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Bioprospection of Bacterial Strains from Chromite Process Industry Residues from Mexico for Potential Remediation

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Featured Application: The potential applications of this work include the reconstruction of artificial bacterial consortia that are able to resist and/or reduce chromium and are robust over time for a bioremediation application at the Quimica Central site (Mexico).

Abstract: Industrial residues with high concentrations of hexavalent chromium [Cr(VI)], characterized by an alkaline pH (between 9 and 13) and high salinity (around 100 psu), were used as a source for extremophilic chromium-resistant and -reducing microorganisms. An investigation of biodiversity through MiSeq showed the presence of 20 bacterial classes, with *Bacilli* (47%), *Negativicutes* (15%), *Bacteriodia* (8%), *Gammaproteobacteria* (7%) and *Clostridia* (5%) being the most abundant. The bioprospection allowed the cultivation of 87 heterotrophic bacterial colonies and 17 bacterial isolates at the end of the isolation, and screening procedures were obtained. The isolates were related to *Cellulosimicrobium aquatile*, *C. funkei*, *Acinetobacter radioresistens*, *Staphylococcus equorum*, *S. epidermis*, *Brachybacterium paraconglometratum*, *Glutamicibacter creatinolyticus*, *Pseudomonas songnenensis*, *Microbacterium algeriense* and *Pantoea eucalypti*, most of them being resistant to Cr(VI). Resistances of up to 400 mg.L^{-1} of chromate were obtained for four related strains (QReMLB55A, QRePRA55, QReMLB33A and QReMLB44C). The *C. aquatile* strain QReMLB55A and the *P. songnenensis* strain QReMLB33A were exposed to $K_2Cr_2O_7$ (200 mg.L^{-1}) under optimal conditions, diminishing 94% and 24% of the Cr(VI) in 6 days, respectively. These strains exhibited a high potential for chromium remediation biotechnologies.

Keywords: bioprospection; hexavalent chromium; chromite residues; extremophile; alkaline; hypersaline

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1. Introduction

Chromium is a transition metal that exists in various oxidation states (from −2 to +6). Among them, the hexavalent [Cr(VI)] and trivalent [Cr(III)] states are the ones of higher environmental significance due to their abundance, persistence and stability. Also, Cr(VI) is of major concern due to its industrial applications and impacts on the environment and human health. In fact, it is on the list of priority pollutants of the World Health Organization (WHO) because of its carcinogenicity and mutagenicity [1]. Furthermore,

chromate ions, generated as by-products of a large number of industries (for example, welding, paper and pigment, leather tanning, chrome plating and thermonuclear weapons manufacturing industries), are still discharged into the environment without any treatment [2,3]. This means that, although there are some methods that can be used to remove metal ions from wastewater [4], they are not always implemented. In the case of Cr(VI), its removal from wastewater may be carried out chemically by using large amounts of acid to transform it into Cr(III) [5]. Since the conventional treatments for metal-contaminated industrial residues are quite expensive, the search for more eco-friendly strategies, such as those based on microbial processes, has been encouraged [6]. These efforts have pointed to the Cr(VI)-resistance and -uptake capacities of some microorganisms, as well as the biological capacity for extracellular Cr reduction [7–9], as promising tools for chromium contamination mitigation. Chromium bacterial resistance involves a combination of several mechanisms, such as biosorption, bioaccumulation, biotransformation, efflux, enzymatic and non-enzymatic reduction, precipitation, and cytosolic binding. In the case of adsorption, adsorbed Cr (VI) may be reduced to Cr (III) since most chromium-resistant bacteria exhibit membrane-bound or intracellular chromium reductase activity.

The Bajio region in Guanajuato State (Mexico) is an important industrial area whose major industrial activities are the production of automobiles and automobile parts and shoes and leather items. To supply the chromium requirements for these industries, a chromite processing company was installed locally, in 1970, near Leon city. In 2014, this unit was closed by environmental edicts. Unfortunately, the company abandoned tons of chromite residues (already pointed out by Armienta et al. in 1993 [10]), increasing the environmental problem.

In a previous study [11], two natural microbial consortia were cultivated from the lixiviates of these residues, with bacterial populations composed mainly of representatives of the *Pseudomonadota* phylum (*Pseudomonas*, *Stenotrophomonas*, *Halomonas* and *Enterobacter* genera). Both consortia were able to reduce Cr(VI) to Cr(III), but at low chromium concentrations (50 to 100 mg.L^{-1}), neutral pH and low salinities. In another study, a chemical and biological characterization of these residues and their lixiviates was carried out [12]. Chemically, the residues had a pH above 9 and the lixiviates had a pH near 14. The salinity of the lixiviates was about 100 psu, and they contained extremely high concentrations of iron and chromium. Concerning biological characterization, some microorganisms were identified from the residues and the lixiviates by a cloning approach based on 16S rDNA. The most abundant microorganisms, based on 99 clones that were sequenced, belonged to the following species: *Lysobacter enzymogenes*, *Thiobacillus thioparus* (both belonging to the *Pseudomonadota* phylum) and *Bacillus akibai*, now known as *Alkalihalobacillus akibai* (belonging to the *Bacillota* phylum). The minority representatives found by this method belonged to the *Actinomycetota*, *Acidobacteriota*, *Deinococcota* and *Bacteroidota* phyla, in addition to the already mentioned phyla.

Due to the chemical characteristics and bacterial biodiversity of these residues, it has been assumed that they may be a source of microorganisms physiologically adapted to both alkaline and saline conditions, resistant to chromate ions, and able to biologically deplete chromate. The main objective of this work was to isolate aerobic bacterial strains resistant to Cr (VI) with the ability to diminish these ions in liquid media. Abandoned waste from industries and mines represents a serious and frequent socio-environmental problem. Environmental mitigation bioprocesses can be an economic option for underdeveloped countries. However, obtaining extremophilic microorganisms in axenic cultures is a major challenge for microbial ecology, since under stress conditions the microorganisms live and maintain synergistic relationships with themselves, which makes their isolation and maintenance in laboratory conditions difficult to carry out.

Here, we report a new study of the global bacterial diversity through MiSeq sequencing and the process used to obtain chromate ion-resistant consortia/bacteria from industrial wastes for use in a bioprocess to mitigate Cr(VI) contamination. These bacteria were isolated and identified and their ion resistance to chromate was evaluated, and the ability

of two of these strains to diminish chromate ions in media was verified under optimal conditions of salinity, pH and temperature. The results and advances of this project can have a direct impact on further biotechnological investigations, i.e., the development of bio-processes based on artificial consortia for the bioremediation of sites heavily contaminated by chromite residues.

