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Functional genomics and taxonomic insights into heavy metal tolerant novel bacterium *Brevibacterium metallidurans* sp. nov. NCCP-602^{T} isolated from tannery effluent in Pakistan

Sadia Manzoor · Saira Abbas · Sobia Zulfiqar · Hong-Chuan Wang · Min Xiao · Wen-Jun Li · Muhammad Arshad · Iftikhar Ahmed

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Abstract The strain designated NCCP-602^{T} was isolated from tannery effluent, and displayed aerobic, gram-positive, rod-shaped cells that were characterized by oxidase negative, catalase positive, and non-motile features. The most favourable growth conditions were observed at a temperature of 30°C, pH 7.0, and NaCl concentration of 1% (w/v). It tolerated heavy metals at high concentrations of chromium (3600 ppm), copper (3300 ppm), cadmium (3000 ppm), arsenic (1200 ppm) and lead (1500 ppm). The results of phylogenetic analysis, derived from sequences of the 16S rRNA gene, indicated the position of strain NCCP-602^{T} within genus *Brevibacterium* and showed that it was closely related to *Brevibacterium ammoniilyticum* JCM 17537^{T}. Strain NCCP-602 ^{T} formed a robust branch that was clearly separate from closely related taxa. A comparison of 16S rRNA gene sequence similarity and dDDH values between the closely related type strains and strain NCCP-602^{T} provided additional evidence supporting the classification of strain NCCP-602^{T} as a distinct novel genospecies. The polar lipid profile included diphosphatidylglycerol, glycolipid, phospholipids and amino lipids. MK-7 and MK-8 were found as the respiratory quinones, while anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, and anteiso-$C_{17:0}$ were identified as the predominant cellular fatty acids (> 10%). Considering the convergence of phylogenetic, phenotypic, chemotaxonomic, and genotypic traits, it is suggested that strain NCCP-602 ^{T} be classified as a distinct species *Brevibacterium metallidurans* sp. nov. within genus *Brevibacterium* with type strain NCCP-602^{T} (JCM 18882^{T}=CGMCC1.62055^{T}).

Keywords Tannery effluent · *Brevibacterium metallidurans* · Heavy metal tolerance · Whole-genome sequencing

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S. Manzoor · S. Zulfiqar · I. Ahmed (✉)
National Culture Collection of Pakistan (NCCP), Land Resources Research Institute (LRRI), National Agricultural Research Centre (NARC), Islamabad, Pakistan
e-mail: iftikhar.ahmed@parc.gov.pk

S. Manzoor · M. Arshad (✉)
Institute of Environmental Sciences and Engineering, School of Civil and Environmental Engineering, National University of Sciences and Technology (NUST), Islamabad, Pakistan
e-mail: marshad@iese.nust.edu.pk

S. Abbas
Department of Zoology, University of Science and Technology, Bannu, Pakistan

H.-C. Wang · M. Xiao · W.-J. Li (✉)
State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, People's Republic of China
e-mail: liwenjun3@mail.sysu.edu.cn

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Introduction

Breed laid the foundation for the identification of the genus *Brevibacterium* in 1953, with the inaugural type species designated as *Brevibacterium linens*. This bacterium was first isolated from surface-ripened cheese and was characterized as a gram-positive microorganism that is nonspore-forming, nonbranching, and possessing short rods, along with a notably high G+C content. The genus name "*Brevibacterium*" originates from the fusion of the Latin term "*breviation*" (signifying short) with the Greek term "bakteria" (meaning rod), which captures its unique morphological characteristics. Subsequently, genus *Brevibacterium* was expanded to include a variety of species with diverse morphological, physiological, and biochemical traits (Breed 1953). In a subsequent revision, the genus description was refined to encompass only those species that demonstrated chemotaxonomic and morphological traits in alignment with the characteristics of type species *Brevibacterium linens* (Collins et al. 1983). The venerable genus *Brevibacterium* stands as the exclusive representative of the family *Brevibacteriaceae*, residing within the order *Micrococcales* and the distinguished class *Actinobacteria* (Forquin-Gomez et al. 2014). However, the genus exhibits remarkable diversity in physiological, biochemical, and chemical characteristics, with a total of 37 species reported with valid published names https://lpsn.dsmz.de/genus/brevibacterium. Several novel species within genus *Brevibacterium* (sensu stricto), including *B. spongiae* (Huang et al. 2022; Zhang et al. 2023b), *B. limosum, B. atlanticum* (Pei et al. 2021), and *B. rongguiense* (Deng et al. 2020; Pei et al. 2020), have been found in recent reports. The members of this genus are frequently identified on human skin and are prominently found in cheese and dairy products (Collins et al. 1983; Roux and Raoult 2009; Wauters et al. 2004). In diverse environments, sporadic occurrences of *Brevibacterium* strains, including those characterized by elevated salinity levels (Bhadra et al. 2008) and poultry (Pascual and Collins 1999), have been detected. *Brevibacterium picturae* and *Brevibacterium celere* were isolated by Heyrman et al. (2004) and Ivanova et al. (2004) during examination of a ruined mural artwork and a brown alga's deteriorated thallus.

The genus members have generic recognition in biotechnological industry patents, encompassing applications in antibiotic production, wastewater treatment, and the art of making cheese. Moreover, in addition to indigenous *Agrobacterium* and *Corynebacterium* strains, specific native *Brevibacterium* strains found in heavy metal-contaminated soils in Kuwait have demonstrated impressive resilience to both hydrocarbon and heavy metal contamination (Ali et al. 2012).Additionally, it has been demonstrated that certain strains of *Brevibacterium linens* can grow when provided with n-alkanes as carbon substrates. Leveraging these metabolic capabilities for remediation of crude oil pollution is another evident application. The influence of specific pollutants, such as cadmium sulfate or sodium arsenate, on enhancing this metabolic pathway remains uncertain; however, there is a positive indication of an increased rate of direct crude oil consumption. These findings underscore the potential of certain native *Brevibacteriaceae* species as valuable resources for bioremediation of complex organic–inorganic pollutants. Moreover, in addition to detoxifying pentachlorophenol, *B. casei* was also utilized for synthesis of gold and silver nanoparticles (Kalishwaralal et al. 2010; Ng et al. 2010; Verma and Singh 2013). *Brevibacterium metallicus* (Román-Ponce et al., 2018) and *Brevibacillus parabrevis* (Wani et al., 2023) exhibited tolerance to high concentrations of heavy metals, showed markable potential for bioremediation by accumulating high concentrations of copper, cadmium and zinc.

