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Genome mining and metabolite profiling illuminate the taxonomy status and the cytotoxic activity of a mangrove-derived *Microbacterium alkaliflavum* sp. nov

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Abstract

The genus *Microbacterium* in the phylum *Actinomycetota* contains over 100 species to date that little is known about their bioactive metabolites production. In this study, a mangrove sediment-derived strain B2969^T was identified as a novel type strain within the genus *Microbacterium* due to the low 16S rRNA gene sequence similarity (< 99%), and low overall genome relatedness indices (ANI, 75.4%-79.5%; dDDH, 18.5%-22.7%, AAI, 68.7%-76.3%; POCP, 48.3%-65.0%) with the validly named species of the genus. The type strain B2969^T (= MCCC 1K099113^T = JCM 36707^T) is proposed to represent *Microbacterium alkaliflavum* sp. nov.. The crude extracts of strain B2969^T showed weak cytotoxicity against NPC cell lines TW03 and 5-8F, with IC_{50} values of ranging from 3.5 μg/μL to 2.4 μg/μL respectively. Genome analysis of strain B2969^T found 8 clusters of genes responsible for secondary metabolite biosynthesis, including cytotoxic compounds desferrioxamines. In addition, the application of liquid chromatography tandem mass spectrometry (LC–MS/MS)-based molecular networking strategy led to the identification of 10 compounds with potent cytotoxic activity in ethyl acetate extracts of strain B2969^T. Results from the cytotoxicity assay, genome mining, and metabolite profiling based on LC–MS/MS analysis revealed its ability to produce bioactive compounds.

Background

Mangrove ecosystems are largely unexplored sources of *Actinomycetota*, which represent potential important reservoirs of bioactive compounds. The genus *Microbacterium* in the phylum *Actinomycetota* contains over 100 species to date that little is known about their bioactive metabolites production. In this study, a novel species, namely B2969^T, within the genus *Microbacterium* that showed cytotoxicity against nasopharyngeal carcinoma (NPC) cell

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lines was isolated from mangrove sediments. Genome mining and metabolic profiling analyses were explored here to assess its biosynthetic potential of metabolites with cytotoxic properties.

Results

Here, a mangrove sediment-derived strain B2969^T was identified as a novel species within the genus *Microbacterium* due to the low 16S rRNA gene sequence similarity (< 99.0%), and low overall genome relatedness indices (ANI, 75.4%-79.5%; dDDH, 18.5%-22.7%, AAI, 68.7%-76.3%; POCP, 48.3%-65.0%) with the type strains of this genus. We proposed that strain B2969^T represents a new species, in which the name *Microbacterium alkaliflavum* sp. nov. is proposed. The strain showed weak cytotoxicity against NPC cell lines TW03 and 5-8F, with IC_{50} values of ranging from 3.512 μg/μL to 2.428 μg/μL respectively. Genome analysis of strain B2969^T found 8 clusters of genes responsible for secondary metabolite biosynthesis, including desferrioxamines. In addition, the application of liquid chromatography tandem mass spectrometry (LC–MS/MS)-based molecular networking strategy led to the identification of 10 potent cytotoxic compounds in ethyl acetate extracts of strain B2969^T.

Conclusions

This study confirmed the taxonomy status of type strain B2969^T (= MCCC 1K099113^T = JCM 36707^T) within the genus *Microbacterium*, in which the name *Microbacterium alkaliflavum* sp. nov.. Results from the cytotoxicity assay, genome mining, and metabolite profiling based on LC–MS/MS analysis revealed its ability to produce bioactive substances, providing sufficient evidence for the potential of *Microbacterium* species in the discovery of novel pharmaceuticals.

