytoplasm, Cr(V) is oxidized to Cr(VI) and the process produces a reactive oxygen species, referred as ROS, that easily combines with DNA-protein complexes. On the other hand, Cr(IV) is able to bind to cellular materials, altering their normal physiological functions [24,25]. It is known that Cr(VI) species and hydroxyl radicals cause DNA lesions *in vivo* [26]. The intermediates that originated from the action of Cr(VI) are dangerous to cell organelles, proteins and nucleic acids [27,28,29]. Cr(VI) is a very dangerous chemical form on biological systems as it can induce mutagenic, carcinogenic and teratogenic effects. Moreover, Cr(VI) is able to induce oxidative stress in cells, damaging its DNA [30]. Inside of cells, the Cr(III)-DNA adducts and related hydroxyl radical oxidative DNA damages have a central role in originating the genotoxic and mutagenic effects [31]. Moreover, the formation of Cr(III)-DNA binary adducts and L-cysteine-Cr(III)-DNA and ascorbate-Cr(III)-DNA ternary adducts likely increase both genotoxicity and mutagenicity in human cells [32,33]. Again the formation of DNA protein cross-linking, a process favoured by Cr(VI), induces a significant promutagenic effect [33].

Considering the dangerous effects Cr(VI) can cause to human health, Cr(VI) has been comprised among priority pollutants and listed as a class A human carcinogen by the US Environmental Protection Agency (USEPA) [34].

The cell membrane is nearly impermeable to Cr(III), Cr(III) has thus only about one thousandth of the toxicity of Cr(VI) [35,36]. Taking into account these considerations, it is possible to conclude that, depending on its oxidation state, chromium can have different biological effects, with Cr(VI) that is highly toxic to most organisms, and Cr(III) that is relatively innocuous [37,38].

## 2. Use of chromium and environmental contamination

Chromium enters in the anthropogenic activities, it is used in stainless steel plant, preparation of alloys, chrome plating, leather tanning, production of refractories, dye industry, industrial water cooling, paper pulp production, petroleum refining, wood preservation and nuclear power [1,39].

As consequence of its broad use, chromium is present in effluents originated from the different activities and represents a serious pollutant of sediments, soil, water and air [40]. Wastewaters have resulted in significant quantities of Cr(VI) in the environment, which may constitute toxicological risk to humans, animals, and plants [41]. Cr(VI) is introduced in the environment mainly as a consequence of its industrial use, while chromium in its trivalent form, Cr(III), naturally predominates in the environment [42].

Cr(VI) is highly dispersed in sediments and surface waters, and it is characterized by a much greater solubility, mobility and bioavailability than Cr(III) and all the forms of chromium [43,44]. As consequence of this high water solubility and elevated mobility, Cr(VI) diffuses easily away from the native site of contamination. Moreover, the increase in soil pH increases the leachability of Cr(VI). Cr(III) shows a low mobility and is relatively inert, and easily absorbable on mineral surfaces and solid-phase organic ligands, thus resulting less bioavailable in the environment. Additionally, Cr(III) is quite insoluble at environmentally significant pH values, since in these conditions there are formation of insoluble hydroxide and oxide compounds. Mobility of Cr(III) decreases with absorption of clays and oxide minerals below pH 5. Binding of Cr(III) by iron oxides can be considered an example of these mechanisms, as this feature can decrease the solubility of this form of chromium [43,45]. Again, the characteristic of insolubility of Cr(III) diminish its bioavailability and mobility of Cr(III) toxicity in saltwater exposures [46].

In the presence of oxidizing conditions Cr(VI), in forms of the anions chromate ( $\text{CrO}_4^{2-}$ ) and bichromate ( $\text{HCrO}_4^-$ ), is extremely soluble and mobile (Barnhart 1997). In anaerobic environments, under reducing conditions, in the presence of reducing agent as sulfides, ferrous iron, and organic matter, that are several of the organic and inorganic constituents, Cr(VI) may rapidly convert to Cr(III) [47]. Again, bacterially mediated reduction of Cr(VI) has also been considered in the chromium biogeochemical cycle [48].

Cr(III) is stable in aquatic environments and its oxidation to Cr(VI) is improbable, even in the presence of dissolved oxygen [49,50,51]. Different factors affect Cr(III) oxidation to Cr(VI), depending on the presence and mineralogy of Mn(III, IV) hydroxides, pH, and the form and solubility of Cr(III) [52]. Oxidation of Cr(III) is improbable to occur in aquatic environments because aged waste materials containing Cr(III) are typically less soluble and more inert to oxidation, and  $\text{Cr}(\text{OH})_3$  precipitates may form on surfaces of Mn(III, IV) hydroxide [53]. Besides, possible Cr(III) oxidants are scarcer and less abundant than potential Cr(VI) reductants in natural sediments, and Cr(III) oxidation is slower than Cr(VI) reduction [54].

### 3. Microbial resistance to Cr(VI) and microbial Cr(VI)-reduction

Despite the toxicity of Cr(VI), some microorganisms evidence resistance to this heavy metal, showing the capability to reduce Cr(VI) to Cr(III), as was first reported for *Pseudomonas* spp., and a characterization of bacteria capable of reducing the Cr(VI) was reported successively in 1979 [55,56]. Since then it has been evidenced the presence of numerous bacteria capable of transforming the Cr(VI) to Cr(III) under different conditions [57,58,59]. Recent isolation and purification of Cr(VI) reductases from aerobic bacteria and the fact that the process involved in Cr(VI) reduction occurring under anaerobic conditions is starting to be understood, allowed knowledge of biological processes of chromium resistance [60]. Numerous bacteria have then been reported evidencing their capability to reduce Cr(VI) to Cr(III) as a mechanism of resistance to Cr(VI) [61,62]. Further studies evidenced different bacteria able to reduce Cr(VI), including *Escherichia coli* [63], *Pseudomonas putida* [64], *Desulfovibrio* sp. [65], *Bacillus* sp. [66], *Shewanella* sp. [67], *Arthrobacter* sp. [68], *Streptomyces* sp. MC1 [36] and *Microbacterium* sp. CR-07 [69].

The *chrBCAF* operon from transposable elements confers resistance to Cr(VI), synthesizing for ChrA and ChrB, a protein acting as chromate-sensitive regulator [70]. Enzymatic reduction of Cr(VI) was evidenced in an *Halomonas* sp. strain TA-04, isolated from polluted marine sediments, in the presence of 8.0% NaCl suggesting new insights for metal reduction at halophilic conditions [71]. Investigations on mechanisms of resistance to chromate, in particular at level of the bacterial cells have been evidenced. The best characterized mechanisms that have been reported include the efflux of chromate ion from the cell cytoplasm and the reduction of Cr(VI) to Cr(III). The efflux by the transport protein CHRA has been identified in *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) and consists of an energy-dependent process driven by a membrane potential. Moreover, the reduction of chromate is completed by chromate-reductase from different bacterial species generating Cr(III) that may be the object of detoxification due to other mechanisms. The most specific enzymes belong to the large family of flavoprotein reductase NAD(P)H-dependent. Other mechanisms of bacterial resistance to the chrome were evidenced, and these mechanisms were related to the expression of components of the systems of mechanism for the DNA repair and are related to the mechanisms of homeostasis of iron and sulfur [72].