2. Materials and Methods

2.1. Sampling Site

The samples were taken from a chromite processing site located in Guanajuato State, Mexico (21°02′32″ N, 101°47′29″ W; Figure 1). Sub-samples were taken from two structures: one from the surface layer (1 mm thick) of residue tailings (photos a and b of Figure 1) and one from sediment deposits (about 0.5 cm thick) in the lixiviation channels (photos C and D of Figure 1). The pH and conductivity were checked using a Hanna portable conductivity meter (model: HI 8733). A mixed sample obtained in January 2012, collected and transported in sterile conical tubes™ and stored at 4 °C, was used for the isolation and resistance analyses. Another sample of the residue tailings, obtained in September 2014, was stored at −80 °C for the biodiversity analysis.

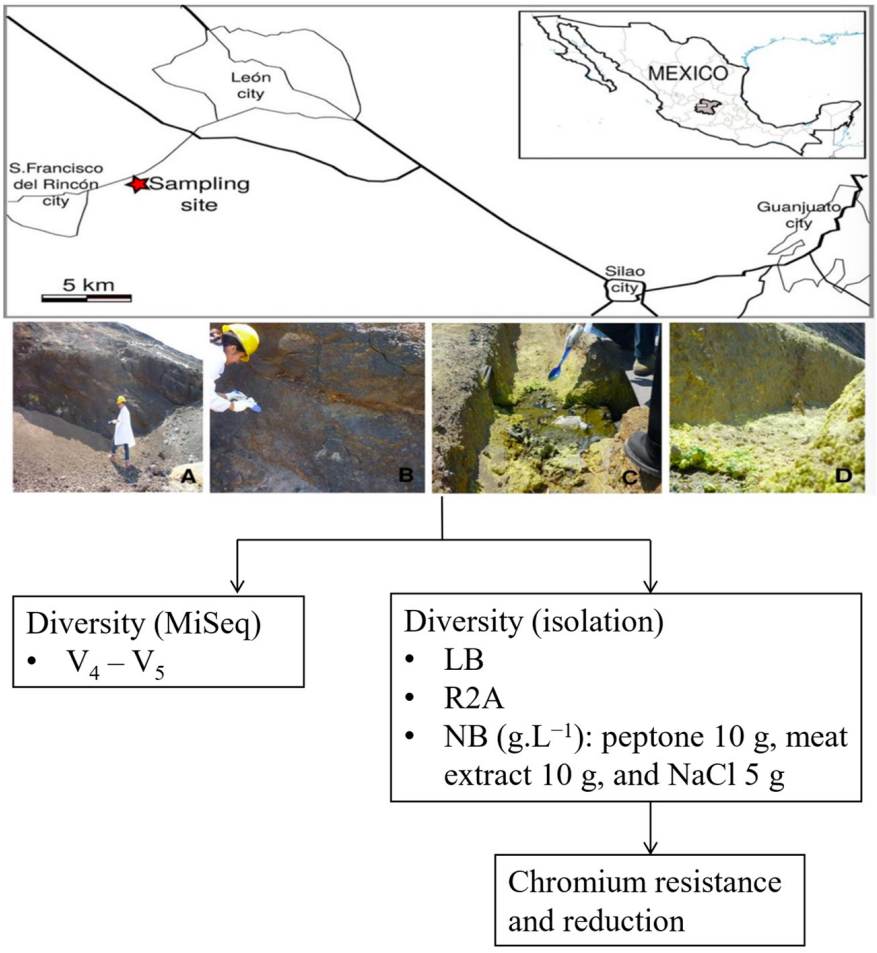

Figure 1. Sampling site. The panel on the top indicates the location of the site (red star) in the state of Guanajuato, between the cities of Leon and San Francesco del Rincon. The middle panel illustrates details of sample collection: photos A and B show the solid residues, and photos C and D show details of the lixiviate samplings. The bottom panel represents the strategies used: diversity analysis through MiSeq (using V4–V5 region); culture and isolation using LB, R2A and NB media; and the assessment of the reduction of and resistance to Cr(VI).

2.2. Biodiversity Analysis

Total environmental DNA was extracted directly from the 2014 sample with a Dneasy PowerSoil Kit (QIAGEN, Courtaboeuf, France) following the manufacturer's instructions. PCR amplifications were performed with the universal eubacterial 16S rRNA gene primers 515 F (5′-GTGYCAGCMGCCGCGGTA-3′) and 928 R (5′-CCCCGYCAATTCMTTTRAGT-3′) containing the adapter sequences (CTTTCCCTACACGACGCTCTTCCGATCT and GGAGTTCAGACGTGTGCTCTTCCGAT, respectively) used in Illumina sequencing technology. PCR reactions included different steps: a short denaturation for 10 min at 95 °C, followed by 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 65 °C and elongation for 40 s at 72 °C. The last step consisted of an extension for 10 min at 72 °C. 16S rRNA gene fragments were amplified from the extracted total DNA with AmpliTaq Gold™ 360 Master Mix (Applied Biosystems, Waltham, MA, USA) in the presence of 0.5 µM of primers in a final volume of 50 µL. Amplicons were sequenced by the Get-PlaGe sequencing service (INRA, Toulouse, France) using Illumina MiSeq 250 bp paired-end technology. The bioinformatics processing of the data was performed using the method described by Esdudié [13] on the Galaxy FROGS pipeline [14]. After a pre-processing step (merging, denoising and dereplication of the reads), the sequences were clustered into operational taxonomic units (OTUs) with an aggregation distance of three bases. OTUs containing less than 0.005% of the total sequences were removed along with chimeric OTUs. Taxonomic assignments were performed using the Silva database v.128 [15]. The raw sequence data of this project were deposited in the Sequence Read Archive (SRA) database of the NCBI associated with the Bio-Project PRJNA933578.

2.3. Colony Isolation Procedure

From the sample obtained in 2012, 1 g was suspended in 10 mL of phosphate buffer ($g.L^{-1}$: $(NH_4)_2SO_4$ (20), K_2HPO_4 (140), KH_2PO_4 (60), Sodium citrate (10) and $MgSO_4 \cdot 10H_2O$ (2)) and shacked at 1000 rpm for 10 min. Then, 1 mL of this suspension was used as a bacterial source for 9 mL of liquid media (R2A, LB or NB). The R2A medium [16] contained the following components ($g.L^{-1}$): yeast extract (0.50), protease peptone (0.50), casamino acids (0.50), glucose (0.50), starch (0.50), Na-pyruvate (0.30), K_2HPO_4 (0.30) and $MgSO_4 \cdot 7H_2O$ (0.05). The LB medium contained the following components ($g.L^{-1}$): tryptone (10), yeast extract (5) and NaCl (10). The NB medium contained the following components ($g.L^{-1}$): peptone (10), meat extract (10) and $NaCl_5$ (5).

After three sequential inoculations in liquid media, several dilutions (up to 10^{-6}) were prepared, cultivated by the streak plate technique and incubated at 33 °C. The last procedure was repeated until individual bacterial colonies were obtained. All media had a settled pH of 8. Due to the large number of colonies obtained (see Table S1), a screening procedure was carried out, both to complete the isolation process and to select isolates with potential for bioremediation. First, we took all colonies of the three media and probed their growth on LB solid media only. Afterward, all cultures containing yeast were disposed of. Then, the ability of the colonies to form chromium reduction halos on solid media was verified, and, finally, their resistance to Cr(VI) and the Cr(VI) depletion time on liquid media were determined.