Keywords *Microbacterium*, Cytotoxicity, Genome mining, Metabolite profiling

Background

Actinomycetes are considered as remarkable source of bioactive compounds with significant therapeutic applications [1]. To date, more than 350 genera of *Actinomycetota* were reported [2], and *Streptomyces* is the most studied genus. Although *Streptomyces* species produced approximately two-thirds of clinically used antibiotics between 1950 and 1970s [3], they have recently become more difficult for researchers to obtain novel compounds for the development of new drugs. Compared with *Streptomyces*, other members in the phylum *Actinomycetota* with lower isolation rates were usually termed as "rare actinomycetes". The discovery of antibiotics erythromycin, vancomycin, rifamycin, gentamicin, and salinosporamide A from "rare actinomycetes" have brought great attention for these genera [4]. As the isolation frequency of rare actinomycetes has increased over the past few decades, the number of bioactive compounds derived from them has risen from 125 to more than 2500 [4–6]. Thus, the isolation of novel taxa of rare actinomycetes could definitely expand the possibility of discovering potentially novel bioactive compounds.

The genus *Microbacterium* belongs to the family *Microbacteriaceae* in the phylum *Actinomycetota* that comprised 154 species with validly published name at present [2]. Members of this genus are typically Gram-positive and rod-shaped, and many of them produce yellow colonies on agar plates [7, 8]. *Microbacterium* species have been detected from diverse habitats and play vital roles in the environment, such as soil organic substances degradation [9, 10], plant growth promotion [11], and polysaccharide-production [12]. Despite the fact that *Microbacterium* species produced a very limited number of secondary metabolites [13–15], their biosynthetic potential remains vastly understudied. This point can be illustrated by the fact that crude extracts of some *Microbacteirum* species isolated from macroalgae *Laminaria ochroleuca* [16], deep-sea [17], and mangrove sediments [18] exhibited antimicrobial and cytotoxic activities.

During the screening of rare actinomycetes with anti-nasopharyngeal carcinoma (NPC) cell lines activity from mangrove sediments, a novel *Microbacterium* species, designated as strain B2969^T (= MCCC 1K099113^T = JCM 36707^T) was isolated. The taxonomic position of the isolated strain within the genus *Microbacterium* was determined using polyphasic approach. To gain insight into the potential cytotoxic compounds derived from strain B2969^T, genome mining and metabolite profiling based on LC–MS/MS analysis were applied in this study. This is of particular interest as it may provide valuable insights into the biological and genome characteristic of these rare actinomycetes associated with antibiotics chemicals.

Results and discussion

Phylogenetic analysis

The strain B2969^T exhibited high sequence similarities to the type strains of species within the genus *Microbacterium*, showing similarity of 98.8% to *M. ginsengisoli* DSM 18659^T and similarities of 96.7%–98.7% to other *Microbacterium* members. The 16S rRNA gene phylogenetic tree also showed that strain B2969^T formed a stable

monophyletic cluster with *M. ginsengisoli* DSM 18659 ^{T} (Fig. S1). However, the ANI and dDDH values between strain B2969^{T} and *M. ginsengisoli* DSM 18659 ^{T} were 76.97% and 20.70%, respectively, which were lower than the highest ANI and dDDH values of this strain with *M. ulmi* JCM 14282 ^{T} (ANI, 79.5% and dDDH, 22.7%). Values of ANI and dDDH between strain B2969^{T} and its closely related species all were well below the threshold for species demarcation (Table S1, 95–96% for ANI values and 70% for dDDH values) [19, 20], indicating a significant divergence between B2969^{T} and other *Microbacterium* species. The strain B2969^{T} shared the highest AAI (76.3%) with *M. ulmi* JCM 14282 ^{T}, while it exhibited the highest POCP (65.0%) with *M. pumilum* JCM 14902 ^{T} (Table S1). These values were higher than the proposed genus boundaries with a POCP of > 50% and an AAI of > 60–65% [20], suggesting that the strain B2969^{T} should be placed in the genus *Microbacterium*. The taxonomic position of strain B2969^{T} was further confirmed to be a novel species due to the distinct lineage within the genus *Microbacterium* in the phylogenomic analysis (Fig. 1).