Both aerobic and anaerobic microorganisms are able to reduce Cr(VI) to Cr(III). In aerobic conditions it is possible to observe the bio-reduction of Cr(VI) that can be obtained directly as a result of microbial metabolism [73]. In the presence of oxygen, microbial reduction of Cr(VI) is commonly catalyzed by soluble enzymes, except in *Pseudomonas maltophilia* O-2 and *Bacillus megaterium* TKW3, which utilize membrane-associated reductases. Soluble Cr(VI) reductase ChrR was purified from *Pseudomonas putida* MK1 and reductase YieF purified from *Escherichia coli*. Enzyme ChrR catalyzes an one-electron shuttle followed by a two-electron transfer to Cr(VI), with the formation of intermediate(s) Cr(V) and/or Cr(IV) before further reduction to Cr(III). Reductase YieF displays a four-electron transfer that reduces Cr(VI) directly to Cr(III). The membrane-associated Cr(VI) reductase was isolated from *B. megaterium* TKW3, without any characterization of related reduction kinetics. In the absence of oxygen, Cr(VI) reduction

was evidenced both by soluble and membrane-associated enzymes, and Cr(VI) functions as the terminal electron acceptor of an electron transfer chain that frequently involves cytochromes. Researches on Cr(VI) reductases focusses on enzymes with higher reductive activity. In anaerobic conditions, the reduction processes uses a broad range of compounds, involving carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves, that can function as electron donors in the reduction processes [74]. According to the advancement in technology for enzyme immobilization, the direct application of Cr(VI) reductases could be an important approach for bioremediation of Cr(VI) in different environments, in particular where whole cells are difficult to apply [75].

#### 4. Bioremediation of Cr(VI) by microorganisms

Conventional methods for removing metals from contaminated sites include chemical precipitation, oxidation/reduction, ion exchange, filtration, use of membranes, evaporation and adsorption on activated coal, alum, kaolinite, and ash [15,41]. However, most of these methods require high energy or large quantities of chemical reagents, with possible production of secondary pollution [76,77]. Concerning removal of Cr(VI), conventional approaches include chemical reduction followed by precipitation, ion exchange and adsorption on activated carbon, alum, kaolinite and of ashes, and most of these methods require a high energy and large amounts of chemical reagents [76]. Moreover, costly safe disposal of toxic sludge, incomplete reduction of Cr(VI) and high cost for Cr(VI) reduction, especially for the removal of relatively low concentrations of Cr(VI) are non-convenient from the economical point of view [78,79].

An innovative technology is represented by bioremediation, which uses the metabolic potential of microorganisms to remove toxic metals, in order to decontaminate the polluted areas. Bioremediation techniques can be classified as *in situ* or *ex situ* depending, respectively, on whether the intervention is carried out with suitable bacteria directly on the polluted site, or on portions of environmental matrices, such as water, sediment or soil, after being removed and transported in appropriate facilities for treatment [76,79].

Cr(VI)-resistant microorganisms represent an important opportunity to have safe, economical and environmentally friendly methods for reducing Cr(VI) to Cr(III), for possible bioremediation applications [27]. The reduction of Cr(VI) to Cr(III) is then a potential useful process for the recovery of sites contaminated by Cr(VI) [36]. Cr(VI)-removal based on microorganisms is now considered to be an effective alternative method to the conventional processes, and is receiving great attention for potential application in bioremediation [76,80]. Taking into account that the insolubility of Cr(III) facilitates its precipitation and removal, the biotransformation of Cr(VI) to Cr(III) has been considered as an alternative process for treating Cr(VI)-contaminated wastes [81,82]. Among biotechnological approaches, microbial reduction of Cr(VI) is cost-effective and eco-friendly and can offer a viable alternative [80,83,84].

Chromium resistant microorganisms are responsible of the biological reduction of Cr(VI) into the less mobile Cr(III), and its consequent precipitation, could represent an effective method for detoxification of Cr(VI) contaminated sites and have a potential use in bioremediation [85].

Included in the bioremediation technologies, phycoremediation is the use of photosynthetic microorganisms as microalgae, macroalgae and cyanobacteria for the removal of pollutants as metals. Furthermore, it is essential to understand the distribution of the metal adsorbed onto the surface in relation to the metal accumulated inside the cell, in order to understand the predominant removal mechanisms and to make decisions of the viability of the recovery of the adsorbed metals [86].

Biosorption and bioaccumulation of chromium for bioremediation purposes have been demonstrated. Yeasts and especially molds have been most widely investigated from this aspect, and the mechanisms of chromium tolerance or resistance of selected microbes are of particular importance in bioremediation technologies. The mechanisms of chromium toxicity and detoxification have been studied extensively in yeasts and fungi, and some promising results have emerged in this area [87].

The ability existing in a number of environmental microorganisms, known for their capability to bind metals, can be evidenced in human gastrointestinal bacteria. Bacterial species belonging to the genus *Lactobacillus*, resident in different districts as the human body and in fermented foods, have the ability to bind metals, including Cr(VI), and to detoxify them from different districts [88].

A method for bioremediation of sites contaminated by metals, including chromium, is represented by bioaugmentation-assisted phytoextraction, in which bacteria and fungi, associated with plants able to accumulate metals were analyzed on the basis of a proposed as bioprocess for a bioremediation approach. The implementation of bioaugmentation to favour the microbial survival, was suggested in order to enhance the microbial-plant association and the efficiency of the process [89].

The process of biomineralization is a process by which microorganisms transform aqueous metal ions, including chromium, into amorphous or crystalline precipitates. Biomineralization is regarded as a promising and cost-effective strategy for remediating chromium contamination. An example of arsenic precipitation was considered as a possible mechanism for arsenic bioremediation of sediments contaminated by arsenic [90]. Biologically mediated transformation, immobilization, and mineralization of toxic metals may represent an important perspective for bioremediation [91].

## **5. Case study: Cr(VI)-reduction by Actinobacteria isolated from polluted sediments near a stainless steel plant**

Wastes from stainless steel plants produce soluble Cr(VI) contaminating sediments, soils and water bodies. Chromium at high concentrations are widespread in sediments of industrialized areas because of industrial discharges [92]. In a previous study, carried out from polluted

marine sediments near a stainless steel plant in Southern Italy, near the industrialized area of Taranto, an halophilic Cr(VI)-resistant bacterial strain *Halomonas* sp. TA-04 was isolated. The isolated strain showed a MIC at 200  $\mu\text{g ml}^{-1}$  Cr(VI), and the reduction of Cr(VI) in the presence of 80  $\text{g l}^{-1}$  NaCl. Cr(VI) was removed from sediment leachate by immobilized cells and the cell free extract reduced Cr(VI) with a maximum of activity at pH 6.5, at 28°C. These results suggest the possible use of the isolated strain in bioremediation processes, in particular concerning detoxification of saline polluted environments [71].