2.4. Screening by Formation of Chromium Reduction Halos

LB solid media (20 $g.L^{-1}$ agar) were prepared and sterilized, and, before their solidification, sterile $K_2Cr_2O_7$ solution was added to obtain different Cr(VI) concentrations (15, 25, 50, 75 and 100 $mg.L^{-1}$); then, quantities of nearly 15 mL were poured over dish plates. Each dish plate was divided into four parts: one as a positive control, one as a negative control, one as a control with no culture, and the last one with the tested microorganisms. The controls used were taken from a personal bacterial collection whose members were previously tested for their Cr(VI) reducing ability. To obtain fresh cultures, the colonies were first cultivated in LB liquid media, and, once in the exponential phase, a little drop was spread over the last 1/4 of the agar plate surface and incubated at 35 °C. The same

procedure was performed for the control strains. When the colonies were visible (after 2 or 3 days), an agar ortho-dianisidine solution was prepared and immediately poured over the colonies [7,17]; briefly: 0.035 g of ortho-dianisidine (Sigma Aldrich, Toluca, Mexico) was dissolved in 5 mL ethanol (25%), after which 4.23 mL of distilled sterile H_2O, 194 µL HCl 0.124 M and 580 µL H_2O_2 0.89 M were added. Then, 10 mL of a sterile agar solution was poured over this solution (0.6%, liquid but not hot) and immediately poured over the bacterial colonies grown on the LB agar plates. The plates were incubated at room temperature (27–33 °C), protected from the light. After 4 and 24 h, the plates were checked to verify the halo development around the colonies.

2.5. Determination of Chromium Resistance

Once with the colonies in axenic culture, a micro-culture technique with 96 well-microplates was used for a fast screening of the Cr(VI) resistance of the isolates. These were inoculated in LB liquid medium, and, once in the exponential phase, 2 mL was used to inoculate 8 mL of fresh LB or MS media. The MS medium contained the following components ($g.L^{-1}$): Tris (6), NaCl (4.7), HCl (1.5), NH_4Cl (1.7), $NaSO_4$ (0.43), $MgCl{\cdot}6H_2O$ (0.2), $CaCl_2{\cdot}H_2O$ (0.03), $Na_2HPO_4{\cdot}2H_2O$ (0.04), Fe(III)NH_4-citrate (0.0048) and oligo-element solution (1 mL) containing the following components ($mg.L^{-1}$): $FeCl_2{\cdot}4H_2O$ (1500), $CoCl_2{\cdot}6H_2O$ (190), $MnCl_2{\cdot}4H_2O$ (100), $ZnCl_2$ (70), H_3BO_3 (62), $NaMoO_4{\cdot}2H_2O$ (36), $NiCl_2{\cdot}6H_2O$ (24) and $CuCl_2{\cdot}H_2O$ (17) (pH 7.2). Then, 225 µL of the suspension was added to the first line (A-1 to H-1), while the rest of wells were filled with 125 µL quantities of cells suspension. A solution of $K_2Cr_2O_7$ (4 $g.L^{-1}$) was prepared in sterile medium (LB or MMS), and 25 µL was added only to the first line to obtain a final concentration of 400 $mg.L^{-1}$ $K_2Cr_2O_7$. Then, a serial dilution was carried out: 125 µL quantities from the first line (A-1 to H-1) were transferred to the second line (A-2 to H-2); from these, 125 µL quantities were transferred to the next line (A-3 to H-3), and so on. The final Cr(VI) concentrations of the plate by line were as follows: 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 $mg.L^{-1}$ $K_2Cr_2O_7$. The last line was used as a control, which was filled with culture media without $K_2Cr_2O_7$. The plates were incubated at 35 °C for 5 days, and the bacterial growth was observed.

2.6. Chromium VI Quantification

Cr(VI) was quantified by the colorimetric method, employing diphenyl-carbazide (DPC; [18]). Briefly, 100 µL of a sample was acidified with 500 µL H_2SO_4, after which 4.450 mL of distilled water was added and then 50 µL DPC 5 $mg.mL^1$. After 10 min under obscurity, the absorbance was measured at λ 540 nm. The Cr(VI) concentration was measured using a standard curve.

2.7. Phylogenetic Analyses of Isolated Strains

The total DNA of bacterial isolates was extracted using an UltraClean Microbial DNA Isolation Kit® (Mo Bio Laboratories Inc., Carlsbad, CA, USA) and verified by electrophoresis (1% agarose gel). DNA solutions were preserved at −20 °C. The 16S rRNA encoding genes were amplified by PCR using the primers 8F (5′−AGAGTTTGATCCTGGCTCAG−3′) and 1489R (5′−TACCTTGTTACGACTTCA−3′). PCR was carried out with highly thermostable DNA polymerase (Fermentas Life Science®, Waltham, MA, USA) in the PTC 200 thermocycler (MJ Research, St. Bruno, QC, Canada). The purified products were Sanger sequenced in a specialized laboratory. For details of these protocols, see Brito [19]. The PCR amplicons from the isolates were sent to GATC (Konstanz, Germany) or to LANGEBIO, CINVESTAV-Irapuato (Irapuato, Mexico). The 16S rRNA gene sequences and the closest references were aligned with the MAFFT program (Multiple Alignment using Fast Fourier Transforming; [20]). A phylogenetic tree was constructed with the MEGA 11 (Molecular Evolutionary Genetics Analysis version 11; [21]) software using the maximum-likelihood method. The confidence of the phylogenetic tree was assessed by bootstrap using 1000 resamplings.

The sequences obtained in the present study were uploaded to the GenBank® database (assigned accession numbers: OQ456364 to OQ456381).

3. Results

3.1. Diversity

Comparing the bacterial biodiversity determined via the metagenomic approach (this study) with the cloning library data obtained from the same site in 2008 [12], differences emerged (Figure 2). In both cases, a similar number of major classes (abundance larger than 1%) was detected (8 and 12, respectively for the cloning library and the metagenome, 4 of which were shared). The cloning data (n = 99) obtained from both lixiviates and tailings revealed the following classes: *Gammaproteobacteria* (with an abundance of 41%), *Bacilli* (20%), *Alphaproteobacteria* (15%), *Betaproteobacteria* (14%), *Actinomycetes* (4%), *Terriglobia* (3%), *Deinococci* (2%) and *Cytophagia* (1%). From the MiSeq data (38,649 reads after cleanup and 120 OTUs), the majority classes detected in the tailings were as follows: *Bacilli* (with 47.0% abundance), *Negativicutes* (14.8%), *Bacteroidia* (7.5%), *Gammaproteobacteria* (6.8%), *Clostridia* (5.4%), *Cloacimonadia* (4.4%), *Synthrophomonadia* (3.4%), *Actinomycetes* (2.7%), '*Oxyphotobacteria*' (2.2%), *Fusobacteriia* (1.4%), *Alphaproteobacteria* (1.3%) and SAR324-Clade (1.1%), while the other 2% corresponded to the sum of the minor classes. The Hill numbers (Exp(Shannon) and Inverse Simpson) decreased rapidly (27 and 12, respectively), showing a low evenness.

