New Plant Growth-Promoting, Chromium-Detoxifying Microbacterium Species Isolated From a Tannery Wastewater: Performance and Genomic Insights

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Hexavalent chromium [Cr(VI)], widely generated by tannery activities, is considered among the most toxic substances and causes a serious damage for the environment and for human health. Interestingly, some microorganisms have a potential of bioremediation of chromium-contaminated wastewaters and soils through the reduction of Cr(VI) (soluble and harmful form) into Cr(III) (stable and non-toxic form). Here, we present the full genome sequence of a novel heavy-metal-resistant, plant growth-promoting bacterium (PGPB), Microbacterium metallidurans TL13, which was isolated from a Tunisian leather industry. The strain TL13 was resistant to many heavy metals, such as chromium, copper, nickel, cobalt, and arsenic. The 50% TL13 growth inhibitory concentration (IC50) values of HgCl2, CoCl2, K2Cr2O7, CuSO4, NiCl2, FeSO4, and Na2HAsO4 are 368, 445, 676, 1,590, 1,680, 4,403, and 7,007 mg/L, respectively, with the following toxicity order: HgCl2 > CoCl2 > K2Cr2O7 > CuSO4 > NiCl2 > FeSO4 > Na2HAsO4. This new strain was also able to promote the growth of the hybrid tomato (Elika F1) under chromium metal stress. Its whole genome sequence length was estimated to be 3,587,460 bp (3,393 coding sequences) with a G+C content of 70.7%. Functional annotation of the genome of TL13 revealed the presence of open reading frames (ORFs) involved in adaptation to metal stress, such as the chromate transport protein, cobalt–zinc–cadmium resistance protein, copper resistance protein, copper responsive transcriptional regulator, multidrug resistance transporters, arsenical resistance operon repressor, arsenate reductase, arsenic resistance protein, mercuric resistance operon regulatory protein, mercuric ion reductase, and organomercurial lyase. Moreover, genes for the production of glutathione peroxidase, catalase, superoxide dismutase, and thioredoxin reductase, which confer a higher tolerance to oxidative/metal stresses, were identified in TL13 genome. In addition, genes for heat shock tolerance, cold shock tolerance, glycine-betaine production, mineral phosphate solubilization, ammonia assimilation, siderophores,
INTRODUCTION

The leather industry plays an important role in the world’s economy. This industry uses a raw material that is originally a livestock coproduct. Leather making consists of converting putrescible hides and skins into a more resistant commercial leather (Fang et al., 2017). In fact, this process comprises three main operations: (i) beamhouse operation, which consists of several steps aimed to eliminate all components other than collagen; (ii) tanning operation, which provides resistant leather using chromium; and (iii) finishing operation, which consists of giving the treated leather its last aspect ready for commercialization. During the process of leather making, several baths containing chemicals harmful to the environment are used in order to treat hides and skins. Indeed, spilled water has a significant pollutant load (Thanikaivelan et al., 2004). Around 30 m$^3$ of effluents is generated for every ton of hides and skins, which have significant impacts on environments and human health due to the presence of heavy metals especially chromium (Suthanthararajan et al., 2004). The toxic form of this metal [Cr(VI)] seems to be the cause of the contamination of aquatic and terrestrial ecosystems and also the cause of several diseases such as cancer, particularly lung cancer (Pradhan et al., 2016).

Despite its toxicity (Balmer, 2018), hexavalent chromium [Cr(VI)] could be removed with an ecofriendly method using microorganisms, a process called bioremediation. Many studies have proven the effectiveness of bacteria and fungi for the reduction of Cr(VI) in chromium-contaminated effluents and soils and the ability to transform it into Cr(III) (Chai et al., 2019). Several bacterial strains are known as potential chromium reducers, such as Enterobacter sp. HU38, Pantoea stewartii ASI11 (Ashraf et al., 2018), Cellulosi microbium sp. (Bharagava and Mishra, 2018), Brucella sp. K12 (Maqbool et al., 2015), Escherichia coli (Learman et al., 2011), Amphibacillus sp. KSUCr3 (Ibrahim et al., 2012), Pseudomonas aeruginosa CCTCC AB93066 (Kang et al., 2014), and Bacillus firmus KUCr1 (Sau et al., 2010). Microbacterium strains, being able to tolerate or resist various heavy metals such as chromium, arsenic, nickel, and cadmium, have been found to be suitable bacteria for bioremediation (Soni et al., 2014; Gutiérrez et al., 2015; Wu et al., 2015; Lun et al., 2016; Elahi et al., 2019; Kumar and Saini, 2019). Microbacterium species have been recently described as efficient plant growth-promoting bacterium (PGPB) although under biotic and abiotic stresses (Manzanera et al., 2015; Jana et al., 2017; Passari et al., 2019a).

In recent years, several bacterial whole genomes were sequenced in order to identify loci involved in metal resistance, particularly chromium reduction and resistance. Genomic annotations indicated that chrR and yieF are implicated in the mechanism of chromate reduction. In fact, some bacterial strains, like E. coli, use YieF reductase to reduce Cr(VI) into Cr(III). Other species, like Pseudomonas putida, are known for their chromium reduction through the ChrR reductase (Ramirez-Diaz et al., 2008; Learman et al., 2011; Ahemad and Kibret, 2014; Henson et al., 2015; Baldiris et al., 2018). Otherwise, M. laeuviformans strain OR221 was able to resist some heavy metals, but the annotation of its genome after sequencing suggested the absence of both ChrR and YieF (Brown et al., 2012). In this paper, we describe the identification of a novel heavy-metal-resistant, PGPB M. metallidurans TL13, isolated from a tannery effluent. TL13 could prove to be a suitable candidate for the bioremediation of heavy-metal-contaminated soils and wastewaters and also could be used as a biofertilizer due to its traits. The complete genome sequencing and annotation of TL13 were conducted, and experimental data were collected, in order to better understand its mechanisms of chromium resistance and reduction and genes involved in stress resistance and PGP activities.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals and reagents used in this study are of pure analytical grade and available commercially.