The aim of the present investigation was the isolation of bacterial strains from chromium-polluted sediments and their characterization in terms of phylogenetic and physiological features. The description of two Cr(VI)-reducing microorganisms isolated from polluted sediments and included into Actinobacteria was carried out, for their possible use in bioremediation applications.

## 6. Materials and methods

### 6.1. Study area

The microbiological study with the isolation of the bacterial strains investigated in this study was conducted in sediment samples collected from a polluted site near a stainless steel plant in the Bagnoli area, Naples (Southern Italy). The site was characterized by a total chromium content corresponding to  $34 \pm 0.23 \text{ mg kg}^{-1}$ .

### 6.2. Sediment sampling

Sediment samples for microbiological analyses were collected manually using Plexiglas tubes (i.d. 10 cm), in June 2008. Collected samples were maintained at 4 °C and transported to the laboratory. Sterile sediment subsamples (0-10 cm) were collected and processed within twelve hours for microbiological analyses.

### 6.3. Enrichment cultures and isolation of the bacterial strain

Enrichment cultures were grown in flasks containing the complex YPEG medium, containing 5.0 g of tryptone, 2.5 g of yeast extract, and 1.0 g of D-glucose per litre of distilled water, in the presence of 5.0 mM of Cr(VI), inoculated with 0.5 g of sediment samples, and incubated at 28 °C in the dark. From flasks showing turbidity, a 100  $\mu\text{l}$  aliquot was spread on Petri dishes containing the complex solid medium in the presence of the same initial concentration of Cr(VI) and incubated at 28°C for 48 hours. Colonies showing different morphologies were selected and subcultured at least three times. Isolated strains were stored in the presence of 30% sterile glycerol (v/v) in liquid nitrogen.

### 6.4. Isolates characterization and identification by 16S rRNA gene sequencing

The bacterial isolates were observed under a stereomicroscope (Optika, mod 620). Gram reactions were determined following the standardized method of bacterial cells staining (Gram



stain kit, Carlo Erba). Catalase and oxidase activities were determined following Smibert and Krieg [93]. For 16S rDNA sequencing of the isolated bacterial strain, a single colony was suspended in 50  $\mu$ l double-distilled water and treated for 5 min at 100°C. Amplification and sequencing of 16S rRNA gene was performed as previously reported [94]. Partial 16S rDNA sequences were determined for the bacterial isolates chr 2 and chr 3, and the sequences were deposited in the GenBank database with the accession numbers: HQ609600 and HQ609601, respectively. The consensus sequences of the isolates were compared with those deposited in GenBank using the BLAST program [95].

### **6.5. Analysis of sequence data**

The 16S partial sequences were compared at the prokaryotic small subunit rDNA on the Ribosomal Database Project II website [96]. The 16S rDNA sequences retrieved from the databases were aligned using ClustalW included in the MEGA software, version 4.1 [97]. The phylogenetic trees were inferred by MEGA 4.1 (neighbour-joining method) [98]. Sequence divergences between strains were quantified using the Kimura-2-parameter distance model [99]. The "Complete Deletion" option was chosen to deal with gaps. Bootstrap analysis (1000 replicates) was used to test the topology of the neighbour-joining method data. The trees were unrooted.

### **6.6. Minimum Inhibitory Concentrations (MICs)**

One ml aliquots of overnight cultures were incubated in 99.0 ml of YEPG-NaCl broth, and 10 ml were distributed in 18 ml test tubes sealed with radial caps. MIC tests were carried out at different concentrations of Cr(VI). Tubes were incubated in a rotary drum at 30°C for 24 hours. The optical density of the cultures, used as a measure of microbial growth, was detected at a wavelength of 600 nm by an UV-visible spectrophotometer (Jenway, mod. AC30); a blank with the culture medium alone (without bacteria) was also analysed. Experiments were carried out in duplicate.

### **6.7. Chromium (VI) assay**

Hexavalent chromium was determined colorimetrically using the 1,5-diphenylcarbazide (DPC) (Sigma-Aldrich, Milan, Italy) method [100].

### **6.8. Effect of chromium concentration on bacterial growth and Cr(VI)-reduction**

The Cr(VI)-resistant isolates were grown over-night in YEPG-NaCl medium, in the presence of 0.2 mM Cr(VI). The pre-culture were used for inocula in different cultures at the same conditions, and incubated at 28°C in the presence of Cr(VI) concentrations: 0, 10, 25, 50, 75, 10 and 150  $\mu$ g ml<sup>-1</sup>. At different times (0, 0.5, 3, 6, 12, 18 and 24 hours), aliquots were harvested in order to measure the absorbance at 600 nm spectrophotometrically, and to evaluate Cr(VI) reduction according to the DPC method. For each series, experiments were conducted in triplicate.

### 6.9. Effect of temperature on bacterial growth and Cr(VI)-reduction

Cultures of the isolates were incubated in a temperature range from 4 to 42°C, with an inoculum prepared by an overnight pre-culture in YEPG medium containing 25 µg ml<sup>-1</sup> of Cr(VI). After different times of incubation of (0, 6, 12 and 24 hours), the effect of different temperatures was detected by harvesting two aliquots of 1 ml for each series, one to evaluate the biomass, revealing absorbance at 600 nm spectrophotometrically, the other to estimate Cr(VI) reduction according to the method of DPC. Experiments were conducted in triplicate.

### 6.10. Extraction of plasmids from the cells of the bacterial strains Cr (VI)-resistant isolates

The two isolated bacterial strains Cr(VI)-resistant were grown in liquid medium YEPG-NaCl in the presence of 25 µg ml<sup>-1</sup> of Cr(VI). Aliquots of 2 ml were centrifuged at 15,000 × g and the pellet washed twice in saline (0.8% NaCl). The pellets were resuspended in 200 µl of solution I (25 mM Tris, 50 mM Glucose, 10 mM EDTA, pH 8.0) in the presence of 4 µg ml<sup>-1</sup> of lysozyme (Sigma, Milan). The pellets were kept at room temperature (RT) for 5 min. Then were added 200 µl of solution II [0.2 M NaOH, 1% (w/v) sodium dodecyl sulphate (SDS)], and the pellets were homogenized gently and kept on ice for 5 min. Then were added 300 µl of solution III (5M potassium acetate, glacial acetic acid 11.5%, deionized water to make up to 100 ml) [101]. The suspension was then centrifuged at 15,000 × g for 5 min. and the supernatant was transferred to a new tube and were added 0.6 volumes of isopropanol, mixed and left at RT for 10 min. The suspension was then centrifuged at 15,000 × g for 5 min., and the pellet was washed using 400 µl of 70% ethanol, centrifuged at 15,000 × g for 5 min. The pellet was dried, then resuspended in 30 µl of deionized water, filtered and sterilized. Four microliters were run on agarose gel at 1,2% (w/v) (Flash Gel® System, Lonza) for testing the purity and quality of the plasmid DNA extracted. One milliliter of standard DNA Marker 100-4000 bp (Flash Gel®, Lonza) was also added to the gel. The gel image was acquired using the system Flash® Gel Room (Lonza).