In this study, strain NCCP-602^T was isolated from tannery effluent to characterize heavy metal tolerant potentially novel bacteria from Pakistan and its functional genomics for potential application in bioremediation. This study evaluated its potential application in bioremediation, detailed genomic functional analysis for heavy metal tolerance, and xenobiotic degradation-encoding genes of strain NCCP-602^T, which were found to effectively tolerate high concentrations of heavy metals. Through polyphasic taxonomic analysis, strain NCCP-602^T was identified as a novel heavy metal-tolerant species. This study focused on the strain NCCP-602^T, which was initially isolated from a sample of tannery effluent. The primary objective was to establish its taxonomic classification by thoroughly examining its physiological, biochemical genotypic, and phylogenetic attributes. The taxonomic position of strain NCCP-602^T was unequivocally confirmed through this comprehensive investigation, which confirmed its status as a

novel species within genus *Brevibacterium;* for which the name *Brevibacterium metallidurans* sp. nov. was proposed.

Materials and Methods

Strain NCCP-602^T was isolated from samples collected from tannery effluent in Sambrial, Sialkot, Pakistan. The aerobic recovery of strain NCCP-602^T was achieved using a dilution plate method on tryptic soy agar (TSA, Difco), which contained varying concentrations of Pb^{2+}, Cr^{2+}, Cd^{2+}, As^{2+}, and Cu^{2+}. Individual colonies of strain NCCP-602^T were subsequently transferred to fresh plates for purification, after which 16S rRNA gene sequencing was used for identification of the purified colonies. For routine cultivation, TSA medium was used to grow the isolate at 30 °C, while for prolonged preservation, the mixture was stored at −80°C in tryptic soy broth (TSB; Difco) supplemented with 70% (v/v) glycerol.

To meet the essential criteria for characterizing novel aerobic bacterial taxa, comprehensive polyphasic characterization experiments were conducted on the isolated strain. The reference strains from closely related taxa, including *Brevibacterium ammoniilyticum* JCM 17537^T, *Brevibacterium celere* JCM 13521^T, *Brevibacterium casei* JCM 2594^T, and *B. linens* JCM 1327^T, were obtained from the Japan Collection of Microorganisms (JCM), Japan. Unless specified otherwise, the characterization experiments were conducted at a temperature of 30°C under the same laboratory conditions along with the reference strains.

Heavy metal tolerance

The ability of newly discovered strain NCCP-602^T to endure increased levels of heavy metals (Pb^{2+}, Cu^{2+}, As^{2+},Cd^{2+} and Cr^{2+}) was assessed via cultivation on TSA enriched with heavy metals at various concentrations over a period of five-days. In TSA, concentrations of Pb^{2+}, As^{2+}, Cr^{2+}, Cd^{2+} and Cu^{2+} were carefully regulated within the range of 300−3000 ppm by employing specific salts of Pb $(NO_3)_2$, $CuSO_4.5H_2O$, NaH_2AsO_4 $Cd(NO_3)$ and $K_2Cr_2O_7$.

Antibiotic resistance

ATB-Vet Strep (BioMerieux, France) was used to investigate antibiotic resistance. Strain NCCP-602^T and reference strains were inoculated to ATB-Vet strep according to the manufacturer's instructions, and then incubated for 48 h at 37°C. After this incubation period, growth was visually assessed (Guérin-Faublée et al. 1993).

Morphological and physiological characterization

The strain growth was assessed on a range of agar media (Difco, US), which included Luria–Bertani (LB) agar, nutrient agar (NA), tryptic soy agar (TSA), and brain heart infusion agar (BHI) (Ventosa et al. 1982)., Colony morphology of strain NCCP-602^T was examined on TSA after two days of incubation. For detailed cell morphology, a scanning electron microscope (EVO MA10-W) was used following established procedures using cells grown in TSB for 24 h. After immersion in a 2.5% glutaraldehyde solution for 3 h, the cultured cells were sequentially dehydrated in ethanol at concentrations of 30%, 50%, 70%, 90%, and 100% for 15 min each. Subsequently, the samples were subjected to one-hour drying process in a vacuum dryer. Next, a suitable area was selected, and pieces measuring 1 × 1 cm were cut. Finally, gold was sprayed for optimal observation under a scanning electron microscope (EVO MA10-W) (Abbas et al. 2015a). Gram staining was performed using a commercial Gram staining kit (Solarbio, Beijing) following the manufacturer's instructions. Growth temperatures were assessed by growing strains on TSA media and incubated at different temperature conditions (4, 20, 25, 30, 37, 45, 50, 55, or 60°C) for 5 days. To investigate the range of pH for cell growth, TSA was modified to various pH values ranging from 4 to 10. Specific buffer systems, such as 0.1 M citric acid/0.1 M sodium citrate (pH 4.0−5.0), 0.1 M KH_2PO_4/0.1 M NaOH (pH 6.0–8.0), and 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 9–10), were used (Xu et al. 2005) to achieve the desired pH of the media. NaCl tolerance was assess by growing cells on TSA supplemented with different NaCl concentrations in the range of 0−15% w/v and incubation for a period of 5 days. Motility of the strains was determined using TSA as a semisolid medium (Tambalo et al.