Isolation and Characterization of a Novel Heavy-Metal-Resistant Bacterium

In this study, a heavy-metal-resistant bacterium strain TL13 was isolated from tannery (TMM) wastewater using trypptic soy agar (TSA). Water content, total organic carbon (TOC), organic content, and total heavy metal content from the sludge generated in TMM wastewater treatment plants were determined by gravimetry, calcination, colorimetry, and inductively coupled plasma atomic emission spectroscopy (ICP-OES) according to the standards ISO 12880: 2000, Rodier, NF ISO 14235: 1998 and ISO 1188: 2007, respectively (Zeng et al., 2016).

Analysis of bacterial resistance to heavy metals was carried out on TSA medium supplemented with increasing concentrations (50–2,500 mg/L) of iron sulfate (FeSO$_4$), copper sulfate (CuSO$_4$), cobalt chloride (CoCl$_2$), disodium arsenate (Na$_2$ HAsO$_4$), potassium dichromate (K$_2$Cr$_2$O$_7$), mercury chloride (HgCl$_2$), exopolysaccharides, polyketides, and lytic enzymes (cellulase, chitinase, and proteases) production that enable bacteria to survive biotic/abiotic stress and to promote plant growth and health were also revealed. Based on genome analysis and experimental approaches, strain TL13 appears to have evolved from various metabolic strategies and could play a role in ensuring sustainable environmental and agricultural systems.

Keywords: actinobacterium, bioremediation, genome annotation, heavy metals, plant growth promotion, tannery wastewater
and nickel chloride (NiCl₂). The spot inoculation method was used to involve in triplicate 10 µl of bacterial culture suspension (10⁸ CFU/mL) (Fu et al., 2016). Bacterial cultures were incubated for 24 h at 30°C to determine minimum inhibitory concentrations (MIC) (Davis, 2005). A positive control was used by inoculating bacterial culture on agar plates without metal. The half maximal inhibitory concentration (IC₅₀) was determined using experiments conducted in tryptic soy broth (TSB) medium at 30°C with shaking at 120 rpm (Fassy et al., 2017). Bacterial growth was measured after 24 h on a UV-visible (UV-Vis) spectrophotometer at 600 nm. All experiments (solid and liquid) were performed under alkaline conditions (pH 9). Metal reduction was determined by inductively coupled plasma mass spectrometry. Bacterial cell free extracts were recovered after a 10 min of centrifugation at 10,000 rpm and then filtered using 0.22 µm Whatman filter paper.

**Plant Growth-Promoting Activities**

**In vitro Screening of TL13 for Its Plant Growth-Promoting Activities**
The screening of TL13 strain for various in vitro plant growth-promoting activities was performed by adopting standard methods (Hassen et al., 2018). Inorganic phosphate-solubilizing activity and growth on nitrogen free medium was estimated by the method of Pikovskaya (1948) and Jensen (1942), respectively. To detect the ability of TL13 strain to produce siderophore, chrome azurol sulfonate (CAS) agar solid medium was used (Alexander and Zuberer, 1991). Cellulase activity was tested on minimal medium amended with 1% carboxymethyl cellulose (CMC) (Souii et al., 2018). The protease production was screened by skim milk agar (Ouertani et al., 2018). The ability of TL13 to produce indole-3-acetic acid (IAA) and exopolysaccharides (EPS) was assessed as indicated by Penrose and Glick (2003) and Naseem et al. (2018), respectively.

**In vivo Plant Growth Promotion Assays**

**In vivo** plant growth promotion and tomato seed germination assays were performed as described by Passari et al. (2019a) with slight modification. Elica F1 tomato seeds were surface sterilized with 1% sodium hypochlorite solution for 20 min and subsequently rinsed three times with sterile distilled water. Disinfected Elica F1 tomato seeds were immersed for 2 h in a TL13 bacterial suspension (10⁸ CFU/mL). Seeds immersed in sterile distilled water served as a control. A sterile Whatman paper was soaked in a chromium solution at different concentrations (0, 100, and 500 mg/L) and deposited in Petri dishes. Seeds were then gently deposited on Whatman filter paper under appropriate conditions, incubated in darkness at 30°C for 8 days and irrigated every day with sterile distilled water. Germination percentages were calculated by dividing total germinated seeds to total number of seeds that were planted (Passari et al., 2019a,b). The experiment was repeated three times using 20 Elica F1 tomato seeds per germination condition. Different parameters, including shoot length, shoot fresh, and dry weights, were measured.

**Antibiotic Sensitivity Test**
The Kirby–Bauer test, known as the disk-diffusion method, was performed to determine antibiotic sensitivity of TL13 strain (Bauer et al., 1966). A pure culture of TL13 strain was diluted using saline solution and adjusted to 10⁸ CFU/mL. Bacterial inoculum was then streaked on Mueller Hinton agar using a sterile swab. Antibiotics used were cefoxitin (FOX, 30 µg), pristinamycin (PTN, 15 µg), neomycin (NE, 30 µg), rifampicin (RD, 30 µg), norfloxacin (NOR, 5 µg), streptomycin (S, 10 µg), erythromycin (E, 15 µg), cefotaxime (CTX, 30 µg), tetracycline (TET, 30 µg), vancomycin (VAN, 30 µg), ceftazidime (CAZ, 30 µg), spiramycin (SP, 100 µg), and oxytetracyclin (OT, 30 µg). Antibiotic impregnated disks were placed on agar plates and incubated for 72 h at 30°C. Bacterial resistance or sensitivity was determined by measuring the zone of inhibition.