## 7. Results and discussion

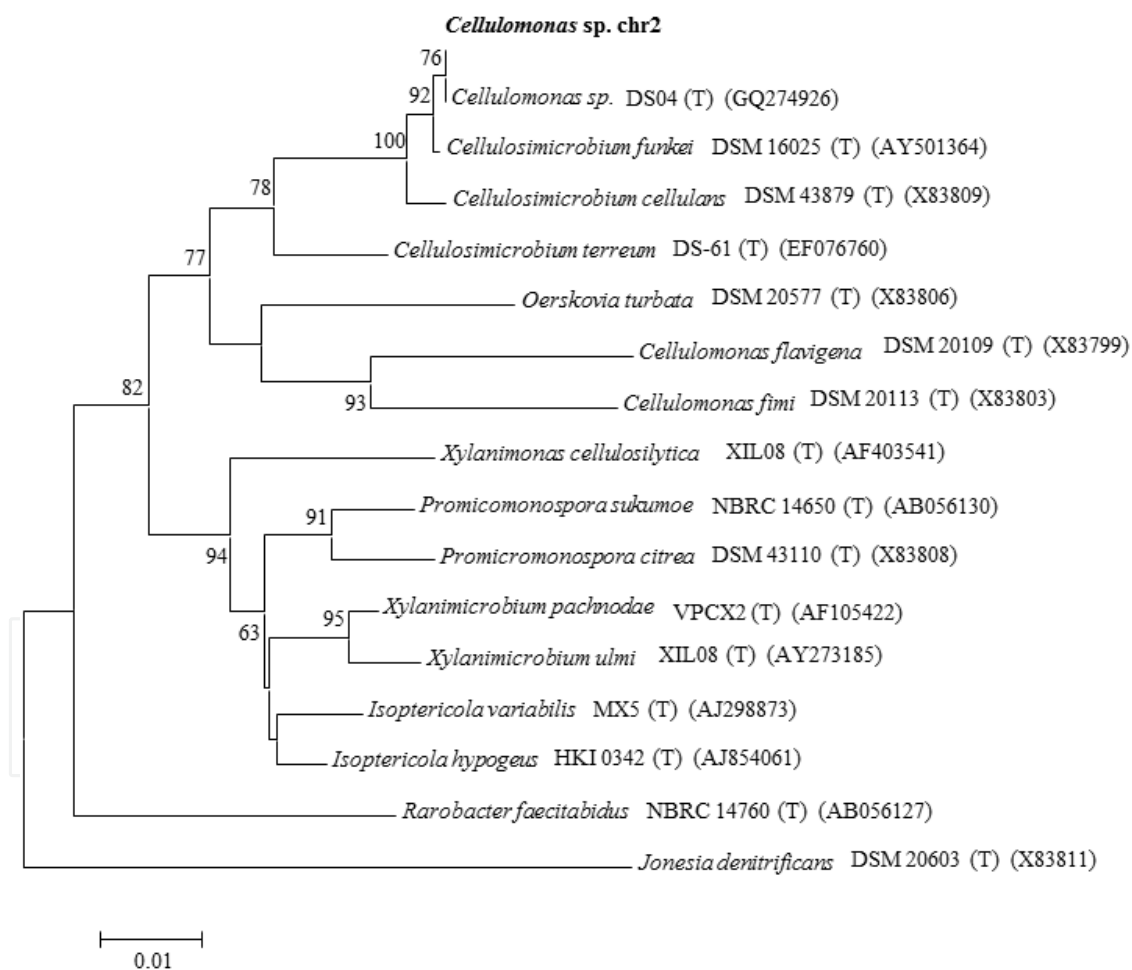
From enrichment cultures arranged from samples of polluted sediments collected near the industrial area, including metallurgical plants, of Bagnoli (Naples, Italy), two Cr(VI)-resistant bacterial strains were isolated and named chr2 and chr3. A microbiological characterization of the isolated strains is reported in Table 1.

Bacterial strain	Gram staining	Oxidase test	Catalase test	Colony morphology
chr2	positive	+	+	Ø 1.0 mm; beige color; regular margins; flat; moist
chr3	positive	-	+	Ø 1.5 mm; yellow color; regular margins; convex; mat

**Table 1.** Characterization of the Cr(VI)-resistant isolated bacterial strains.

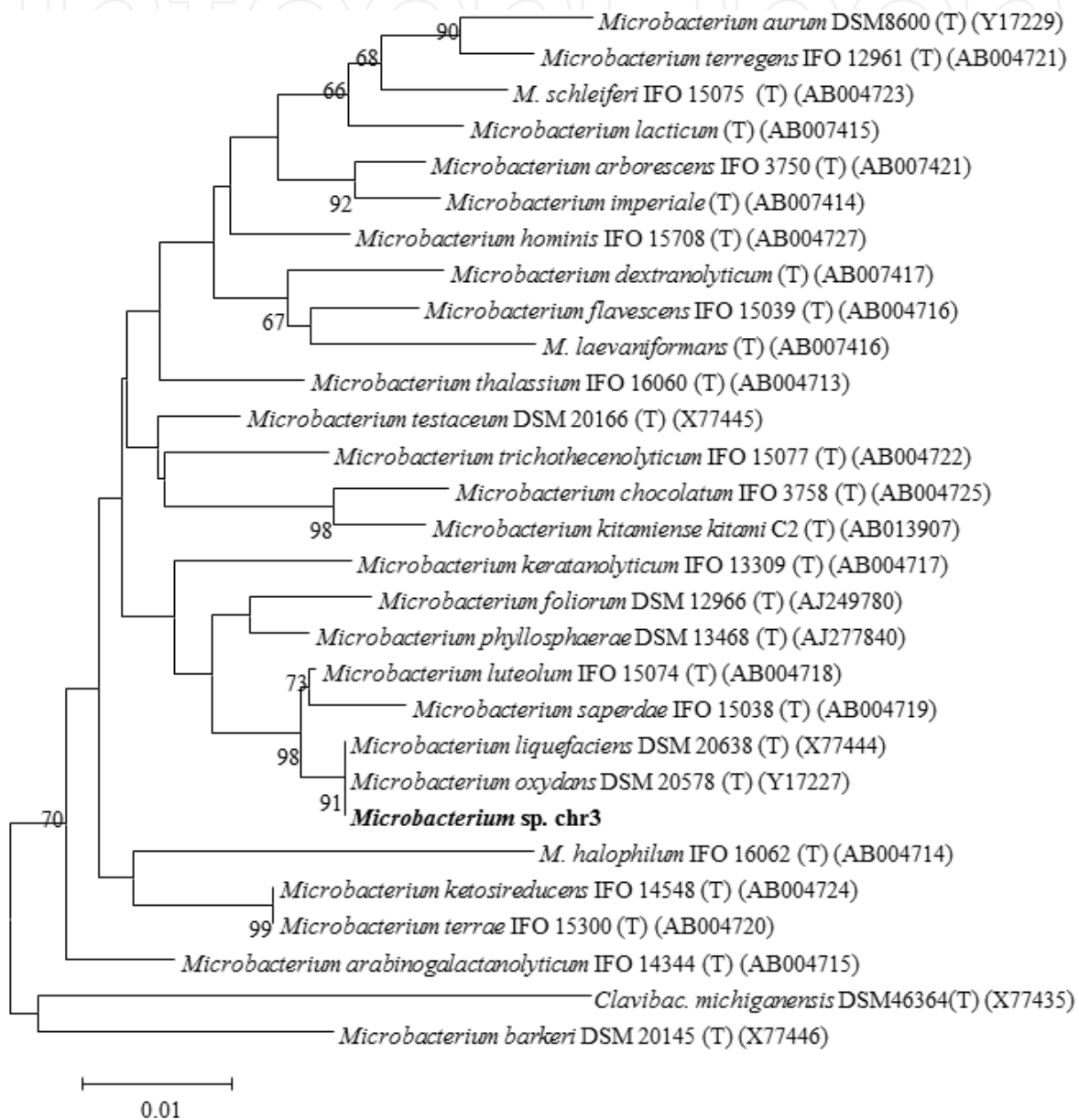
BLAST analysis evidenced a similarity of 100% for strain chr2 with strains *Cellulomonas* sp. DS04-T (GQ274926), able to produce a lactic acid depolymerase; *Cellulosimicrobium cellulans* strains DQ-4 (EU816697) and *C. cellulans* AS4.1333 (AY114178), isolated from soil, and *Cellulosimicrobium* sp. 87N50-1 (EU196469) originating from marine sediments. Strain *Cellulomonas* sp. chr2 did not show a proximity with the Cr(VI)-reducing *Cellulomonas* spp. WS01 (AY617101), ES6 (AY617099) and ES5 (AY617098) [102]. Moreover, BLAST analysis evidenced a similarity of 100% of the strain chr3 with strains *Microbacterium oxydans* spp. XH0903 (GQ279110), isolated from soil; WT141 (GQ152132) and with clones of non-cultivated bacteria nbw 291fa2c1 (GQ086586), nbw 289a11c1 (GQ086396) and nbt 38e04 (FJ894305), obtained from samples of animal origin.