2010). Oxidase activity was analysed by utilizing an oxidase reagent; catalase activity, 3−10% hydrogen peroxide; degradation of tweezers (1% each of Tweens 20, 40, 60, and 80); and starch degradation (0.1% soluble starch) following conventional procedures as outlined in previous descriptions by Odds (1981). The physiological and biochemical properties of the strains were determined using API 50CH, API 20E, and API 20NE galleries (bioMerieux, France), whereas the API ZYM strip (bioMerieux, France) was utilized to analyse enzyme production API Kits were used following the manufacturer's instructions. Biolog Universal Growth (BUG) medium was used for cultivation of the strains in Biolog experiments and BIOLOG test was performed using a BIOLOG GEN III microplate system (Inc. Hayward, CA, USA) in accordance with the manufacturer's recommendations.

Phylogenetic analysis

Following the protocol described previously (Ahmed et al. 2007), 16S rRNA gene of strain NCCP-602^T was amplified through PCR amplification using universal forward and reverse primers, namely, 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). PCR product was sequenced using 16S rRNA gene-specific universal primers by commercial service of Macrogen (http://dna.macrogen.com/eng), Korea. The obtained sequences were assembled using BioEdit software, and the strain was identified using the EzTaxon server (http://eztaxon-e.ezbiocloud.net). The sequences of 16S rRNA gene of closely related reference strains were obtained via Ez-Taxon server database and aligned through CLUSTAL W following the Tamura–Nei model (Tamura and Nei 1993) for calculating evolutionary distances. MEGA 11 software was used to construct phylogenetic trees via the maximum-parsimony, neighbor-joining, and maximum-likelihood methods in accordance with protocols detailed conducted by Ahmed et al. (2014). The robustness of associations was evaluated through 1000 iterations of the bootstrap technique involving resampling of the tree topology (Kumar et al. 2016).

Genome features

Whole-genome sequencing of strain NCCP-602^T was performed using Illumina NovaSeq platform, employing commercial service of Sangon Biotech Co., Ltd., in Shanghai, China. Trimmomatic software (version 0.38) was used to filter out low-quality data and remove barcodes from the original reads (Bolger et al. 2014). The assembly of clean data was carried out using SPAdes software (version 3.11.1) (Bankevich et al. 2012). Contigs with lengths exceeding 500 bp were chosen for the prediction of genes via Prodigal software (version 2.6.3) (Besemer et al. 2001). We utilized the COG classifier (version 1.0.5, accessible at https://github.com/moshi4/COGclassifier) to annotate the protein-coding sequences of strain NCCP-602^T alongside closely related type strains. The genomes of NCCP-602^T were examined for resistance and virulence factors by searching the databases of the Pathosystems Resource Integration Center (PATRIC) and the Comprehensive Antibiotic Resistance Database (CARD) available at their respective websites: https://www.patricbrc.org and https://card.mcmaster.ca. Additionally, the annotation of enzymes related to carbohydrate metabolism and carbon metabolism was conducted using the HMM search tool (version 10) (Johnson et al. 2010) from the dbCAN2 database (Zhang et al. 2018). Using CheckM, the completeness and contamination of the genome assembly were assessed (Parks et al. 2015), resulting in a completeness of 98.97% and a contamination of 5.93%. For construction of phylogenomic tree, draft genome of 23 related type strains belonging to genus *Brevibacterium* were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) database. The phylogenomic tree was inferred using EasyCGTree with default options. Evolutionary relationships were inferred using amino acid sequences of the bac120 gene set as default parameter in FastTree version 4.0 (Zhang et al. 2023a). The Type Strain Genome Server (TYGS) was utilized to construct a phylogenomic tree based on genomic sequences, including those of NCCP-602^T and closely related type strains (Meier-Kolthoff and Göker 2019). The average nucleotide identity calculation was used to calculate the genomic distance between the NCCP-602^T strain and related strains (Yoon et al. 2017). The calculation of digital DNA−DNA hybridization (dDDH) value involved the application of formula 2 using genome−to-genome

distance calculator (GGDC, v. 3.0) (Meier-Kolthoff et al. 2022).

Gene network/pathway analysis

The genome sequence was annotated with KEGG (Kyoto Encyclopedia of Genes and Genomes) enzyme database. Each gene was assigned to a KEGG pathway. Physiological features of strain NCCP-602^T were demonstrated by biochemical pathway maps constructed by result of individual analysis of KEGG pathways.

Chemotaxonomic analysis

For chemotaxonomic analysis, strain NCCP-602^T was grown on TSA for 24 h to obtain necessary biomass. Subsequently, isoprenoid quinones were extracted from 100 to 150 mg of lyophilized cells. The method outlined by Minnikin et al. (1984) was followed for purification of quinones using TLC. The analytical method involved HPLC, employing a Cosmosil column (4.6×150 mm; Nacalai Tesque; reversed-phase silica gel; 5C18) and a mobile phase comprising methanol: 2-propanol (2:1) (Collins et al. 1977; Tamaoka 1986), which was used for determination of quinone. Quinone peaks were identified at a UV wavelength of 270 nm using Shimadzu equipment (Kyoto, Japan). Polar lipids were investigated through two-dimensional TLC (silica gel 60, Merck) plates (10×10 cm).Lyophilized cells (10 mg) were used to analyse amino acids in cell wall peptidoglycan. The cells were hydrolysed with 6 N HCl at 100°C for 18 h. Thin-layer chromatography (80:26:4:10) was performed on an HPTLC cellulose plate (10×10 cm, 1.05787.001 Merck, Germany) with a solvent combination of methanol-distilled water and 6 N HCl-pyridine for the analysis of whole-cell hydrolysate. To aid in identification, a TLC plate was also concurrently run with a standard solution of diaminopimelic acid (0.01 M, including meso-a2pm, DD-, and LL-) (Fig. 1).

Whole-cell fatty acids analysis was performed by growing strain NCCP-602^T and reference strains on TSA media. Fatty acid methyl esters were prepared following the procedures described by Sasser (1990), andcellular fatty acids analysis was conducted using a gas chromatograph (model 6890; Hewlett Packard), with identification performed using the MIDI Sherlock version 4.5 system and the MIDI database TSGA40 4.10.