**Genome Sequencing, Assembly, and Annotation of TL13 Strain**
Genomic DNA extraction of TL13 strain was performed by sodium dodecyl sulfate–protease K treatment (Neifar et al., 2019). Purified genomic DNA was sequenced on an Illumina MiSeq platform (MRDNA, USA) yielding 2 × 250 bp paired-end reads. The Prinseq-lite software was used for trimming the 4,320,030 paired reads by discarding low-quality sequences with a score lower than 23 (Mahjoubi et al., 2019). Genome assembly was then performed using the SPAdes algorithm (Bankevich et al., 2012). Genome annotation was done using the Integrated Microbial Genomes/Expert Review (IMG/ER) database (Markowitz et al., 2009) and the Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al., 2008). Annotated protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) (Galperin et al., 2015) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999) databases.

**Phylogenetic Analysis**
The 16S ribosomal DNA (rDNA) gene sequence was annotated within the genome and compared to sequences of bacterial strains from the NCBI and EzBioCloud databases (Kim et al., 2012). The phylogenetic tree was inferred by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA 6) software (Kumar et al., 2016). A 95–96% average nucleotide identity (ANI) value was used as a genomic measure for prokaryotic species delineation (Richter et al., 2016) and was measured using the JSpecies Web Server (JSpeciesWS) available at http://jspecies.ribohost.com/jspeciesws. A circular genome map was drawn using CGView software (Grant and Stothard, 2008).

**Structural Analysis**
The structures of enzymes involved in chromate reduction were predicted by the online structure prediction tool, Iterative Threading ASSEmbly Refinement server (I-TASSER) (Yang and Zhang, 2015; Ouertani et al., 2018). The confidence score (C score) was used to identify the best generated model, and MolProbity was applied to evaluate the refined model coordinates.
TABLE 1 | Physicochemical characteristics of a TMM raw sludge sample.

| Parameters          | Values   |
|---------------------|----------|
| Moisture (%)        | 68.2     |
| Organic content (g/kg-dry sludge) | 601      |
| TOC (mg/kg-dry sludge) | 353      |
| Cadmium (mg/kg-dry sludge) | <0.6    |
| Cobalt (mg/kg-dry sludge) | <5.0     |
| Copper (mg/kg-dry sludge) | 7.11     |
| Iron (mg/kg-dry sludge) | 16.4 x 10³ |
| Lead (mg/kg-dry sludge) | 7.52     |
| Nickel (mg/kg-dry sludge) | 47.05    |
| Zinc (mg/kg-dry sludge) | 561.7    |
| Chromium (mg/kg-dry sludge) | 26.2 x 10³ |
| Arsenic (mg/kg-dry sludge) | <1.0     |
| Selenium (mg/kg-dry sludge) | 4.6      |
| Antimony (mg/kg-dry sludge) | 302.7    |
| Tin (mg/kg-dry sludge) | <1.0     |
| Silver (mg/kg-dry sludge) | 12.86    |
| Boron (mg/kg-dry sludge) | <1.0     |
| Baryum (mg/kg-dry sludge) | 33.04    |
| Molybdenum (mg/kg-dry sludge) | <1.0     |
| Titanium (mg/kg-dry sludge) | 17.47    |
| Mercury (mg/kg-dry sludge) | <1.0     |

(Davis et al., 2007). Enzyme models were assessed using COACH software (Yang et al., 2013). A final analysis with CCP4mg molecular-graphics software was performed to validate the quality of the structures (McNicholas et al., 2011).

Statistical Analysis

Experimental results were expressed as the mean value of three independent replicates ± the standard deviation (SD). Data were analyzed using one-way ANOVA with post-hoc Duncan’s tests (SPSS 16.0 for Windows, SPSS Inc., USA). Statistical significance was determined as p < 0.05.

Nucleotide Sequence Accession Number

The Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession SZZQ00000000. The version reported in this work is SZZQ01000000 (https://www.ncbi.nlm.nih.gov/nuccore/SZZQ00000000).

RESULTS

Characterization of a Heavy-Metal-Resistant, Plant Growth-Promoting Actinobacterium Isolated From a Tannery Wastewater

The physicochemical composition of the sludge collected from TMM industry is shown in Table 1. The content of chromium in sludge exceeds the thresholds for agriculture, according to national and international standards (Jakov, 2005). Qualitative analyses indicated that TL13 strain has the capacity to grow in the presence of K₂Cr₂O₇ concentrations that could exceed 1,000 mg/L (Figure 1A). The bacterial growth was followed in the presence of K₂Cr₂O₇ with variable concentrations ranging from 50 to 2,500 mg/L on TSB medium. As shown in Figure 1B, TL13 strain tolerated more than 1,000 mg/L of K₂Cr₂O₇. The TL13 IC₅₀ of K₂Cr₂O₇ is equal to 676 mg/L (Figure 1C). ICP-MS analysis revealed that TL13 strain was able to reduce 71.68% of chromium [Cr(VI)] with initial concentration of 500 mg/L (Figure 1D). Heavy metal resistance experiments indicated also that TL13 strain has the capacity to grow under high concentrations of FeSO₄ and Na₂HAsO₄ (up to 2,500 mg/L), NiCl₂ and CuSO₄ (up to 1,000 mg/L), and CoCl₂ and HgCl₂ (up to 500 mg/L). IC₅₀ values of HgCl₂, CoCl₂, K₂Cr₂O₇, CuSO₄, NiCl₂, FeSO₄, and Na₂HAsO₄ are 368, 445, 676, 1,590, 1,680, 4,403, and 7,007 mg/L, respectively (Table 2).

The in vitro study of plant growth-promoting activities showed that TL13 strain was able to fix atmospheric nitrogen and to solubilize phosphate. The strain TL13 has also the capacity to produce siderophores, EPS, and cellulase as materialized by the presence of transparent halo around the colony in the corresponding culture media. Furthermore, the development of a pink color following the addition of Salkowski’s reagent to the culture supernatant showed the capacity of TL13 to produce IAA (Figure S1). The results of antibiograms (Figure S2) showed the resistance of TL13 strain to cepotaxime, ceftazidime (antibiotics of β-lactam group), and norfloxacin (antibiotic of fluoroquinolone group). M. metalldurans TL13 was sensitive against all other tested antibiotics. Tomato in vivo experiments revealed positive effect of the TL13 strain on seeds germination under chromium stress. In fact, with simple visual inspection, the toxic effect of high levels of chromium and the promoting effect of TL13 on seeds germination were well distinguishable (Figure 2A). Tolerance to higher chromium levels was observed with TL13-treated seeds compared to control seeds since a significant increase in shoot fresh and dry weights and shoot length were recorded (Figures 2B–D).