Phylogenetic analysis of the strain *Cellulomonas* sp. chr2 highlighted its position close to the strain *Cellulomonas* sp. DS04-T (GQ274926), separated from neighboring strains *Cellulosimicrobium cellulans* DSM 43879 (X83809) and *C. funkei* DSM 16025 (AY501364) (Fig. 1).



**Figure 1.** Unrooted phylogenetic tree based on 16S rDNA sequence comparisons showing the position of the isolate *Cellulomonas* sp. chr2. The sequence of *Jonesia denitrificans* DSM 20603(T) has been used as *outgroup*. The branching pattern was generated by neighbour-joining methods. Bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap values lower than 60% are not shown. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S rDNA sequences are given in parentheses after the strain.

Strain *Microbacterium* sp. chr3 evidenced a proximity with strain *M. oxydans* DSM 20578, formerly known as *Brevibacterium oxydans*, included in a cluster comprehending strain *M. liquefaciens* DSM 20638 also (Fig. 2). Moreover, phylogenetic analyses showed that the position of the bacterial strains *Microbacterium* sp. chr3 was located quite far from the bacterial strains known for their capability to grow in the presence of Cr(VI) as the Cr(VI)-resistant strain *M. foliorum* DSM 12966 (AJ249780) [103] (Fig. 2)



**Figure 2.** Unrooted phylogenetic tree based on 16S rDNA sequence comparisons showing the position of the *Microbacterium* sp. chr3 isolate and representative species of the genus *Microbacterium*. The sequence of *Clavibacter michiganensis* DSM 46364 has been used as *outgroup*. The branching pattern was generated by neighbour-joining methods. Bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap values lower than 60% are not shown. The scale bar indicates substitutions per nucleotide. The GenBank accession numbers for the 16S rDNA sequences are given in parentheses after the strain.

The Cr(VI)-resistant bacterial strains isolated in this study were assigned to the genera *Cellulomonas* and *Microbacterium*, known for including Cr(VI)-resistant bacterial strains. Eight Cr(VI)-reducing bacterial strains belonging to the genus *Cellulomonas* were isolated from polluted sediments [102]. A dissimilative reduction of Cr(VI) was evidenced in three bacterial strains *Cellulomonas* spp. isolated from environment [104]. A bacterial strain of the genus *Microbacterium* was able to reduce Cr(VI), included in a mixed culture [105], moreover, immobilized cells of a strain *Microbacterium* sp. showed the capability to reduce Cr(VI) [106] [56]. The bacterial strain *Microbacterium* sp. CR-07, isolated from a mud sample of iron ore, evidenced its characteristic of resistance to Cr(VI) its capability to reduce chromate [69].

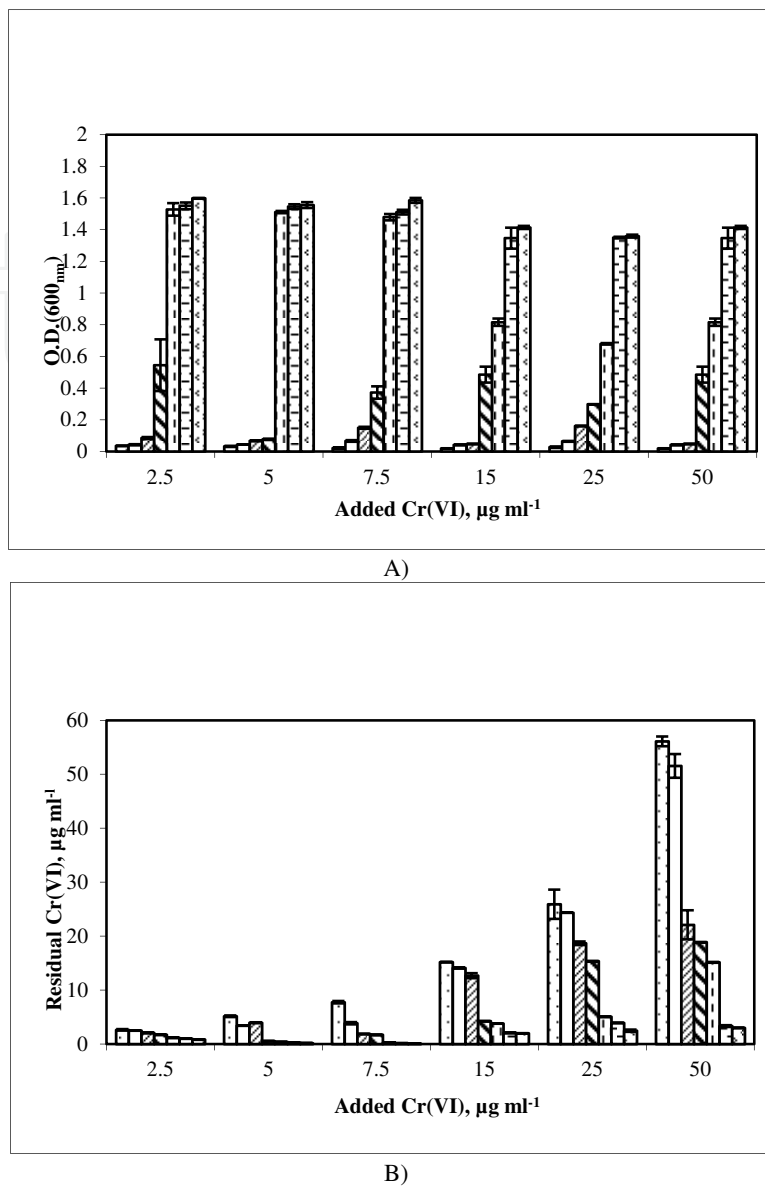
The two genera *Cellulomonas* and *Microbacterium* are included into the Actinobacteria, that represents a significant component of microbial communities present mostly in soils, nevertheless they were isolated also from sediments of marine areas [107]. It is known that bacterial strains belonging to these two genera evidenced their capability to resist to heavy metals, in conjunction with particular growth characteristics, as a rather rapid colonization of selective substrates, suggesting them as right microorganisms to be used for bioremediation processes [36,85].