Results

Following incubation at 30°C for 24−48 h, strain NCCP-602^T exhibited small, smooth, whitish colonies with glossy surfaces, a convex and spherical shape, entire margins, and a somewhat adhesive texture. The cells exhibited aerobic, nonmotile, and gram-positive staining and were rod-shaped (1.577−0.448 µm diam.) (Fig. 2). Cells growth was monitored over a temperature range of 20−42°C, revealing optimal growth between 30°C and 37°C within a span of 3 days. Strain NCCP-602^T demonstrated no growth at pH 5.0 but exhibited robust growth within the pH range from 6.0 to 10.0, with a pH ranging from 7 to 8 for optimal growth.

Heavy metal tolerance

Genomic analysis of strain NCCP-602 ^{T} revealed an array of genes dedicated to maintaining heavy metal homeostasis. The *copC* gene has been shown to confer resistance to copper. The periplasmic proteins encoded by the *copC* gene exhibit a high affinity for copper atoms. The periplasmic accumulation of copper is regulated by four genes: *copA, copC, copB* and *copD* (Banci et al. 2003). The genome of strain NCCP-602^T revealed the presence of the *corA* gene, which is responsible for conferring resistance to cobalt and magnesium (Table S5). The *corA* gene encodes for the transport of Mg^{2+} ions. The *corA* gene, responsible for Mg^{2+} transport, has been reported to exhibit a wide phylogenetic distribution among bacterial species (Groisman et al. 2013; Hmiel et al. 1989, 1986).

In the present study, proteins involved in zinc homeostasis, including Zn-dependent hydrogenase, Zn-binding hydrogenase, and neutral zinc metallopeptidase, were reported. The *chrA* gene, which is responsible for chromium resistance through encoding of a chromate-transporting protein, has been identified. The presence of Cr (IV) induces the expression of the chromate transporter gene *chrA* (He et al. 2010). Genome analysis revealed that the resistance of strain NCCP-602^T to Mn (II) is mediated by the

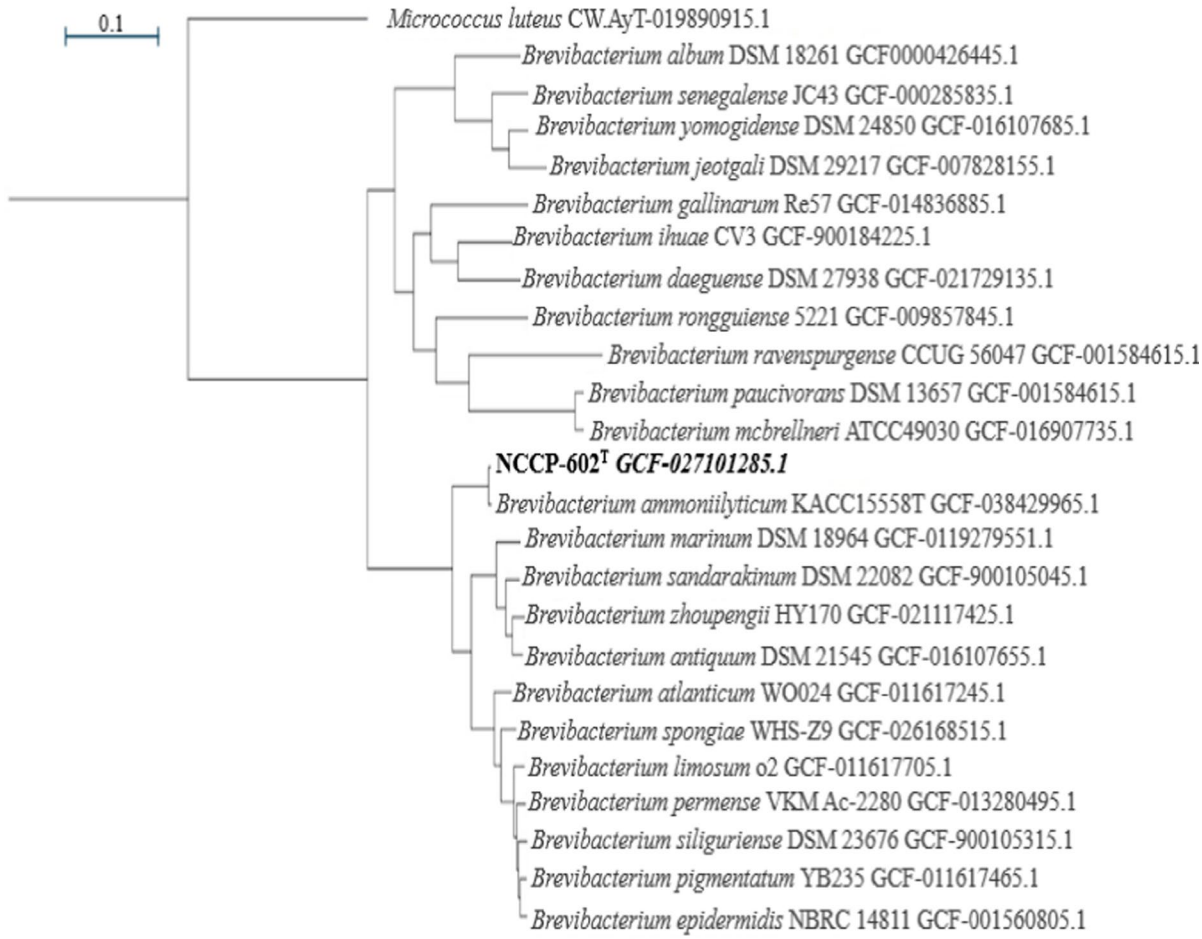

Fig. 1 Phylogenomic tree showing the evolutionary relationship of strain NCCP-602 ^{T} with its closest relatives in genus *Brevibacterium*. The Bac120 gene set protein sequence (as the default parameter) was used to infer the maximum-likelihood tree

MnmtR and *MnmA* genes, which regulate the Mn (II) transport system.