Morphological and physiological properties

The morphological properties of strain B2969^{T} were typical of members of the genus *Microbacterium*, such as rod-shaped, non-spore-forming, and non-motile (Fig. 2) [21, 22]. Colonies were pale yellow on modified ISP2 agar (Fig. 2) and marine agar 2216 plates. Growth could be observed at temperatures of 28, 30, 35, 37, and 50 °C (optimum 28 °C), and pH 4–11 (optimum pH 7). The strain exhibited growth across a NaCl concentration ranging from 1 to 3% NaCl (w/v, optimal at 1%) on modified ISP2 agar. According to the API ZYM assay, the strain B2969^{T} had the following enzyme activities: esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase (weak), β-galactosidase (weak), α-glucosidase, β-glucosidase, and N-acetyl-β-glucosamidase. Activities of alkaline phosphatase, valine arylamidase, cystine arylamidase, trypsin, β-glucoronidase, α-manosidase, and α-fucosidase were negative. It was able to produce acid from L-arabinose, D-ribose (weak), D-xylose, methyl β-D-xylopyranoside, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyl-D-glucosamine (weak), amygdalin (weak), arbutin (weak), esculin, salicin (weak), D-cellobiose, D-maltose (weak), D-melibiose (weak), D-saccharose, D-trehalose (weak), D-raffinose, glycogen (weak), and 5-ketogluconate in API 50CH test. Assimilation of glycerol, meso-erythritol, D-arabinose, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-lactose, inulin, D-melezitose, starch, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, and 2-ketogluconate were negative. The cellular fatty acids (> 10%, Table S2) of strains B2969^{T} were $C_{15:0}$ anteiso (42.2%), $C_{16:0}$ iso (16.1%), $C_{17:0}$ anteiso (11.1%), and $C_{17:0}$ iso (10.4%). The strain B2969^{T} could be differentiated from its closely related species *M. arborescens* DSM 20754 ^{T} and *M. ginsengisoli* DSM 18659 ^{T} due to different phenotypic and chemotaxonomic characteristics listed in Table 1.

Cytotoxic activity of strain B2969^{T}

Nasopharyngeal carcinoma (NPC) was one of the most prevalent malignancies that occurs in the head and neck region [23]. NPC showed a remarkable geographic and ethnic prevalence that is endemic to southern China (Guangdong, Guangxi, Hainan, and Fujian) [24]. A majority of natural products with NPC cells inhibition activity were derived from plant, and only a few discovering from fungi and *Streptomyces* [25–27]. Extracts from some *Microbacterium* species had been reported to display low cytotoxic activity against HepG2, HT-29, Ca Ski, and T47-D cells [17, 28, 29]. However, the anti-NPC activity of rare actinomycetes, including *Microbacterium*, has not been investigated. In the Cell Counting Kit-8 (CCK-8) cell viability assay, the ethyl acetate extracts of strain B2969^{T} showed inhibition against NPC cell lines TW03 and 5-8F with half maximal inhibitory concentration (IC_{50}) values of 3.512 μg/μL and 2.428 μg/μL, respectively (Fig. 3). The viability of 5-8F cells was significantly reduced at the concentration of 1 μg/μL, while there was no remarkable reduction of the viability TW03 cells at this concentration (Fig. 3). Similarly, the extracts of *M. mangrovi* MUSC 115 ^{T} was found to be showed low cytotoxicity towards human cervical carcinoma cell lines (Ca Ski) and human colon cancer cell lines (HT-29) (> 200 μg/mL) [29]. In general, the low cytotoxicity activity could be due to the low production and variety of bioactive secondary metabolites generated by *Microbacterium* species.

Genome features of strain B2969^{T}

The genome assembly of strain B2969^{T} contained 35 scaffolds with a draft genome size of 5,169,222 bp and a GC content of 69.92%. A total of 4,586 protein-coding genes, 47 tRNAs, and 4 sets of rRNA genes (1, 5S; 2, 16S; 1, 23S rRNAs) were predicted in this genome. Classification based on the Clusters of Orthologous Group (COGs) of proteins analysis revealed that more than 10% of the sequences were attributed to carbohydrate transport and metabolism, transcription, general function prediction only, and amino acid transport and