The bacterial strains showed MIC values in the presence of Cr(VI) of 150  $\mu\text{g ml}^{-1}$  for the bacterial strain *Cellulomonas* sp. chr 2, and of 250  $\mu\text{g ml}^{-1}$  for *Microbacterium* sp. chr 3 (Tab. 2). The isolated bacterial strains evidenced levels of resistance to Cr(VI) similar to those reported in literature [108,109]. Levels of resistance to Cr(VI) similar to those evidenced in this study were highlighted in different Cr(VI)-resistant bacteria isolated from polluted sediments, that evidenced values of resistance to Cr(VI) corresponding to 250  $\mu\text{g ml}^{-1}$  [110].

Bacterial strain	MIC, $\mu\text{g ml}^{-1}$
chr2	150
chr3	250

**Table 2.** MIC values expressed as  $\mu\text{g ml}^{-1}$  of Cr(VI) evidenced in the isolated Cr(VI)-resistant strains.

The mechanism of resistance to Cr(VI) was investigated in the isolated bacterial strains, evidencing their capability to reduce Cr(VI), the most toxic and extremely soluble form of chromium, as revealed by the tests evidencing the depletion of the Cr(VI) content in cultures in conjunction with the increase of the bacterial biomass. The bacterial strain Cr(VI)-resistant *Cellulomonas* sp. chr2 evidence high levels of growth which, after 24 hours of incubation, reached an absorbance of 1.6 without Cr(VI) added, and an absorbance of 1.4 in the presence of 50  $\text{mg ml}^{-1}$  of Cr(VI), evidencing a high adaptability of the bacterial strain to the presence of the toxic chromate (Fig. 3 A). Parallel to the growth of bacterial cells, a correspondent Cr(VI) reduction in the presence of different Cr(VI) concentrations was evidenced, with a residual 8% of Cr(VI) after 24 hours, when the toxic anion was added at a concentration of 50  $\text{mg ml}^{-1}$  (Fig. 3 B). The behaviour of the bacterial isolate *Cellulomonas* sp. chr2 suggested its possible involvement in Cr(VI) removal from polluted sites.

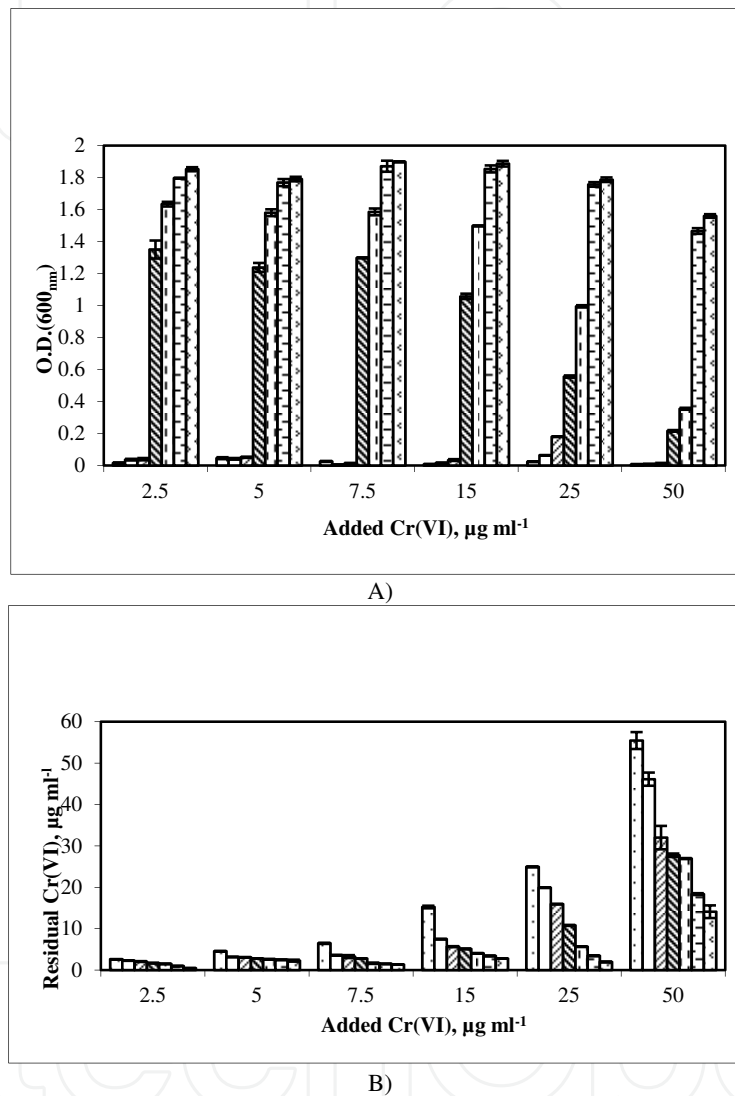


**Figure 3.** Growth of strain *Cellulomonas sp. chr2* in the presence of different concentrations of Cr(VI) (A) and correspondent reduction of Cr(VI) (B). Values were detected at different times: 0 (□), 0.5 (▤), 3 (▥), 6 (▦), 12 (▧), 18 (▨) and 24 (▩) hours.

The bacterial strain *Microbacterium sp. chr3* evidenced higher levels of growth if compared to those of the bacterial strain *Cellulomonas sp. chr2*. In fact, after 24 hours of incubation the strain chr3 evidenced values of absorbance corresponding to 1.8 and 1.6 in the presence of 0 and 50  $\text{mg ml}^{-1}$  of Cr(VI), respectively (Fig. 4 A). In the same experiment, the capability to reduce Cr(VI) added at different concentrations was evidenced for strain *Microbacterium sp. chr3* in the presence of a concentration of 50  $\text{mg ml}^{-1}$ , with a residual concentration of Cr(VI) equal to 32% after 24 hours of incubation (Fig. 4 B).

The isolated bacterial strain *Cellulomonas sp. chr2* evidenced an higher efficiency in reducing Cr(VI) added at a concentration of 50  $\text{mg ml}^{-1}$  as respect to the isolate *Microbacterium sp. chr3*.