The arsenic resistance of strain NCCP-602^{T} is attributed to the presence of an arsenic efflux pump membrane protein and a metalloregulator belonging to the *ArsR*/*SmtB* family of transcription factors, which encode mechanisms for arsenic resistance. The presence of an arsenic operon, as well as genes conferring tolerance to copper and chromium, within the genome of strain NCCP-602^{T} potentially contributes to its survival in natural wastewater environments (Flores et al. 2022).

Antibiotic susceptibility testing revealed that strain NCCP-602^{T} was resistant to various antibiotic classes, including cephalothin, cefoperazone (a cephalosporin), tetracycline (tetracycline), lincomycin (lincosamide), oxacillin, penicillin (ampicillin), amoxicillin, amoxicillin-clavulanic acid (abeta lactam-beta lactamase inhibitor), colistin (polymyxin), cotrimethoprim, sulfonylens (sulfonamide), flumequin, oxolinic acid, enrofloxacin (quinones), nitrofurantoin (nitrofuran) and fucoxanthin (xanthophylls), however, it was found sensitive to gentamicin (aminoglycoside), rifamcin (rifampicin), spectinomycin (aminocyclitol), erythromycin (macrolides) and chloramphenicol (phenicol). Furthermore, the phenotype analysis indicated the presence of 49 antibiotic resistance genes, including antibiotic resistance efflux pump complexes**.** These genetic elements

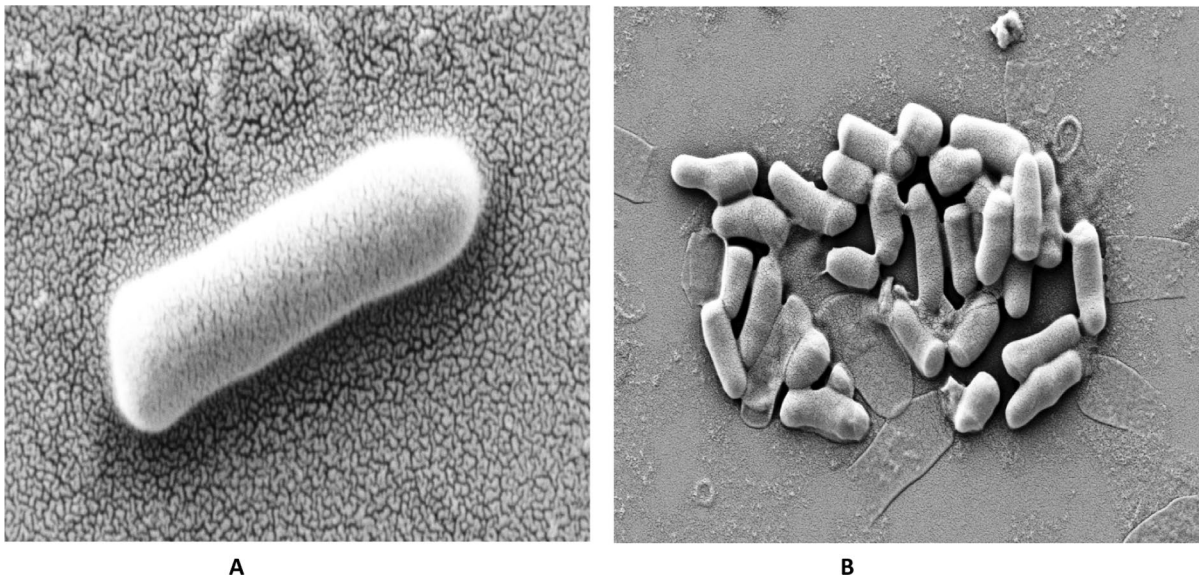

Fig. 2 Scanning electron micrographs illustrating *Brevibacterium metallidurans* sp. nov. NCCP-602^T: **A** a single cell and **B** a colony

are responsible for conferring resistance to multiple antibiotic classes through molecular mechanisms (Fig. S7).

Biochemical characterization

Strain NCCP-602^T shared numerous phenotypic traits with closely related strains; however, this strain also showed unique physiological and biochemical traits that distinguish it from the reference species. Table 1 provides a comprehensive overview of the distinct physiological and biochemical characteristics of strain NCCP-602^T. Notable distinctions include its ability to utilize citrate, voges-proskauer, release N_2 from nitrate, no nitrate reduction to NO_2, trisodium citrate, gelatin hydrolysis, malic acid, glucose, N-acetyl-glucosamine, potassium gluconate, phenylacetic acid, and adipic acid (API 20E, API 20NE). The strain demonstrated high enzyme activity for α-glucosidase and acid phosphatase (API ZYM); use of glycerol, arabinose, glucose, maltose, melezitose, and L-arabitol as sources of carbon (API 50 CH); resistance against lincomycin, streptomycin, apramycin, mutactimycin, cephalothin, and kanamycin; and sensitivity against chloramphenicol, tetracycline and erythromycin, setting it apart from closely related species such as *B. ammoniilyticum* JCM 17537^T, *B. celere* JCM 13521^T and *B. casei* JCM 2594^T. Strain NCCP-602^T did not degrade Tween 20, 40, 60, or 80, and starch and can utilize L-alanine, citrate, inosine, and D-serine as carbon sources. Strain NCCP-602^T was found to be positive for nalidixic acid, aztrename, sodium bromate and potassium tellurite (Biolog).

Furthermore, strain NCCP-602^T exhibited notable resistance to typically harmful concentrations of heavy metals and can survive in TSA media supplemented with As (1200), Pb (1500 ppm), Cd (3000 ppm), Cu (3300 ppm), and Cr (3600 ppm). Compared to previously documented heavy metal-tolerant bacteria, strain NCCP-602^T displays a relatively greater tolerance to these harmful concentrations of heavy metals, especially when contrasted with numerous other bacterial strains (Abbas et al. 2014, 2015b; Elahi et al. 2019; Mustapha and Halimoon 2015).