Phylogenetic Analysis and Genomic Insights Inferred From TL13 Genome Sequence

Based on the proposed rule of using 16S rDNA sequence similarity threshold value of 98.65% for species delineation (Kim et al., 2014), TL13 and Microbacterium proteolyticum (RZ36) should belong to the same species as their 16S rDNA sequences exhibited an identity of 99.3%. However, this taxonomic result was not corroborated by the phylogenetic tree (Figure 3) as both strains are not sister taxa and do not form a distinct clade for M. proteolyticum species. The previously mentioned taxonomical 16S rDNA value has been equated to the species delineation threshold ANI values of 95–96% [DNA–DNA hybridization (DDH) value of 70%] (Richter and Rosselló-Móra, 2009). TL13 exhibited 82.4 and 75.2% ANI with Microbacterium enclense NIO-1002 and Microbacterium hominis TPW29, respectively, based on JSpeciesWS. Consequently, TL13 should not belong neither to M. enclense nor to M. hominis species. Accordingly, TL13 is a novel species within the genus Microbacterium,
for which the name *M. metallidurans* is proposed. Figure 4 shows the graphical representation map of the genome of TL13 strain compared with the genomes of *M. enclense* NIO-1002 and *M. hominis* TPW29. The complete genome of *M. metallidurans* TL13 measured 3,587,460 bp with 70.70% G + C content (Table 3). *De novo* assembly of TL13 strain genome sequencing data is represented by 5 contigs and 3,393 coding sequences.

Based on the annotation of the RAST pipeline, TL13 genes were classified into 380 subsystems revealing its high metabolic diversity. Analysis of the predicted protein sequences showed that most of the annotated genes were involved in carbohydrates.
(399), amino acids and their derivatives (354), cofactors, vitamins, prosthetic groups, pigments (214), protein metabolism (217), fatty acids, lipids and isoprenoids (129), cell membrane and capsule (91), DNA (88) and RNA (85) metabolism and response to stresses (74), virulence, defense (55), respiration (55), metabolism of aromatic compounds (47), and phosphorus metabolism (40).

Analysis of *M. metallidurans* TL13 genome revealed the presence of several genes contributing directly or indirectly to PGP activities (Table 4). Indeed, the annotation study showed the presence of genes involved in the solubilization of inorganic phosphate, assimilation of ammonia, production of levan (EPS), synthesis of IAA, siderophores and polyketides, as well as genes encoding the key cell-wall-degrading enzymes (Table 4).

The genome of *M. metallidurans* TL13 contains 32 genes related to heavy metal resistance. Based on genomic analyses, enzymatic and non-enzymatic mechanisms could be proposed to explain hexavalent chromium resistance and removal by this strain (Figure 5).

The first chromium resistance mechanism to mention is the chromate transporter protein (ChrA) encoding by fig|69370.7.peg.2330 gene and localized in contig 29. The predicted model for ChrA indicated that the enzyme is composed of seven α-helices (α1, Gly24–Val37), α2 (Asp45–Ala62), α3 (Ser64–Ala75), α4 (Phe77–Thr88), α5 (Ser91–Ala97), α6 (Gly101–Leu104), α7 (Gly107–Gly131), α8 (Asp139–Ala159), α9 (Gly164–Ala180), α10 (Gly192–Leu209), α11 (Ala227–Glu251), α12 (Ser259–Val273), α13 (Pro276–Gly285), α14 (Val295–Val303), α15 (Leu307–Leu323), α16 (Gly335–Leu348), α17 (Pro360–Phe363), α18 (Ala366–Phe376), and α19 (Val384–Leu396) (Figure 5B).

The second gene potentially involved in the bacterial chromium reduction by TL13 strain is the superoxide dismutase with a molecular weight of 23,177.87 Da, encoded by the gene fig|69370.7.peg.2482. The superoxide dismutase predicted model indicated that this enzyme belongs to the metalloenzyme family (EC1.15.1.1), which is composed of seven α-helices (α1, Thr23–Lys53; α2, Arg61–Thr81; α3, Asp94–Asp100; α4, Asp107–Gly119; α5, His164–Tyr170; α6, Ala175–Asn183; and α7, Asp189–Arg198), three-stranded antiparallel β sheets (β1, Gly124–Asp131; β2, Asn136–Phe143; and β3, Val154–Asp160), and loops joining these secondary structure elements. The monomer structure of the enzyme contains two domains: the rod-shaped N-terminal domain (N-terminal extended region and two α-helices) and the globular (α + β) type C-terminal domain (Figure 5C).

The active site is composed of His28, His76, Asp160, and His164 (Figure 5D).

According to genome analyses, the glutathione peroxidase could also be involved in chromium reduction. This enzyme, with a molecular weight of 17,787.16 Da and an isoelectric point of 5.66, is encoded by the gene fig|69370.7.peg.2340. The gene is localized on the contig 4. The predicted model for glutathione peroxidase indicated that its structure is composed of seven β strands (β1, Pro7–Thr10; β2, Gly13–Thr17; β3, Ala26–Val32; β4, Phe57–Pro63; β5, Met91–Ser92; β6, Lys128–Phe132; and β7, Gly135–Phe140), four α-helices (α1, Gln41–Arg55; α2, Glu74–Trp85; α3, Pro104–Thr112; and α4, Pro149–Pro160), and loops joining these elements together. The structural analysis demonstrated that (i) the N-terminal end is formed by two β strands (β1 and β2), (ii) the enzyme contains central twisted β sheets (β3, β4, β5, β6, and β7) boarded by helices α1, α2, and α4 on one side and helix α3 on the other side, and (iii) the C-terminal end is composed of helix α4, which is nearly parallel with the α1. The model contains 161 residues from Met1 to His161, and the amino acid residues of the catalytic triad are Cys36, Gln70, and Trp124 (Figure 5E).