In fact, at the end of the 24 hours of incubation a reduction equal to 92% of Cr(VI) was recovered in strain *Cellulomonas* sp. chr2, whereas strain *Microbacterium* sp. chr3 showed a percentage of reduction of 68%. The higher levels of Cr(VI) reduction evidenced in cultures of strain *Cellulomonas* sp. chr2 suggested that it could be a better element for possible processes of Cr(VI) bioremediation.

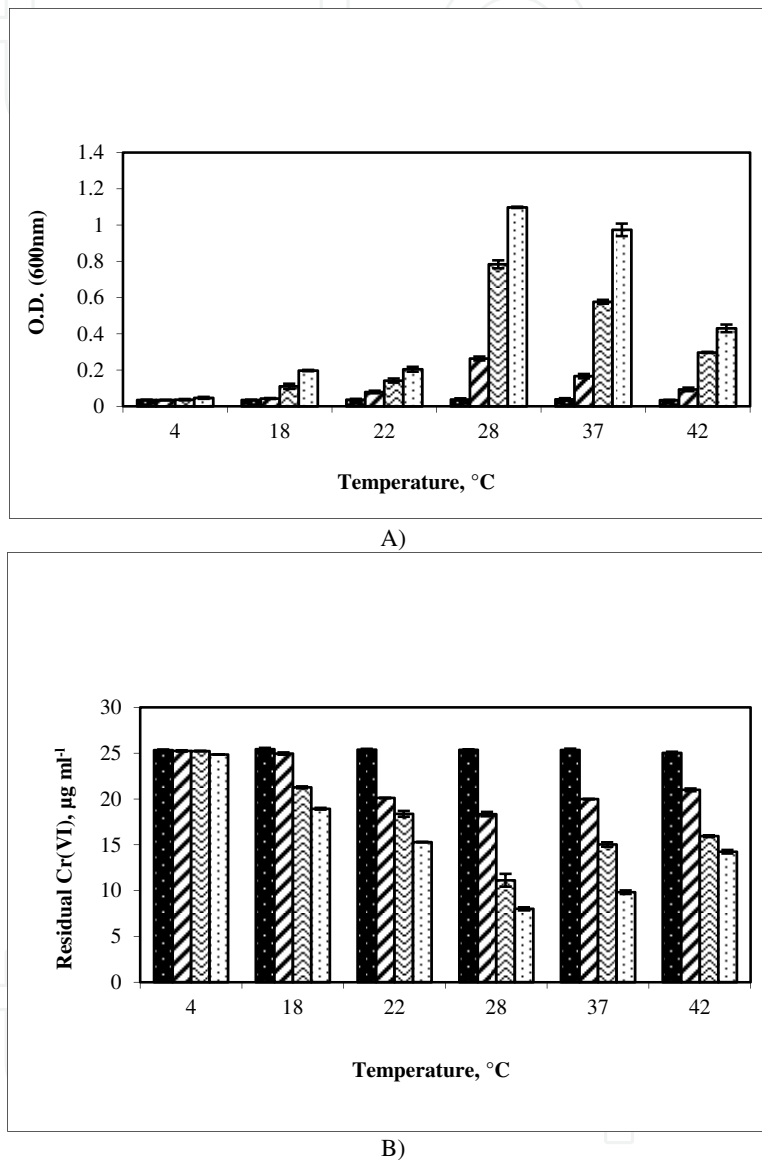


**Figure 4.** Growth of strain *Microbacterium* sp. chr3 in the presence of different concentrations of Cr(VI) (A) and correspondent reduction of Cr(VI) (B). Values were detected at different times: 0 (□), 0.5 (▤), 3 (▨), 6 (▩), 12 (▧), 18 (▦) and 24 (▣) hours.

The two isolated Cr(VI)-resistant strains belonging to Actinobacteria were investigated for their capability to grow and to reduce Cr(VI) added at different concentrations, tested in a range of temperatures included from 4°C to 42°C. Concerning the strain *Cellulomonas* sp. chr2, growth was absent at 4°C, and scarce at 18 and 22°C, with an absorbance equal to 0.2 at a wavelength of 600 nm. The optima levels of growth was detected at a temperature of 28°C, with a value of absorbance of 1.1. A similar value, equal to 1.0 of absorbance was evidenced

at 37°C, whereas a decrease of growth was detected at 42°C, with a value of growth corresponding to an absorbance of 0.4 (Fig. 5 A).

Cr(VI)-reduction in the same cultures of the isolate *Cellulomonas* sp. chr2 followed the pathway of bacterial growth, with a maximum level of reduction evidenced at a temperature of 28°C where a residual level of Cr(VI) of 30% was showed (Fig. 5 B).



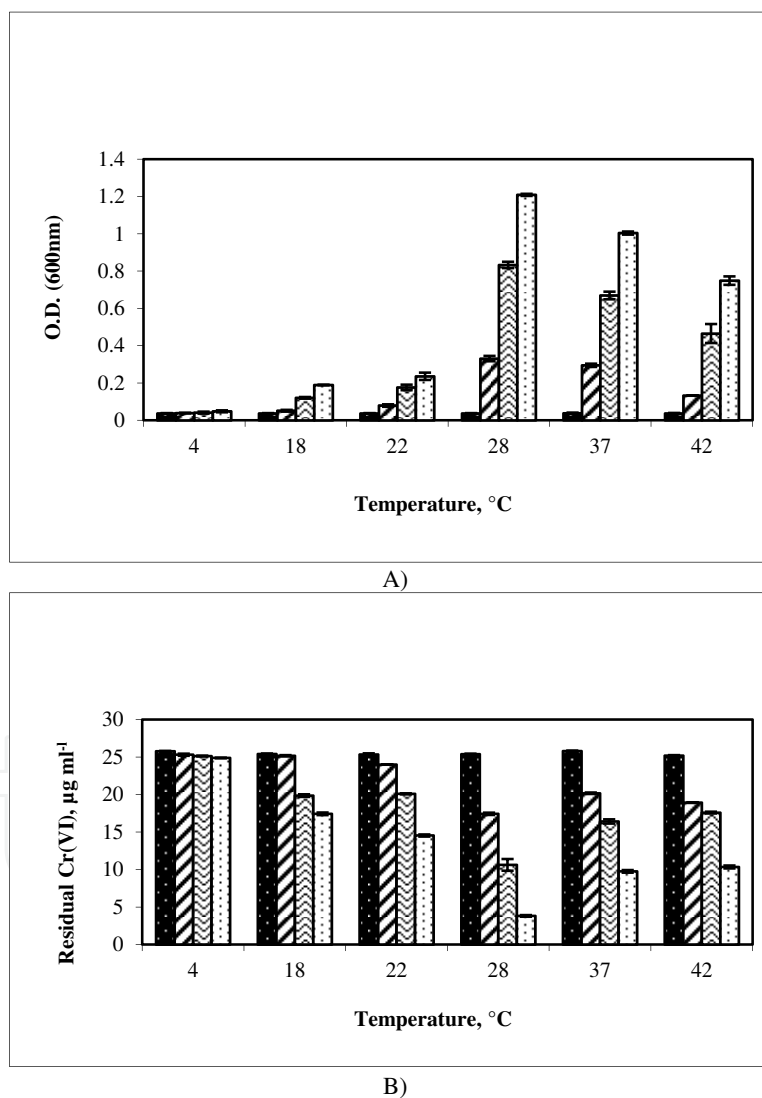
**Figure 5.** Growth of the strain *Cellulomonas* sp. chr2 in medium YEPG-NaCl in the presence of different temperatures, and a fixed concentration of Cr(VI) pair to 25 µg ml<sup>-1</sup> (A). Related Cr(VI) reduction revealed at different temperature (B). Growth and Cr(VI)-reduction were revealed after different times of incubation: 0 (■), 6 (▨), 12 (▧) and 24 (□) hours.