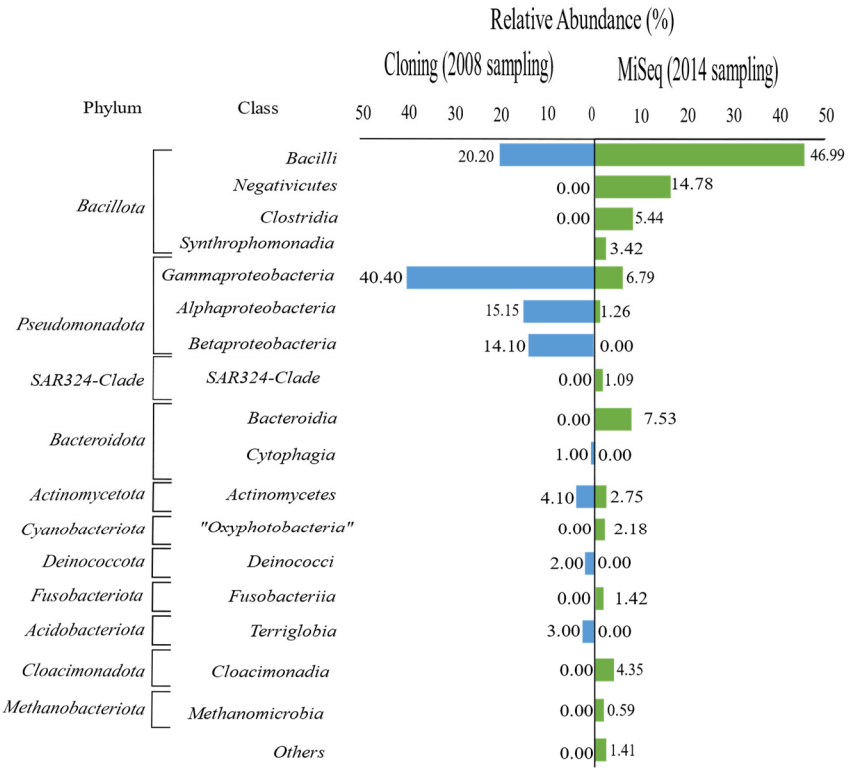

Figure 2. Major bacterial phyla and classes of the community structure obtained by MiSeq sequencing (this work, samples collected in 2014) as compared to the cloning approach (in 2008, from Brito et al., 2013 [12]).

3.2. Colonies and Sequenced Strains

Comparing the three culture media used, LB medium gave the best results (38 colonies) for bacterial growth from our samples, followed by NB and R2A media (28 and 21 colonies, respectively). Nevertheless, only 19 colonies among the 38 that grew in LB medium were considered because they presented morphological (colonial and/or cellular) differences. Among the colonies that grew originally in NB and R2A media, all the ones that also grew

in LB medium were selected: nine from NB medium and eight from R2A medium. Since some colonies were revealed to be still consortia at this point, the isolation procedure was continued when necessary, and another six colonies were obtained from LB consortia, with another two from NB and another three from R2A, giving an overall total of 47 colonies (see Table S1).

The final isolation process resulted in 17 bacterial strains from the 47 colonies described above, which showed higher similarity to the following genera: *Staphylococcus* (QRePLB13, QRePLBC31 QRePLBC34 and QReMLB55B), *Microbacterium* (QRePLB01), *Cellulosimicrobium* (QRePRA55 and QReMLB55A), *Brachybacterium* (QRePLBE33 and QRePLB21), *Glutamicibacter* (QRePLB02), *Pantoea* (QRePLB00E), *Acinetobacter* (QRePRA31 and QRePNB53) and *Pseudomonas* (QReMLB33A, QReMLB33C QReMLB33D and QReMLB44C) (Figure 3).

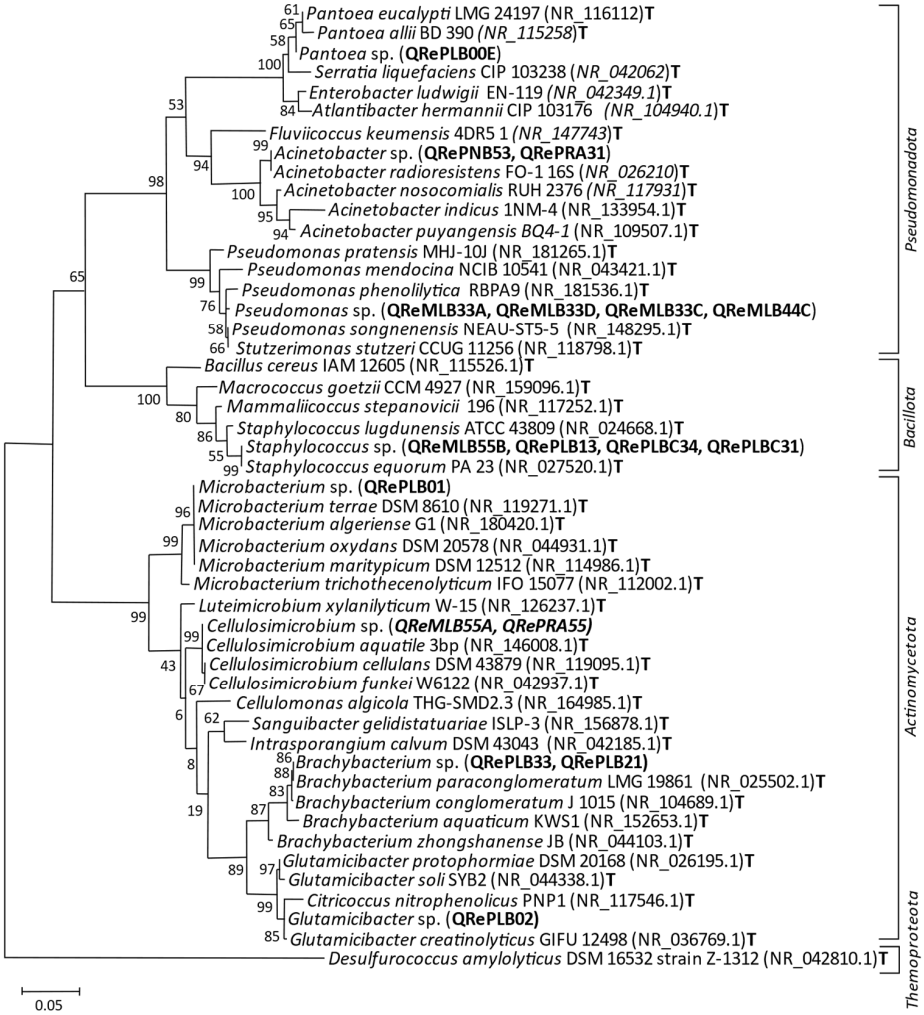

Figure 3. Maximum-likelihood tree based on the alignment of 720 pb of 16S rRNA gene sequences showing the phylogenetic positions of isolated strains. The strains are indicated in parentheses with the name of the respective species. Bootstrap values (1000 resamplings) are indicated at the nodes.