The TL13 genome analysis revealed also the presence of three genes encoding thioredoxin reductases (fig|69370.7.peg.1744 and fig|69370.7.peg.1847 localized on the contig 29 and fig|69370.7.peg.2708 localized on the contig 45). The predicted model for the thioredoxin reductase encoded by gene (fig|69370.7.peg.1847) indicated that the structure is composed of seven α-helix α1 (Gly10–Met23) α2 (Leu57–Ala67), α3 (Ala119–Arg124), α4 (Gly134–Phe137), α5 (Pro149–Val161), α6 (Phe210–214), and α7 (Leu256–Pro277) and 15 β strands β1 (Ala4–Ile6), β2 (Thr27–Leu29), β3 (Val71–Ile73), β4 (Ser79–Ala82), β5 (Val88–Val92), β6 (Glu95–Ala99), β7 (Tyr101–Leu104), β8 (Val126–Ala127), β9 (Ala143–Val144), β10 (Val166–Val167), β11 (Val173–Arg175), β12 (Thr186–Leu181), β13 (Gly190–Thr192), β14 (Ala198–Phe199), and β15 (Leu238–Ala239). The structure is composed of the flavin adenine dinucleotide (FAD)-binding domain (Ser32, Glu34, Tyr35, Val40, Ala43, Val 78, Thr106, Gly 107, Cys 132, Arg 207, Thr 255, Leu 256) and the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-binding domain (Pro114, Ala147, Ser148, His 150, Lys153, Val203, His 248) (Figure 5F).

The predicted model of the thioredoxin indicated that the N-terminal domain is composed of three β strands β′ (Ala17–Val18), β′′ (Arg25–Arg26), and β′′′ (Gln28–Gly29). The central structure is composed of five β strands [β1 (Glu38–Ile39), β2 (Thr55–Ser60), β3 (Arg85–Asp90), β4 (Thr108–Asp113), and β5 (Gly116–Phe122)] surrounded by four α-helices (α1 (Pro42–Leu50), α2 (Arg66–Asp80), α3 (Leu91–His100), and α4 (Gly127–Ala143). It displays a strictly conserved CXXC catalytic active-site motif (Figure 5G). In addition to the abovementioned mechanisms, *M. metallidurans* TL13 can protect itself from the infiltration of toxic chromium ions by covering its peripheral surface with a shield of levan, a fructose homopolysaccharide, which can be synthesized by levan suscace
FIGURE 2 | Effect of TL13 inoculation on the germination of tomato seeds under 0, 100, and 500 mg/L of Cr(VI). (A) View of the plantlets after 8 days of germination; (B) shoot fresh weight; (C) shoot dry weight; (D) shoot length. Values are means of \( n = 3 \) per treatment; for each treatment, 20 tomato seeds were considered; error bars represent standard deviation. For each parameter, panels with different letters indicate significant differences at \( P < 0.05 \) (ANOVA, Duncan post-hoc test).

**DISCUSSION**

Bacteria of the genus *Microbacterium* (phylum of Actinobacteria) include more than 110 cultivable species (http://www.bacterio.net/microbacterium.html) isolated from aquatic and terrestrial ecosystems and from food and clinical samples. Figure S3 illustrates the microbial world (according to the Gold and DSMZ databases) with each point representing a type of bacterial strain, and if applicable, the associated ongoing sequencing projects (colored red). As shown in Figure S3, many taxonomic groups still need to be explored at the genomic level and, in particular, the *Microbacterium* group for which only few genomes are available (https://microbial-earth.namesforlife.com/). Several *Microbacterium* members have been isolated from extreme environments (Li et al., 2008) or contaminated with organic pollutants (Schippers et al., 2005) and metals such as uranium (Reardon et al., 2008; Gallois et al., 2018), plutonium and iron(III) (John et al., 2001), arsenic (Cai et al., 2009), and chromium (Pattanapipitpaisal et al., 2001; Humphries et al., 2005).

There are only few studies reported in the literature on the predominance and ecological importance of bacterial populations of the genus *Microbacterium* as PGPB (Alves et al., 2015; Gao et al., 2017). Although such bacteria directly stimulate...
plant growth by increasing soil nutrient uptake, inducing and producing plant growth regulators, and activating induced resistance mechanisms in plants, special attention has recently been given to bioremediation of organic and inorganic pollutants by PGPB (Bishnoi, 2015). Therefore, the present study aimed to characterize a new heavy-metal-resistant PGPB isolated from a Tunisian tannery wastewater for potential application as a green and sustainable tool for decreasing accumulation of metals in industrial sludges and wastewaters and in plant rhizosphere and tissues.

The actinobacterium TL13 exhibited resistance to all of the tested heavy metal ions. The half inhibitory concentration values of mercury, cobalt, chromium, copper, nickel, iron, and arsenic were 368, 445, 676, 1,590, 1,680, 4,403, and 7,007 mg/L, respectively, and their toxic order was HgCl₂ > CoCl₂ > K₂Cr₂O₇ > CuSO₄ > NiCl₂ > FeSO₄ > Na₂HAsO₄. TL13 possesses in vitro PGP traits including nitrogen fixation and inorganic phosphate solubilisation as well the production of PGP metabolites like IAA, siderophore, EPS, and cellulases. The growth-promoting capacity of TL13 strain was confirmed in vivo by measuring shoots length and fresh and dry weight of tomato seedlings in the presence of increasing chromium concentrations. Inoculation of TL13 to tomato increased plant growth by 22.77% of shoot length, 12.79% of fresh weight, and 34.62% of dry weight.