Phylogenetic analysis

Highest similarity (98.5%) of 16S rRNA gene sequence of strain NCCP-602 ^{T} was found with *Brevibacterium ammoniilyticum* (GenBank accession no. JF937067), while it displayed less than 98.5% similarity with that of other *Brevibacterium* species. Results of phylogenetic analysis indicated that strain NCCP-602^T clustered within a specific clade alongside *Brevibacterium casei* and *B. ammoniilyticum* as its closest neighbours (Figure S2). Phylogenetic trees

Table 1 Distinctive features of *Brevibacterium metallidurans* sp. nov. NCCP-602^T and reference strains from genus *Brevibacterium*

Characteristics	NCCP-602^T	*B. ammoniilyticum* JCM 17537^T	*B. celere* JCM 13521^T	*B. casei* JCM 2594^T	*B. linens* JCM 1327^T
Colony color	White	Creamy white	Whitish yellow	Gray white	Pale yellow
Growth at (°C) range (optimum)	20−42(30−37)	20−42 (30)	12−42(25)	15−40 (30−37)	(20−25)
pH for Growth range (optimum)	6−10(7−8)	6−10 (8−9)	5−10 (8.5−9)	5−10 (8−9)	6−10 (7−8)
Oxidase	−	−	+	−	+
NaCl tolerance (%)	0−15(1)	0−11(1)	0−15(1)	0−15(1)	10(1)
Enzyme activity: API Coryne test					
Reduction of nitrates	−	+	−	−	+
α-Glucosidase	+	+	+	+	−
API 20E					
Gelatin	+	−	+	+	+
Glucose	+	+	−	−	−
Citrate	+	+	+	−	−
Voges-Proskauer	+	+	+	+	−
Nitrate reduction to NO_2	−	+	−	−	+
API 20NE					
Nitrate reduction to NO_2	+	+	+	−	−
Gelatin hydrolysis	+	−	+	+	+
Assimilation of:					
Glucose	+	+	+	+	−
N-acetyl-glucosamine	+	+	+	+	−
Potassium gluconate	+	+	+	+	−
Adipic acid	w+	w+	−	−	−
Malate	+	w+	+	+	−
Trisodium citrate	+	+	+	+	−
Phenylacetic acid	+	w+	w+	+	−
Enzyme activity (API ZYM)					
Acid phosphatase	+	+	+	+	−
α-glucosidase	+	+	+	+	−
API 50 CH					
Glycerol	−	−	w+	−	−
D-Arabinose	w+	+	−	−	+
D-Glucose	−	−	w+	−	−
D-Maltose	w+	−	w+	w+	−
D-MeLezitose	−	−	w+	−	−
L-Arabitol	+	−	−	−	−
Resistance to antibiotics—API ATB VET					
Cephalothin	R	S	R	R	S
Kanamycin KAN	R	S	S	S	S
Chloramphenicol	S	R	R	R	S
Tetracycline	R	R	S	S	S
Lincomycin	R	S	S	S	S
Streptomycin	R	S	S	S	S

Table 1 (continued)

Characteristics	NCCP-602^T	*B. ammoniilyticum* JCM 17537^T	*B. celere* JCM 13521^T	*B. casei* JCM 2594^T	*B. linens* JCM 1327^T
Apramycin	R	R	S	R	S
PRI (2)	R	S	S	S	S
Erythromycin	S	w+	w+	w+	S

All the strains exhibited common characteristics: gram-positive, nonmotile, aerobic, no spore-forming, and catalase-positive rods. +: positive, −: negative, w: weak, R: resistant, S: sensitive

All the strains were positive for pyrazinamidase and pyrrolidonyl, reducing nitrates, arylamidase, alkaline phosphatase, α-glucosidase, and gelatin but negative for β-glucosidase, urease, β-glucuronidase, N-acetyl-β-glucosaminidase, and esculin. There was no metabolism of xylose, glucose, glycogen, ribose, maltose, lactose, mannitol, or saccharose (sucrose) (API Coryne, bioMerieux, France)

All the strains exhibited negative results for the following genes: β-galactosidase, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease, tryptophan deaminase, H_2S, indole, mannose, inositol, arabinose, sorbitol, rhamnose, melibiose, sucrose, and amygdalin (API 20E, bioMerieux, France). All the strains were negative for tryptophan, glucose fermentation, assimilation of capric acid, hydrolysis of esculin, arginine, urea, and β-galactosidase, and assimilation of arabinose, mannose, and mannitol (API 20NE, bioMerieux, France). Negative results were observed for all the strains for the production of acid from L-arabinose, erythritol, ribose, methyl-βD-xylopyranoside, D-xylose, galactose, L-xylose, adonitol, methyl-αD-mannopyranoside, sorbose, dulcitol, fructose, inositol, mannitol, rhamnose, sorbitol, N-acetylglucosamine, amygdalin, methyl-αD-glucopyranoside, arbutin, D-fucose, cellobiose, melibiose, inulin, esculin, raffinose, lactose, L-fucose, trehalose, starch, sucrose, gentiobiose, turanose, glycogen, lyxose, tagatose, arabitol, salicin, xylitol, potassium 5-ketogluconate, potassium gluconate, and potassium 2-ketogluconate (API CH 50, bioMerieux, France). Robust enzyme activity was noted for C4 and leucine arylamidase, while no enzymatic activity was noted for cystine arylamidase, lipase C 14, β-glucosidase, valine arylamidase, trypsin, β-galactosidase, fucosidase galactosidase, mannosidase, chymotrypsin, β-glucuronidase, or N-acetyl-ß-glucosaminidase (API ZYM, bioMerieux, France)