3.3. Chromium Reduction Halos

From the 47 obtained colonies, a sub-sample of 42 were inoculated on LB solid medium containing different chromium concentrations to study the formation of chromium reduction halos. All of these (100%) grew on LB solid medium with 15 mg.L^{-1} Cr(VI), 26 (62%) grew at 25 mg.L^{-1}, 17 (40%) grew at 50 mg.L^{-1}, 5 (12%) grew at 75 mg.L^{-1} and only 2 (5%) grew at 100 mg.L^{-1} (Table S1). As expected, increasing the Cr(VI) concentration decreased the formation of colorless halos around the colonies. The results for Cr(VI) resistance and ability to reduce the ions highlighted five colonies (33AC, NB53G4, R2A44, R2A55 and 55A)

by the presence of colorless halos on solid medium at least until 75 mg.L^{-1}. We selected some of these colonies and strains isolated from them for the complementary studies that will be described in the next sub-section.

Since our goal was to search for bacteria to be applied in metal bioremediation, the possible pathogen strains were not considered in some of the following experiments, even the ones with some chromium tolerance. This was the case for the QRePLB13 strain (from the LB13 colony), which showed high similarity to *Staphylococcus equorum* (100%) but also to *S. lugdunensis* (98.3%), a pathogenic strain isolated from auxiliary lymph nodes [22,23]. These strains also included the other *Staphylococcus* specimens in the sample, namely, QRePLBC31, QRePLBC34 and QReMLB55B (from colonies LBC3-1, LBC3-4 and 55B, respectively), as well as QRePLB00E (LB00E), which is associated with the *Pantoea eucalypti* (99.6%). The isolate QRePLB01 (similar to *Microbacterium algeriense*, 100%) was also discarded because, although this isolate came from a colony that showed colorless halo reduction (colony LB01), the strain could not grow on Cr(VI). Possibly the original colony was not an axenic culture, and, with the intent of obtaining the isolated microorganisms, the strain resistant to Cr(VI) present in the original colony was lost.

The QRePRA31 and QRePNB53 isolates (from colonies R2A31 and NB53G4, respectively) showed similarity (99.7 and 99.8%, respectively) to *Acinetobacter radioresistens*, whose original colonies exhibited colorless halo reductions of up to 25 mg.L^{-1} and 75 mg.L^{-1} Cr(VI), respectively (see Table S1). *Acinetobacter radioresistens* is a radiation-resistant organism isolated from cotton sterilized by gamma radiation [24]. The QRePLB02 strain (LB02 colony) showed similarity (99.6%) to *Glutamicibacter creatinolyticus*, which showed it to be a halotolerant strain [25]. The colony showed a behavior similar to that of the R2A31 colony when exposed to Cr(VI).

The most promising strains, however, were revealed to be the ones that came from the LB33 and R2A55 colonies and their children. The LB33 colony developed a colorless halo reduction of up to 50 mg.L^{-1} Cr(VI), while its child, 33AC, reached 100 mg.L^{-1} Cr(VI). From this family, we obtained four strains, QRePLBE33, QReMLB33A, QReMLB33D and QReMLB33C (see Table 1). The first showed a similarity of 99.9% to *Brachybacterium paraconglomeratum*, while the others showed similarity (99.4%) to *Pseudomonas songnenensis*. Another strain that showed similarity (100%) to *B. paraconglomeratum* was QRePLB21 (from colony NB21), while QReMLB44C (from colony 44CC, the child of R2A44), which developed a colorless halo reduction of up to 75 mg.L^{-1} Cr(VI), showed (99.3%) similarity to *P. songnenensis*. Similarly, the R2A55 colony developed a colorless halo reduction of up to 75 mg.L^{-1} Cr(VI), while its child, 55A, was the second to reach 100 mg.L^{-1} Cr(VI). The QRePRA55 axenic culture strain was shown to be similar to *Cellulosimicrobium funkei* (99.9%), and QReMLB55A was shown to be similar to *C. aquatile* (99.6%). The other strain that showed high similarity to *C. aquatile* (100%) was QRePNB227 (from colony NB22).

Table 1. Ability of the isolates QReMLB33A and QReMLB55A to diminish Cr(VI) at different concentrations of Cr(VI) after 8 days of incubation. All experiments were performed in triplicate, using LB culture medium (pH 7) and incubated at 37 °C. The Cr(VI) was added after 72 h of incubation, which was taken as the initial time.

Isolates	Initial Concentration of Cr(VI) (mg.L^{-1})	Final Concentration of Cr(VI) (mg.L^{-1})	% Diminution after 8 Days
QReMLB33A	105.71 ± 15.32	58.9 ± 10.17	44.8
	232.42 ± 30.60	143.45 ± 16.68	38.28
	294.43 ± 26.97	238.31 ± 26.92	19.06
	368 ± 21.34	313.06 ± 23.70	14.98
QReMLB55A	135.37 ± 52.0	53.50 ± 14.79	60.47
	215.76 ± 11.86	136.59 ± 18.10	36.69
	283.41 ± 24.74	207.18 ± 13.57	26.90
	406.69 ± 18.51	269.44 ± 23.50	33.75

3.4. Chromium Resistance and Depletion

The colony R2A55 was first selected to carry out a study of chromium depletion in liquid culture media. We observed a decrease of 90% in the Cr(VI) after 74 h of incubation at an ion concentration of 50 mg.L^{-1}, a decrease of 75–90% after the same amount of time at 75 mg.L^{-1} and a decrease of 66% at 100 mg.L^{-1} of Cr(VI) after 30 h. At the same time, we verified whether the decrease in Cr(VI) occurred due to its uptake into biomass or due to extracellular reduction. After separation of liquid and biomass fractions, we verified that nearly 75, 67 and 94% of the total chromium was adsorbed on the biomass (for the cultures at 50, 75 and 100 mg.L^{-1} Cr(VI), respectively), suggesting that the colony is a good option for further studies with a focus on applications for chromate remediation.

A complete experiment for determining the chromium resistance was applied to the 17 isolated strains, considering concentrations of up to 400 mg.L^{-1} Cr(VI) in liquid media (Figure 4). These results came from LB liquid medium cultures, since no bacterial growth was observed in MS liquid medium. In this experiment, four isolates revealed high resistance to Cr(VI), all from the genera *Pseudomonas* and *Cellulosimicrobium*.