The whole genome of TL13 strain was sequenced and annotated in order to provide information on genomic elements related to heavy metal resistance and plant growth-promoting traits. The genome consists of 3,587,460 bp with an average GC content of 70.7% and a total of 52 genes encoded RNAs. Of the 3,393 genes predicted, 2,425 were identified as genes encoding proteins (genes had a specific function) and 968 genes (28.53%) were classified as hypothetical proteins (genes without function prediction). The ANI values between the strain TL13 and the
### TABLE 4 | Rapid Annotations using Subsystems Technology (RAST) predicted proteins potentially associated with plant growth promotion traits within the genome of *Microbacterium metallidurans* TL13.

| Protein Name                                      | Start (aa) | Stop (aa) | Length (pb) | Feature ID                  |
|---------------------------------------------------|------------|-----------|-------------|-----------------------------|
| **Ammonia assimilation**                          |            |           |             |                             |
| Ammonium transporter                               | 28,180     | 26,909    | 1,272       | fig|69370.7.peg.113              |
| Glutamine synthetase type I (EC 6.3.1.2)          | 66,639     | 70,063    | 1,425       | fig|69370.7.peg.148              |
| Glutamate-ammonia-lyase adenyltransferase (EC 2.7.7.42) | 77,333     | 75,340    | 2,994       | fig|69370.7.peg.153              |
| Glutamine synthetase type I (EC 6.3.1.2)          | 79,681     | 78,341    | 1,341       | fig|69370.7.peg.154              |
| Glutamate synthase [NADPH] small chain (EC 1.4.1.13) | 256,192    | 254,726   | 1,467       | fig|69370.7.peg.2590             |
| Glutamate synthase [NADPH] large chain (EC 1.4.1.13) | 260,762    | 258,185   | 4,578       | fig|69370.7.peg.2591             |
| **Inorganic phosphate assimilation and solubilization** |           |           |             |                             |
| Probable low-affinity inorganic phosphate transporter | 87,776     | 86,481    | 1,296       | fig|69370.7.peg.2090             |
| Phosphate regulon transcriptional regulatory protein PhoB (SphR) | 11,84,663  | 11,85,424 | 762         | fig|69370.7.peg.2090             |
| Phosphate ABC transporter, periplasmic phosphate-binding protein PhoP (SphP) | 750,067    | 749,384   | 684         | fig|69370.7.peg.2090             |
| Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3) | 751,281    | 750,064   | 1,218       | fig|69370.7.peg.2090             |
| Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3) | 885,447    | 887,435   | 1,989       | fig|69370.7.peg.2090             |
| **Auxin synthesis**                               |            |           |             |                             |
| Tryptophan synthase alpha chain (EC 4.2.1.20) involved in auxin synthesis | 262,821    | 261,830   | 792         | fig|69370.7.peg.2593             |
| Tryptophan synthase beta chain (EC 4.2.1.20) involved in auxin synthesis | 263,952    | 262,618   | 1,335       | fig|69370.7.peg.2594             |
| Anthranilate phosphoribosyltransferase (EC 2.4.2.18) | 68,751     | 69,812    | 1,062       | fig|69370.7.peg.2594             |
| phosphoribosylanthranilate isomerase (EC 5.3.1.24) | 262,821    | 261,830   | 792         | fig|69370.7.peg.2593             |
| Monoamine oxidase (1.4.3.4)                       | 933,234    | 931,207   | 2,028       | fig|69370.7.peg.2018             |
| **Siderophore synthesis**                         |            |           |             |                             |
| Siderophore -interacting protein                  | 179,305    | 180,231   | 927         | fig|69370.7.peg.2511             |
| ABC-type Fe^{3+}-siderophore transport system, permease 2 component | 777,693    | 778,700   | 1,008       | fig|69370.7.peg.2083             |
| ABC-type Fe^{3+}-siderophore transport system, ATPase component | 181,137    | 180,313   | 8,25        | fig|69370.7.peg.2512             |
| ABC-type Fe^{3+}-siderophore transport system, permease 2 component | 182,192    | 181,134   | 1,059       | fig|69370.7.peg.2512             |
| ABC-type Fe^{3+}-siderophore transport system, permease 2 component | 183,235    | 182,189   | 1,047       | fig|69370.7.peg.2512             |
| Putative ABC iron siderophore transport, fused permease and ATPase domains | 405,489    | 403,672   | 1,818       | fig|69370.7.peg.3014             |
| ABC-type Fe^{3+}-siderophore transport system, permease component | 776,716    | 777,696   | 981         | fig|69370.7.peg.831              |
| Putative ABC iron siderophore transport, fused permease and ATPase domains | 587,004    | 587,765   | 762         | fig|69370.7.peg.647              |
| **Potent fungal cell-wall-degrading enzymes**      |            |           |             |                             |
| Carboxymethyl cellulase (EC 3.2.1.4)              | 694,440    | 695,393   | 954         | fig|69370.7.peg.868              |
| Serine protease, subtilase family (EC 3.4.21.-)   | 19,362     | 16,790    | 2,573       | fig|69370.7.peg.1452             |
| Putative serine protease                         | 480,754    | 479,579   | 1,176       | fig|69370.7.peg.534              |
| β-hexosaminidase (EC 3.2.1.52)                    | 602,405    | 603,559   | 1,155       | fig|69370.7.peg.3342             |
|            | 103,467    | 103,277   | 1,881       | fig|69370.7.peg.534              |
| **Exopolysaccharide synthesis**                   |            |           |             |                             |
| Levansucrase (EC 2.4.1.10)                        | 646,212    | 647,819   | 1,608       | fig|69370.7.peg.1075             |
| **Polyketide Synthesis**                         |            |           |             |                             |
| Regulator of polyketide synthase expression       | 95,550     | 96,821    | 1,272       | fig|69370.7.peg.171              |
| Malonyl CoA-acetyl carrier protein transacylase (EC 2.3.1.39) | 96,904     | 97,824    | 921         | fig|69370.7.peg.172              |
| 3-oxoacyl-[acyl-carrier-protein] synthase, KASIII (EC 2.3.1.41) | 97,821     | 98,825    | 1,005       | fig|69370.7.peg.173              |
| Acyl carrier protein                              | 99,900     | 99,148    | 249         | fig|69370.7.peg.174              |
| 3-oxoacyl-[acyl-carrier-protein] synthase, KASIII (EC 2.3.1.179) | 99,214     | 100,452   | 1,239       | fig|69370.7.peg.175              |
| Chalcone synthase (EC 2.3.1.74)                   | 912,875    | 913,969   | 1,095       | fig|69370.7.peg.956              |
FIGURE 5 | (A) Hypothetical pathways involved in chromium resistance and reduction in M. metallidurans TL13: (1) The sulfate permease allows chromium to get into bacterial cell; (2) expression of gene encoding chrome transporter ChrA (Efflux system); (3) reduction of chromium due to thioredoxin reductase and NADPH; (4) oxidative stress due to chromium reduction (Cr(V) and Cr(IV)) induced reactive oxygen species (ROS); superoxide dismutase, catalase, peroxidase, and glutathione; (5) DNA damage: expression of dna repair genes (RecA, RecG, and RecBDC); and (6) exopolysaccharides (EPS) and metal complex. Cartoon representation of 3D structure of TL13: (B) chromate transporter; (C) superoxide dismutase; (D) the active site of superoxide dismutase; (E) glutathione peroxidase with the localization of the active site; (F) thioredoxin reductase with the localization of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) domain, and (G) 3D structure of TL13 thioredoxin with the localization of the active site.
closely related strains were 76.5–82.7%, indicating that TL13 strain is a novel species of the genus Microbacterium.