(Figures S3 and S4) constructed following maximum-likelihood and maximum-parsimony algorithms showed positioning of strain NCCP-602^T within the same clade as *Brevibacterium casei* and *B. ammoniilyticum* suggesting a close relationship, and a robust bootstrap support was further observed in the neighbour-joining tree. The bac120 tree also indicated that strain NCCP-602^T clustered with *Brevibacterium ammoniilyticum* KACC15558^T (GCF-038429965.1) in the clade (Fig. 1). Determining the relatedness values of digital DNA−DNA hybridization between closely related reference strains and the NCCP-602^T strain revealed 64.7% relatedness for *Brevibacterium ammoniilyticum* JCM 17537^T, 23.3% for *B. celere* JCM 13522^T, and 31.1% for *B. casei* JCM 2594^T. The calculated values fall below the defined threshold of 70%, which is desired for classification of the strain as a distinct new species in the genus (Wayne et al. 1987). The determined G+C content of 67.6 mol% in genomic DNA of strain NCCP-602^T supports its taxonomic affiliation within *Brevibacterium* genus. This G+C content is observed to be consistent with that found in closely related strains, including *Brevibacterium ammonylyticum* JCM 17537^T, *B. celere* JCM 13521^T, and *B. casei* JCM 2594^T. Although biochemical data presented in Table 1 reveal certain distinctions from the closely related reference strains, it is worth noting that strain NCCP-602^T shares numerous characteristics with *B. ammonilyticum* JCM 17537^T and *B. celere* JCM 13521^T. The cumulative evidence strongly suggested that strain NCCP-602^T constitutes a distinct and novel species within *Brevibacterium* genus.

Functional genomics

With a cumulative size of 3.9 Mbp and an N50 length of 236,461 Kbp, the definitive draft genome sequence of strain NCCP-602^T included 83 contigs. The phylogenomic tree generated from the genomes of closely related strains clearly demonstrated that strain NCCP-602^T constituted a unique clade within genus *Brevibacterium* (Figure S5), thereby reinforcing its phylogenetic placement. With the use of Prodigal software, the prediction of protein-coding genes yielded a total of 2621 genes. Supplementary Table S1 provides a comprehensive list of genomic features for strain NCCP-602^T alongside the reference strains *B. ammoniilyticum* JCM 17537^T, *B. celere* JCM 13521^T, and *B. casei* JCM 2594^T. Despite the

significantly high average nucleotide identity (ANI) value (95.4%), digital DNA−DNA hybridization (dDDH) values between strain NCCP-602^T and other *Brevibacterium* members remained below the recognized threshold values (70%) critical for distinguishing novel prokaryotic species (Richter and Rosselló-Móra 2009) (Table S1). The extensive protein profile of strain NCCP-602^T, as categorized in Clusters of Orthologous Groups (COGs) database, exhibited significant similarities to those of its three closely related strains, *B. ammoniilyticum* JCM 17537^T, *B. celere* JCM 13521^T, and *B. casei* JCM 2594^T. The principal protein groups encompassed translation, transcription, coenzyme metabolism, and transport, as well as amino acid metabolism and transport. Notably, cell motility-related genes were present in both strain NCCP-602^T and its related strains, although genes associated with flagella were notably absent (Table S2). Moreover, carbohydrate degradation proficiency of the strains was also evaluated within the genomes of closely related type strains and strain NCCP-602^T. The presence of five families, namely, auxiliary activities (AAs), glycoside hydrolases (GHs), carbohydrate-binding modules (CBMs), glycosyltransferases (GTs), and carbohydrate esterases (CEs), across the scrutinized genomes is presented in Table S3. However, in case of strain NCCP-602^T, certain subfamilies, including GH0, GH13_3, GH13_16, GH13_26, GH16_24, GH23, GH33, GH170, GT5, and GT35, were notably absent. Additionally, antiSMASH version 7.0.0 was used to predict clusters of secondary metabolite genes, and analysis revealed that strain NCCP-602^T contains gene clusters associated with ectoine, hydrogen cyanide, and siderophores. These clusters serve a protective function against oxidative stress in bacteria (Table S4) (Giani and Martínez-Espinosa 2020; Graf et al. 2008; Saha et al. 2016)**.** The orthologous genes of closely related strains and strain NCCP-602^T were clustered using the OrthoVenn3 web server (Wang et al. 2015) with the OrthoFinder algorithm (Emms and Kelly 2019). Among 3298 orthologous gene clusters identified, 2254 were shared by the four genomes. A customized version of CGView (http://cgview.ca) was utilized to generate a circular genome map for strain NCCP-602^T (Figure S6A). It is worth noting that genome of strain NCCP-602^T harbored 11 unique gene clusters containing 26 proteins (Figure S6B).

Genetic elements involved in bioremediation and environmental protection

KEEG annotation of the genome of the NCCP-602^T strain confirmed the presence of specific enzymes responsible for encoding the xenobiotic resistance and degradation capability (Table S6). The genes responsible for histidine degradation; Cr (VI) intracellular reduction; the degradation of lethal nitronates; and catechol degradation, ring cleavage and phthalate hydrolysis were identified. Several nitrate, sulphur, and phosphate reductase and transport genes in the NCCP-602^T genome are presented in (Table S6)**.** The NCCP-602^T genome profile confirms that the NCCP-602^T strain uses GABA as a nutritional source in the rhizome, resulting in the regulation of GABA levels in plants (Renault et al. 2011). Various genes responsible for tolerance of strain NCCP-602^T to osmotic stress and temperature were identified from genome of the strain (Table S6). The presence of osmotic stress tolerance genes in the genetic elements of strain NCCP-602^T indicates that this strain has the ability to survive in highly saline environments.