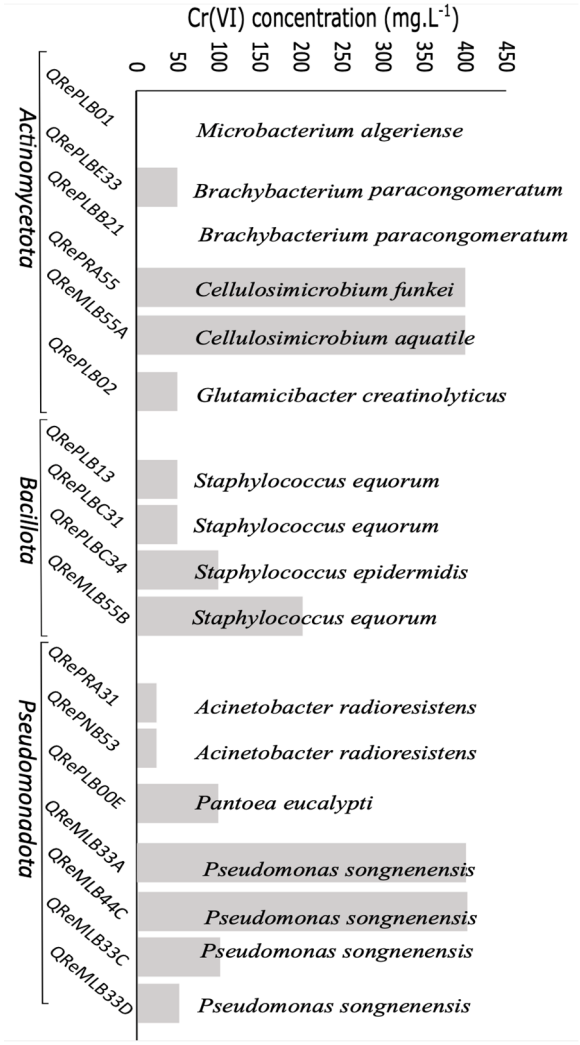

Figure 4. Chromium resistance of the 17 isolated bacterial strains at different Cr(VI) concentrations.

From these, we selected two representatives, QReMLB33A and QReMLB55A (*P. songnenesis* and *C. aquatile*, respectively), to study in detail their ability to diminish chromate ions in the medium over time. In the first experiment, the strains were exposed to different Cr(VI) concentrations, and after 8 days of incubation the Cr(VI) depletion was measured. The strain QReMLB33A diminished 44, 38, 19 and 15% of 100, 200, 300 and 400 mg.L^{-1},

respectively, while the strain QReMLB55A diminished 60, 37, 27 and 34% under the same experimental conditions (Table 1).

Based on these studies, in the next step we carried out a kinetic experiment on Cr(VI) depletion in liquid media. In this experiment, we used the optimal growth conditions for each strain (LB modified for QReMLB33A contained 4% NaCl and had a pH of 7, and for the QReMLB55A strain the medium contained 2% NaCl and had a pH of 9) and 200 mg.L^{-1} $K_2Cr_2O_7$. The media were inoculated with 10^8cells.mL^{-1} and were incubated at 33 °C (Figure 5). In these conditions, the QReMLB33A strain was not as efficient as previously observed, presenting a behavior relatively close to that of the control, with a depletion rate of only –0.00054 ± 0.00019 (%/h), equivalent to a half-life of 1283 h. On the other hand, the strain QReMLB55A achieved a very significant decrease, with a depletion rate of –0.01399 ± 0.00083 (%/h), reaching the half-life in only 49.5 h. These results indicate a great potential for the biotechnological application of the QReMLB55A strain, mainly for Cr(VI) mitigation, but maybe also for the mitigation of other metals.

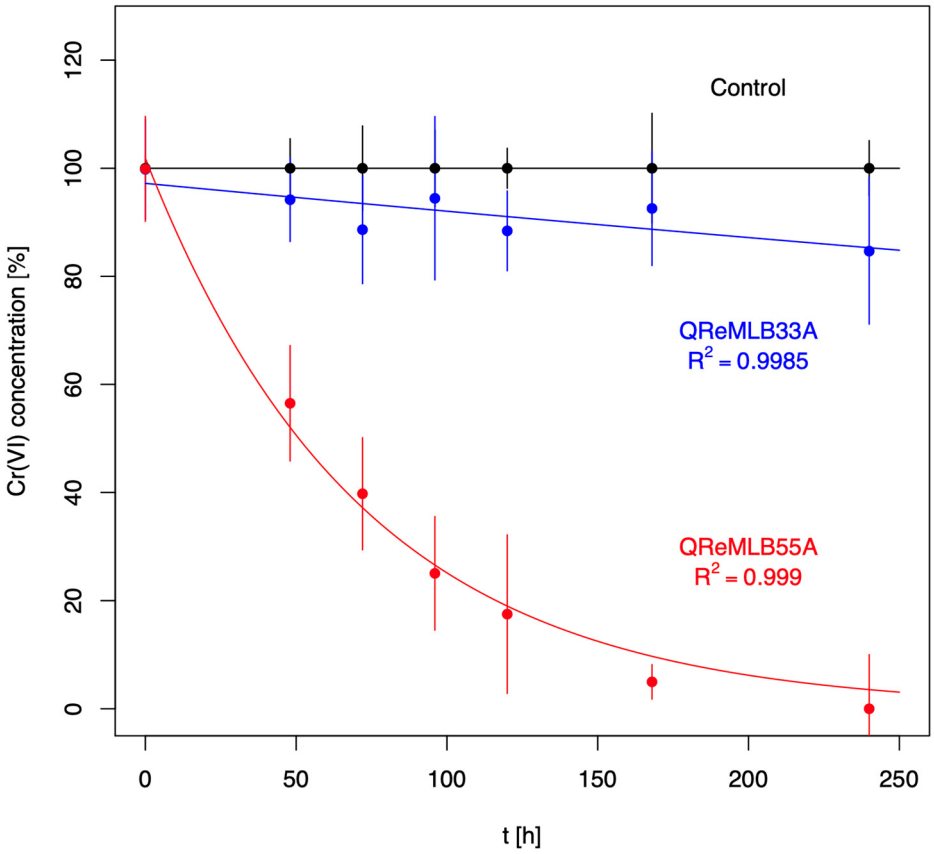

Figure 5. Reduction kinetics of Cr(VI) by the selected isolated bacterial strains, QReMLB55A and QReMLB33A (in LB medium, inoculated with 10^8cells.mL^{-1}, incubated at 33 °C, with optimal pH and salinity). Error bars come from standard deviation of triplicates.