Functional annotation of TL13 genome revealed the presence of genes encoding proteins involved in resistance to metal stress, such as cobalt–zinc–cadmium resistance protein, copper resistance protein, copper responsive transcriptional regulator, arsenical resistance operon repressor, arsenate reductase, arsenic resistance protein, mercuric resistance operon regulatory protein, mercuric ion reductase, and organomercurial lyase. The TL13 genome analysis revealed also the presence of different potential genes involved in chromium reduction such as chromate transporter, superoxide dismutase, glutathione peroxidase, and the thioredoxin reductase. The chrome transporter protein (ChrA) has been reported to play a crucial role in efflux of cytoplasmic chromate (Alvarez et al., 1999; Ahemad and Kibret, 2014). The participation of bacterial enzymes in the defense against oxidative stress induced by chromate represents an additional mechanism of chromium resistance (McCord and Fridovich, 1988; Ackerley et al., 2006; Ramirez-Diaz et al., 2008). The TL13 strain has the potential to have a powerful enzyme system to combat oxidative stress involving catalase (EC 1.11.1.6), glutathione peroxidase, and superoxide dismutase [Mn] (EC 1.15.1.1). The thioridoxin reductase, an Mg²⁺-dependent enzyme, is also involved in the reduction in Cr(VI) (Li and Krumholz, 2009; Collet and Messens, 2010). The two cysteines of the catalytic motif (Cys64, A65, R66, Cys67), located at the amino end of the two-helix, are the key players involved in the reduction of oxidized substrates. Chen et al. (2014) reported that the entire operon of thioredoxin of Streptomyces violaceoruber strain LZ-26-1 was upregulated under the stress of Cr(VI). This result suggests that TL13 strain can use thioredoxin pathway to reduce Cr(VI). Various oxidoreductases have been shown to reduce Cr(VI) such as ChrR from E. coli and Pseudomonas and NfsA from E. coli (Ackerley et al., 2004). Genes coding for Cr(VI)-reducing oxidoreductase were not detected on TL13 genome. This result probably shed light on novel Cr(VI) reductases within the thioredoxin pathway.

M. metallidurans TL13 was able to produce levans based on genomic signature (presence of the gene encoding levansucrase) linked to experimental evidence (mucoid phenotype on agar plate). The anionic property of the EPS enables it to trap the positively charged chromium by electrostatic interaction and hence playing a key role as biosorbents for metal remediation (Harish et al., 2012; Gupta and Diwan, 2017).

TL13 genome encodes an efficient framework for iron acquisition mediated by a siderophore-dependent pathway as similar to that of Cupriavidus metallidurans CH34 (Tables S2, S3). Iron is essential for vital processes including DNA, RNA, and protein synthesis, electron transport, cell respiration and proliferation, as well as gene expression regulation. Iron has the ability to easily acquire and lose electrons from the iron form Fe²⁺ to the iron form Fe³⁺, and vice versa. This rather unique property gives it an essential role in oxidation and reduction processes. The chemical properties of iron make it potentially toxic. When iron is in limited supply, TL13 can produce and secrete siderophores capable of chelating ferric Fe³⁺ with very high affinity. The siderophore–Fe³⁺ complexes are then recovered by the bacterium using specific membrane transporters. The dissociation of iron from its chelator generally requires a reduction in Fe²⁺ to Fe³⁺ and a structural modification of the siderophore, making it less affine for the metal (Payne et al., 2016; Li and Ma, 2017).

Besides these genes conferring resistance to metal and oxidative stresses identified in TL13 genome, the RAST analysis showed the presence of 10 genes involved in osmoadaptation, in particular in the biosynthesis of betaine and choline osmolytes, 14 proteins (operon dnaK) to fight against thermal stress, and 4 against cold (proteins from the CspA family). Analysis of the genome of M. metallidurans TL13 revealed also the presence of 118 genes involved in different pathways of central carbohydrate metabolism like the tricarboxylic acid cycle and the pentose phosphate pathway. It also indicated the presence of 43 proteins involved in anaerobic fermentation processes. The genome of M. metallidurans TL13 codes for exopolyporphosphatase (EC 3.6.1.11), inorganic manganese-dependent pyrophosphatase (EC 3.6.1.1), and low affinity inorganic phosphate carriers, which play a crucial role in phosphorus cycle by solubilizing its inorganic form that is fixed and precipitated in the soil (Al-Kaisi et al., 2013; Bhattacharyya et al., 2017). Moreover, the genome of M. metallidurans TL13 encodes all the genes necessary for ammonia assimilation and plant auxin biosynthesis (Spaepen and Vanderleyden, 2011). Several Microbacterium strains isolated from rhizospheric and non-rhizospheric environments are able to fix nitrogen, as confirmed by acetylene-reducing activity and PCR amplification of nifH gene (Giari et al., 2011). Whereas, some nitrogen-fixing bacteria are able to produce nitorgenases with iron as the only metal cofactor, other bacterial strains incorporate molybdenum or vanadium into the cofactor as well (Johnstone and Nolan, 2015). For the strain TL13, a search for nitorgenase systems merits further investigation at the genome and physiological levels.