Chemotaxonomic analysis

Results of cellular fatty acid analysis depicted the following profile of strain NCCP-602^T: anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, $C_{17:0}$, and anteiso-$C_{17:0}$ (Table S7)., Strain NCCP-602^T differs from other members of *Brevibacterium* genus by variations in quantities of specific components. Nevertheless, predominant components of its fatty acid composition closely mirror those identified in closely related reference strains. Strain NCCP-602^T exhibited elevated concentrations of iso-$C_{15:0}$, iso-$C_{16:0}$, and iso-$C_{17:0}$, constituting 18.65% of fatty acid composition in comparison to the reference strains (Table S7).

The distinctive amino acid *meso*-diaminopimelic acid was identified in cell wall peptidoglycan of strain NCCP-602^T, which is a characteristic feature observed in *Brevibacterium* species. The primary component MK-7 was identified in the respiratory quinone, comprising 67% of its composition, with minor components such as MK-8 (33%). Results of whole-cell composition revealed the sugars such as glucose, ribose, and mannitol. Significantly, occurrence of *meso*-diaminopimelic acid, a notable quantity of iso-C-$_{15:0}$, and prevailing occurrence of major

respiratory quinone as MK-7 closely conforms to the distinctive characteristics observed in members of *Brevibacterium* genus.

Major components of polar lipid profile in strain NCCP-602^T included diphosphatidylglycerol, unknown glycolipids, unknown phospholipids and amino lipids. The strain exhibited a distinctive lipid profile with negative responses to ninhydrin sprays but positive reactions to alpha-naphthol reagents. The glycolipid (GL) exhibited chromatographic mobility typical of a diglycosyldiacylglycerol (Figure S1). This polar lipid composition closely mirrors that of related species, such as *Brevibacterium ammoniilyticum* JCM 17537^T (Kim et al. 2013), which has been analysed at same lab conditions. The polar lipid profile of strain NCCP-602^T, which is similar to that of *B. ammoniilyticum* JCM 17537^T, contains unknown amino phospholipids and phospholipids. Notably, although amino lipid (AL) are present in both NCCP-602^T and the reference species (Figure S1), however, levels of these amino lipids were relatively low.

Based on comprehensive analysis of phenotypic, physiological, phylogenetic, and DNA−DNA relatedness and the chemotaxonomic data, there is clear distinctions established between strain NCCP-602^T and other closely related validly classified members within genus *Brevibacterium*. Therefore, results of these studies support the recognition of this strain as a novel species within genus *Brevibacterium,* for whichas the name *Brevibacterium metallidurans* sp. nov. has been proposed with NCCP-602^T has been designated as the type strain of *Brevibacterium metallidurans.*

Description of *Brevibacterium metallidurans* sp. nov.

Brevibacterium metallidurans (me.tal.li.du'rans. L. neut. n. metallum, metal; L. pres. part. durans, enduring; N.L. part. adj. metallidurans, enduring high metal concentrations).

The cells possess distinctive features characterized by their gram-positive, aerobic, non-motile, absence of spore formation, and with a rod-shaped morphology, (diameters ranging from 1.57 to 0.45 μm). On tryptic soy agar, colonies were circular and small, exhibited smooth and shining surfaces, had a convex structure, and presented a whitish appearance with entire margins and a somewhat sticky texture. After thriving across a broad range of pH values (6–10), cells exhibited optimal growth at pH 7–8. Strain is adaptable to a broad temperature range of 20–42°C, with optimal growth observed at 30–37°C. Additionally, strain has the capacity to withstand NaCl concentrations ranging from 0 to 15% (w/v), with optimal growth achieved at 1% NaCl. Remarkably, the strain also displays resistance to heavy metals, including arsenic (As), chromium (Cr), copper (Cu), lead (Pb), and cadmium (Cd), at high concentrations. Moreover, it can neither hydrolyse starch nor degrade tween 20, 40, 60 or 80, however it showed a positive reaction to α-glucosidase, Voges Proskauer, nitrate reduction to N_2, assimilation of glucose, adipic acid, *N*-acetyl-glucosamine, trisodium citrate, acid phosphatase, potassium gluconate, hydrolysis of gelatin and malate, citrate utilization, arabinose, phenylacetic acid, maltose, and L-arabitol whereas negative reactions for melezitose and glycerol. The strains can use L-alanine, citrate, inosine, and D-serine as carbon sources and are positive for nalidixic acid, aztrename, sodium bromate, and potassium tellurite (Biolog). The predominant polar lipids include diphosphatidylglycerol, glycolipids, and a minor amount of amino lipids and unknown phospholipids. Major cellular fatty acids included anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, and $C_{17:0}$, with anteiso-$C_{17:0}$. MK-7 and MK-8 are identified as dominant respiratory quinones. DNA G + C content was determined to be 67.6 mol%.

Strain NCCP-602^T (JCM 18882^T = CGMCC1.62055^T) was isolated from wastewater collected from Sialkot, Sambrial, Pakistan. Accession number of 16S rRNA gene sequence was AB920787 submitted in DDBJ/EMBL/GenBank. The whole-genome shotgun sequencing project for strain NCCP-602^T has been archived in GenBank under accession numbers BioProject: PRJDB17310, BioSample: SAMD00729978, and WGS: BAAAAF010000001-BAAAAF010000083.

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Author's contribution Credit authorship contribution statement Conceptualization, IA, MA and SA; Methodology, SM, SA, HCW, and IA; Formal analysis, SA, SM and IA; Software, SM, SA, SZ and IA; Data curation, SM, SA and IA; Project administration, Resources and Funding acquisition: IA, WJL; Supervision, IA, WJL. MA; Writing – original draft, SM, and IA; Writing – review & editing, SM, SA, SZ, HCW, MX, MA, WJL and IA.

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Data availability Accession number of 16S rRNA gene sequence of strain NCCP-602^T was AB920787 submitted in DDBJ/EMBL/GenBank. The whole-genome shotgun sequencing project for strain NCCP-602^T has been archived in GenBank under accession numbers BioProject: PRJDB17310, BioSample: SAMD00729978, and WGS: BAAAAF010000001-BAAAAF010000083 .

Declarations

Conflict of interest The authors declare no competing interests.

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