4. Discussion

Extreme sites, natural or anthropic, can act as sources for microorganisms adapted to their surroundings which can be used in the search for new environmentally friendly technologies. In the search for resistant microorganisms capable of chromium bioremediation, we prospected residues from an abandoned chromite mine. These residues are highly alkaline (due to the presence of sodium, carbonate and calcium ions) and contain high levels of metals, mainly Cr(VI) [11,12]. In a previous study, we detected (by a cloning approach) the presence of bacterial populations that could play an important role in the transformation of metals present in these residues [12]. Here, a new biodiversity study using a more modern approach (MiSeq) was carried out.

While cloning results revealed a predominance of the *Pseudomonadota* phylum (69.7%), represented by the classes *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*, followed by *Bacillota* (20.2%, *Baccilli* class) and *Actinomycetota* (4.1%, *Actinomycetes* class), the MiSeq results showed the same phyla, together with *Bacteroidota* and *Cloacimonadota*, as the most abundant ones, but in a slightly different order: *Bacillota* (70.7%, represented by *Bacilli*, *Negativicutes*, *Clostridia* and *Synthrophomonadia* classes), *Pseudomonadota* (8.4%, *Gammaproteobacteria* and *Alphaproteobacteria* classes), *Bacteroidota* (7.5%, *Bacteroidia* class), *Cloacimonadota* (4.4%, *Cloacimonadia* class) and *Actinomycetota* (3.1%, *Actinomycetes* class). The differences between cloning and Illumina results could be expected since both techniques use different approaches, each one with its own bases [26–28]. Additionally, the period between both samplings may also have influenced this difference [29]. The present MiSeq study was carried out to improve the microbial characterization of the studied sampling site, and it will certainly help in future research. The isolation process, while biased towards the selection of chromium-tolerant bacteria, yielded results that were even closer to the cloning results, giving seven strains of *Gammaproteobacteria* class (41.2%), six strains of *Actinomycetes* (35.3%) and four strains of *Bacilli* (23.5%).

The major populations of both studies (2008 and 2014 samplings) showed similarities to other populations examined in studies performed in sites contaminated by chromium or under Cr(VI) stress. For example, Zhang [30] verified *Pseudomonadota*, *Actinomycetota* and *Acidobacteriota* as the predominant phyla present in Cr-contaminated soil samples. Liu [31], in soil samples collected near chromate slag (in Sanchaji, Changsha City, Hunan Province of China), observed *Pseudomonadota*, *Actinomycetota*, *Bacillota* and *Chloroflexota* phyla. Pradhan [32] detected the predominance of *Pseudomonadota*, *Bacillota*, *Bacteroidota* and *Actinomycetota* phyla in soil samples from a chromite mine area in Sikinda Valley (Odisha, India). As can be seen, our results are in agreement with the ones obtained in other sites contaminated by Cr(VI).

The isolates of the *Gammaproteobacteria* class (*Pseudomonadota* phylum) showed affiliation with the *Pantoea*, *Acinetobacter* and *Pseudomonas* genera. *Pantoea* belongs to a diverse bacterial group, with existing species considered as epiphytes [33], endophytes or pathogens to plants, animals and humans [34]. Also, metal resistance was demonstrated for this genus; for example, the *Pantoea* sp. strain TEM18, which was isolated from wastewater (from a petrochemical treatment plant in Izmir, Turkey), was able to decrease a concentration of 25 $mg.L^{-1}$ Cr(VI) at pH 3 [35]. More recently, a genome analysis of the *P. agglomerans* strain C1 showed the presence of genes related to both resistance to multiple toxic metals and plant growth promoters [36]. Our isolate (QRePLB00E) showed similarity to *P. eucalipty* (99%) and *P. allii* (98%), which are cited as occupying different ecological niches. While *P. allii* is recognized as a bacterial plant pathogen, *P. eucalypti* is used to promote plant growth by solubilizing soil phosphate [37–39]. The QRePLB00E isolate also grew on Cr(VI) (at 100 $mg.L^{-1}$).

In this same phylum, two strains (QRePRA31 and QRePNB53) similar to *Acinetobacter radioresistens* were obtained, but their Cr(VI) resistance was limited to 25 $mg.L^{-1}$. Studies have shown some biotechnological applications of microorganisms of this species, for example, the capability to produce alasan, an emulsifying agent [40], and alkaline lipase [41]. Also, the capability to degrade some contaminants, such as methyl parathion and organophosphorus pesticide [42], and aromatic compounds [43,44] was verified for some isolates. Furthermore, the desiccation resistance of this species was indicated as an important attribute for surviving in extreme conditions [45].

However, other of our isolates seem more directly interesting for our purpose because they showed higher resistance to Cr(VI) than these ones. Such strains have interesting features for future research related to biofilm production using metal bioremediation approaches. The genus *Pseudomonas*, also obtained in the present bioprospection, was first described in 1894. The members of this genus are commonly found in aquatic and soil environments and in humans, plants and animals [46]. Some species are free-living, some are plant growth-promoting (N2-fixing symbiotic bacteria), while others are considered

pathogens or opportunistic pathogens. Thus, many members of this genus are of interest for biotechnological research due to their distribution, the large number of described and cultivated species, and their adaptations to a wide variety of environmental conditions [47–50]. Among the species of *Pseudomonas* with some capability of taking up metals (metalloids) are *P. alcaliphila*, *P. aeruginosa*, *P. fluorescens*, *P. koreensis*, *P. mendocina* and *P. stutzeri* (see [50]). In addition, some *Pseudomonas* species have been used to explain the metabolic and physiological mechanisms which they normally carry out, for example, their ability to transform metals [50]. Here, four of our isolates (QReMLB33A, QReMLB33C, QReMLB33D and QReMLB44C) showed higher similarity to the *P. songnenensis* strain NEAU-ST5-5, a strain isolated from a soil sample collected between 5 and 10 cm deep in Songnen Plain, Northeast China, in 2012 [51]. It is an alkaline strain and halotolerant (0–5% NaCl), and, currently, no information about its interaction with metals is available. Among our isolates of this genera, the strain selected as a model (QReMLB33A) showed resistance up to 400 mg.L^{-1} Cr(VI) and an ability to reduce the ions in liquid media (ranging between 45 and 15%, depending on the initial concentration of Cr(VI)), in addition to growing on biofilm. Although another of our strains showed better performance in its interaction with chromate ions, the ability to grow and form a biofilm, combined with its resistance to the ions, could be very useful in biotechnological applications of the research on Cr(VI) removal.