Besides these direct PGP traits, several genes involved in the biocontrol of phytopathogens exist in the genome of M. metallidurans TL13, including genes coding for cell-wall-degrading enzymes (cellulose, β-hexosaminidase, and proteases) (Dutta and Thakur, 2017) and genes coding enzymes responsible for the biosynthesis of polyketides, a class of secondary metabolites with antimicrobial roles (Khanna et al., 2019). M. metallidurans TL13 showed resistance to β-lactams and fluoroquinolones antibiotics leading to improved chance of survival in a competitive rhizospherici soil (Bhattacharyya et al., 2017). All genomic plant growth-promoting features make TL13 strain an excellent actinobacterium for utilization in sustainable agriculture (Table 5). Overall, these PGP results were in line with those reported for Microbacterium sp. strain 3J1 (Manzanera et al., 2015), Microbacterium sp. Yaish 1 (Jana et al., 2017), and Microbacterium hydrothermale BPSAC84 (Passari et al., 2019b).

CONCLUSION

This study reports the characterization of a heavy-metal-resistant, plant growth-promoting actinobacterium, M.
TABLE 5 | Genomic features and biotechnological potential of *M. metallidurans* TL13 compared to other *Microbacterium* species.

| Strain                  | Origin                                    | Genome length (Mb) | GC (%) | Pathways and genes of interest                                                                 | Bioremediation potential                                                                 | References            |
|-------------------------|-------------------------------------------|--------------------|--------|------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|-----------------------|
| *M. oleivorans* strain Wellendorf | Hydrocarbon-contaminated soil             | 2.92               | 69.57  | 4-hydroxyphenylacetate degradation; Nitronate detoxification                                    | Environmental pollutants detoxification                                                   | Avramov et al., 2016  |
| *Microbacterium* sp. A20 | Heavy metal contaminated soil (Indiana, USA) | 3.94               | 68.53  | Co/Zn/Cd efflux system; Tolerance to antibiotics; Chromium reductase (chrR)                     | Reduction of Cr VI into Cr III; Tolerance of cobalt, cadmium, and nickel                 | Learman et al., 2019  |
| *Microbacterium* sp. K19  |                                            | 3.89               | 68.69  |                                                                                                 |                                                                                        |                       |
| *Microbacterium* sp. K21  |                                            | 3.85               | 68.33  |                                                                                                 |                                                                                        |                       |
| *M. oxydans* BEL4b       | Rhizosphere of *Brassica napus* (Belgium)  | 3.8                | 68.27  | Heavy metals resistance; Production of terpenoids Production of polyketides                     | Promoting plant growth in heavy metals contaminated soils                                | Corretto et al., 2015 |
| *M. Azadirachtae* ARN176 | Heavy metals contaminated Soil (Austria)   | 3.91               | 70.14  |                                                                                                 |                                                                                        |                       |
| *M. laevaniformans* Strain OR221 | Field Research Center (Tennessee, USA)      | 3.4                | 68     | Heavy-metal transport proteins                                                                  | Tolerance of heavy metals and acidic conditions                                           | Brown et al., 2012    |
| *M. testaceum* STL037    | Potato leaves (Japan)                      | 3.98               | 70.28  | Lactonases genes                                                                               | Biocontrol agent against phytopathogens                                                   | Morohoshi et al., 2011|
| *M. profundi* Shh49     | Sea sediment (Pacific Ocean)               | 3.36               | 66.54  | Multicopper oxidases (MCOs); Mercuric reductase                                                | Reduction of mercury in contaminated environments                                        | Wu et al., 2015       |
| *M. metallidurans* TL13  | Tannery wastewater (Tunisia)               | 3.58               | 70.7   | Genes involved in heavy metal resistance and plant growth promotion                             | Bioremidation of tannery wastewater and metal contaminated soil; Plant growth promotion under metallic stress | This work              |

*metallidurans* TL13, isolated from a tannery effluent. Genome analyses, as well as experimental studies, indicated that *M. metallidurans* TL13 could promote plant growth, especially by solubilizing inorganic phosphate, synthesizing siderophores, and producing IAA, hydrolytic enzymes, and EPS. Besides, genome annotation revealed the presence of multiple genetic determinants related to the resistance to heavy metals, particularly chromium, arsenic, and mercury. To the best of our knowledge, this is the first report providing new insights into the molecular mechanisms underlying the enhancement of plant growth and the resistance to chromium and other heavy metals in a new species of *Microbacterium*. Further comparative genomic analysis and direct redox experimentation will provide additional information about the variance and pathways involved in the multimetal resistance and rhizosphere bioremediation by the actinobacterium *M. metallidurans* TL13 that is expected to facilitate environmental and agricultural applications in heavy metal-polluted sites.

DATA AVAILABILITY STATEMENT

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession SZZQ00000000. The version reported in this work is SZZQ01000000.

AUTHOR CONTRIBUTIONS

MN, RO, HCho, and AChe conceived and designed the experiments. HChe, YB, and RO performed PGP experiments. ACha and HK performed chemical analysis. AN, HS, RO, AM, MM, and MN performed bioinformatics and structural analysis. HK contributed reagents, material tools. MN, RO, AO, AN, HChe, MM, AM, HS, HCho, and AChe analyzed the data. RO, AO, MM, AN, MN, HS, HChe, and AChe prepared and revised the manuscript. MN and AChe supervised the entire project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00521/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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