The isolated strain *Microbacterium* sp. chr3 did not evidence growth at 4°C, and low levels of growth, corresponding to an absorbance equal to 0.19 and 0.22 at 18°C and 22°C, respectively. Higher levels of growth were reached at 28°C and 37°C, with values of 1.2 and 0.99, whereas at 42°C, growth of the isolate *Microbacterium* sp. chr3 evidenced an absorbance with a value of



0.75 (Fig. 6 A). Reduction of Cr(VI) by strain *Microbacterium* sp. chr3 evidenced maxima levels at temperatures of 28°C and 37°C, with residual values of Cr(VI) corresponding to percentages of 5.0% and 10%, respectively (Fig. 6 B).

This experiment was conducted in the presence of a fixed concentration of Cr(VI) corresponding to 25 mg ml<sup>-1</sup>, and in this case strain *Microbacterium* sp. chr3 resulted more efficient than strain *Cellulomonas* sp. chr2 in reducing Cr(VI) at 28°C. Nevertheless, at an higher concentration of Cr(VI), equal to 50 mg ml<sup>-1</sup>, strain chr2 evidenced a better capability to reduce Cr(VI) and an higher adaptability to Cr(VI), suggesting a probable better use for bioremediation applications. On the other hand it is noteworthy to note that mixed populations of bacterial strains can represent the better solutions in bioremediation, as different microorganisms, often with complementary features, can cope efficiently with contaminants in bioremediation.



**Figure 6.** Growth of the strain *Microbacterium* sp. chr3 in medium YEPG-NaCl in the presence of different temperature, and a fixed concentration of Cr(VI) pair to 25 µg ml<sup>-1</sup> (A). Related Cr(VI) reduction revealed at different temperature (B). Growth and Cr(VI)-reduction were revealed after different times of incubation: 0 (■), 6 (▨), 12 (▩) and 24 (□) hours.

The capability to reduce Cr(VI) to Cr(III) was evidenced in both the bacterial strains isolated in this study. It is known that different bacterial strains were isolated from polluted sites, as a strain of *Streptomyces griseus* able to reduce Cr(VI) both in virtue of the activity of free cells and of the immobilized ones [111]. Autochthonous bacteria resistant to high levels of Cr(VI) were isolated from polluted sediments. Strain *Bacillus* sp. PB2 isolated from polluted soil evidenced an optimal growth and reduction of Cr(VI) at a temperature of 35°C, at pH values from 7.5 to 9.0, and the use of this isolate was suggested in bioremediation processes of terrestrial sites contaminated by Cr(VI) [112]. Strain *Bacillus* sp. ev3 evidenced the capability to reduce Cr(VI) to Cr(III), with an efficiency of 91% of Cr(VI) reduced in 96 hours [113]. Studies on the reduction of Cr(VI) in autochthonous bacteria in soils contaminated by Cr(VI) near stainless steel industries in the province of Hunan, in China, evidenced that in the presence of an adapt concentration of nutrients, a corresponding efficacy in reducing Cr(VI) was highlighted. A bacterial strain isolated from these polluted soils, and assigned to the genus *Bacillus* was characterized and resulted adapt for bioremediation applications [114].

Strain *Streptomyces* sp. MC1, included in the Actinobacteria, showed the capability to reduce Cr(VI) in cultures arranged in mineral medium. This strain evidenced the capability to reduce 94% of bioavailable Cr(VI) at a concentration of 1 mM in one week of incubation. Moreover the activity of the strain *Streptomyces* sp. MC1 was not inhibited by the native microbial communities resident in the native soil. Cr(VI) was almost completely removed from the polluted soil as consequence of the activity of the Cr(VI)-reducing bacteria [115]. Cr(VI)-reduction was characterized in detail in cells of the strain *Streptomyces* sp. MC1 [116]. Related chromate reductase activity in strain *Streptomyces* sp. MC1 was further evidenced and characterized [36]. The same Cr(VI)-resistant strain *Streptomyces* sp. MC1 evidenced its metabolic versatility and the capability to produce a bioemulsifier in the presence of Cr(VI) [117]. Again *Microbacterium* sp. CR-07, a bacterial strain included in the Actinobacteria, showed resistance to Cr(VI) and the capability to reduce the toxic form of the metal [69].

The ability of the isolates to grow at different temperatures evidenced the range of the use of the Cr(VI)-reducing bacterial strains in eventual bioremediation processes, even in conditions of non-controlled temperature.

Recently, the bio-reduction of Cr(VI) to Cr(III) focused more attention for possible use in bioremediation processes of sites contaminated by Cr(VI). This strategy represents an environmentally friendly technology, to be applied *in situ* and acting in a selective way, and with lower costs, as respect to chemical and physical strategies. The produced Cr(III) can then precipitate as insoluble chromium-hydroxides [Cr(OH)<sub>3</sub>] [118].

The two isolates evidenced the presence of plasmids when tested with primers specific for the presence of genes of Cr(VI)-resistance included into plasmids (data not shown). The bacterial strains capable of expressing both the resistance and the reduction of chromate are very useful for bioremediation. Plasmids involved both in resistance and in the reduction of Cr(VI) have been described in a strain of *Bacillus brevis* isolated from wastes of a tanning industry [119]. Plasmids of this type can be a source of genes for resistance to Cr(VI), that can be transferred *via* cloning assays, and possibly used as DNA probes for the detection of chromate-resistant

bacteria in waters and soils highly contaminated with heavy metals, and the same genes can be used in Cr(VI) biosensors construction [119].

## 8. Conclusions

The potentiality of the Cr(VI)-resistant microorganisms in bioremediation of polluted sites was evidenced in this chapter. A case study was moreover reported with the description of bacterial strains isolated from sediments contaminated by Cr(VI), and tests of Cr(VI)-reduction were included. The isolated bacterial strains showed resistance to Cr(VI) and phylogenetic analyses of the 16S rRNA gene assigned them to the genera *Microbacterium* and *Cellulomonas*, of the order Actinomycetales, class Actinobacteria. The mechanism of Cr(VI)-resistance was due to the reduction of Cr(VI) to Cr(III) and the isolates showed the adaptability of resistance to Cr(VI) in the presence of different temperatures. These results suggested the use of the Cr(VI)-resistant isolated bacterial strains for possible bioremediation processes of contaminated sites.

Further studies including investigations on mechanisms of resistance to Cr(VI) in autochthonous microorganisms isolated from polluted sites, and on the adaptability of microorganisms to contaminants, could give insights for new researches, favoring the development of new technologies for environmental recovery.

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