Like the *Pseudomonadota* phylum, the *Actinomycetota* phylum has great metabolic versatility and potential for biotechnological application, playing an important role in recycling substances, in removing xenobiotic compounds and in the synthesis of new substances [52]. They are widely distributed in aquatic and terrestrial ecosystems, including various extreme environments, explaining their ability to survive under stressful conditions, such as starvation, desiccation and toxic chemicals, including toxic metals [52]. Our isolates belonging to the *Actinomycetes* class showed affiliation to the *Microbacterium*, *Glutamicibacter*, *Brachybacterium* and *Cellulosimicrobium* genera. Although our QRePLB01 isolate (a *Microbacterium algeriense* strain) showed no resistance to Cr(VI), there are some microorganisms in this genus, isolated from tannery wastes, in which a capacity to reduce chromate ions has been demonstrated, such as the *M. testaceum* strain B-HS2 [53], the *M. metallidurans* strain TL13 [54] and *M. paraoxydans* [55]. On the other hand, our isolate, QRePLB02 (*Glutaminicibacter creatinolyticus*), showed resistance to 50 mg.L^{-1} Cr(VI), like the QRePLB33 strain (similar to *Brachybacterium paracongromeratum*, although the other strain of this species, QRePLB21, showed no resistance to Cr(VI)). The *Glutaminicibacter* genus was proposed based on a review of the *Arthrobacter* genus [56], which contains some members identified as resistant to metals. For example, cell biomass of *A. protophormia* was indicated as an efficient adsorbent of Pb(II) and Cd(II) ions [57,58]. With respect to chromium, the *A. mysoren* strain AHA was identified as a chromium-resistant bacterium [59], while the *G. nicotianae* strain MSSRF PD35 had its growth completely inhibited by the addition of chromium (50 mg.L^{-1}) [60]. Concerning the *B. paracongromeratum* genus, the strain MSA21 was shown to be resistant to metals and capable of producing a biosurfactant and increased surfactant production in culture supplemented with a pre-treated tannery effluent by 60% [61]. Another example is the *B. paraconglomeratum* ER41 strain, a bacterium isolated from soil taken from an abandoned lead and iron mine in the region of Taza, Morocco [62], which showed high Cr(VI) tolerance (900 mg.L^{-1}), removing up to 200 mg.L^{-1} of the metal completely after 120 h of incubation. Finally, two of our isolates belong to the genus *Cellulosimicrobium* (QReMLB55A and QRePRA55). Out of these, the strain QReMLB55A (similar to *C. aquatile*) presented resistance at up to 400 mg.L^{-1} of Cr(VI), and, in media containing 200 mg.L^{-1} $K_2Cr_2O_7$, decreased 94% of the Cr(VI) after 7 days. The behavior of this isolate in liquid medium amended with Cr(VI) was similar to the one verified in other studies carried out using microorganisms similar to *Cellulosimicrobium* sp. For example, Bharagava and Mishra, in 2018 [63], observed a Cr(VI) reduction of up to 100% after 96 h; Rehman and Faisal, in 2015 [64], showed a strain of *C. cellulans*, CrK16, to be capable of reducing 41% of Cr(VI) at 200 mg.L^{-1} and 18% at 400 mg.L^{-1}; and Karthik et al. (2017) [65] showed a strain of *C. funkei*, AR8, to have the ability to diminish up to 100% of Cr(VI).

Also, it was verified that some strains of this genera (*C. funkei* AR6 and *C. funkei* AR8) can promote plant growth [66–68].

Concerning the strains belonging to the *Bacilli* class (QRePLB13, QRePLB31 and QReMLB55B, similar to *Staphylococcus equorum*, and QRePLBC34, similar to *S. epidermidis*), it is known that such microorganisms, whose ability to form spores and survive in inactive life stages allows them to exist even in very harsh environmental conditions, e.g., in extremely dry environments and under exposure to solar radiation and high concentrations of metals such as those present in mine residues, are omnipresent in extreme sites. With respect to *S. equorum* specifically, strains of this species were isolated from a site contaminated by metals [69], and Cr(VI) resistance was demonstrated for some strains [70].

It is worth reinforcing the importance of working with isolated microorganisms when one is searching for a new bioprocess. While the final bioprocess will perhaps be more operable working with natural or artificial consortia, in the prior experiments, it is easier to carry them out and to analyze the data when working with isolates (in pure cultures). Extremophiles, for example, which have great biotechnological potential for the transformation of xenobionts, are frequently poly-extremophiles (existing under exposure to alkaline pHs, high salinity, radiation and metals)—a condition that needs to be verified. Furthermore, assays with axenic cultures allow straightforward identifications and control of the experimental variables, as well as understanding of mechanisms and system failures, enabling optimal control and the application of modifications when necessary. Axenic cultures also allow for studying the metabolic and physiological mechanisms related to the performed bioprocess. On the other hand, it is difficult to maintain wild strains in axenic cultures, especially extremophiles such as those described in the present work. Thus, accessing microorganisms for axenic cultures is still one of the challenges of current microbial ecology, and even more so for extremophiles [71]. Although metagenomic studies have already provided some insight, we do not know these microorganisms' exact nutritional needs or the best physico-chemical parameters for their growth, in addition to the limitations due to their low relative abundance (usually only the major populations are captured).

In summary, in this work, several Cr(VI)-resistant bacterial strains from an industrial residue were isolated. From these, the QReMLB55A strain showed an impressive ability to diminish Cr(VI) in culture media. Other isolates, like the QRePLB0 strain, although showing minor resistance to Cr(VI) compared to QReMLB55A and QReMLB33A, are also interesting for a chromium transformation process because of their ability to grow on biofilms, together with their ability to diminish Cr(VI). The ability of these strains to reduce Cr(VI) to Cr(III) remains to be determined, but their ability to reduce concentrations of Cr(VI) has been demonstrated. In general, these results indicate the potential of extreme environments, such as the lixiviates of industrial cores, in searching for microorganisms with applied biotechnological potential, particularly in the biotransformation of metals. Constructing consortia on the basis of these results will be the next challenge. This challenge will be easier to overcome because the isolated strains are extremophile microorganisms (haloalkaline), enabling their use in the search for a bioprocess for transforming Cr (VI) using mining effluents with high pHs. It will be quite possible to use a mixture of several of these strains in bioprocesses/bioreactors rather than use them directly in tailing wastes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4020046/s1, Table S1: Overview of bacterial bioprospection. Colonies obtained and strains isolated.

Author Contributions: E.M.S.B. and R.G. designed and conceptualized the research. E.M.S.B., R.G., M.G.-U. and M.M.M. acquired funding. E.M.S.B., P.A.M.-A., M.E.S.-V., R.E.P.-H., C.G., J.C.V.-N. and P.C. performed the experiments. E.M.S.B., P.A.M.-A., M.E.S.-V. and C.A.C. performed the data analysis. E.M.S.B., R.G., P.A.M.-A. and M.E.S.-V. conducted the major part of the investigation. E.M.S.B., R.G., C.A.C. and P.A.M.-A. wrote the original draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: We confirm that all sequence data have been deposited in a public deposit (GenBank): high-throughput sequences were registered under accession number PRJNA933578, while the 16S rDNA sequences (determined by the Sanger approach) have been assigned the accession numbers OQ456364 to OQ456381. Other data not shown in the manuscript are included in Table 1.

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