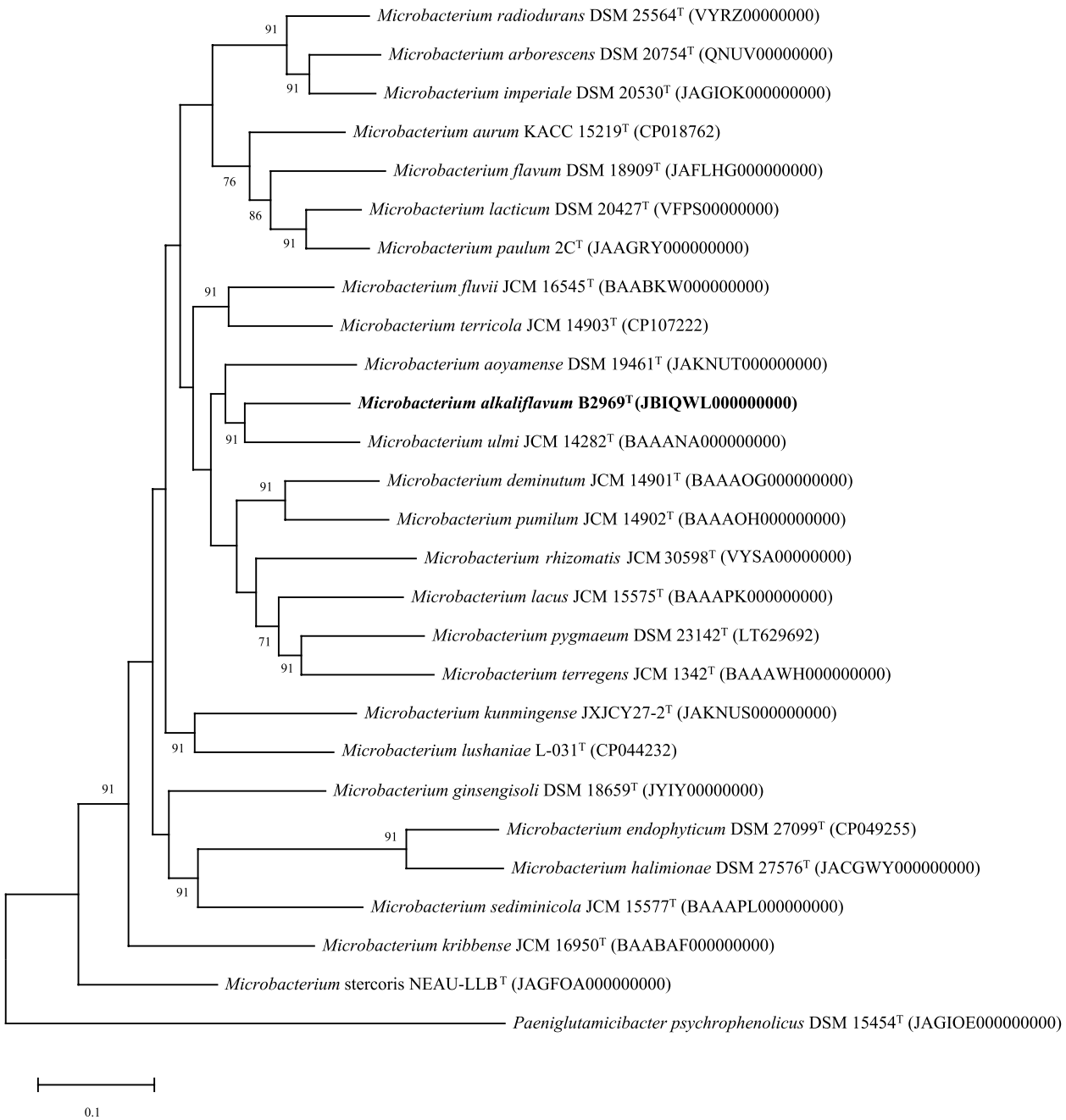

Fig. 1 A core gene phylogenomic tree generated with UBCG, showing the phylogenetic position of strain B2969^T within the genus *Microbacterium*. Gene support indices (GSI) of above 70% are given at the nodes. Strain *Paeniglutamicibacter psychrophenolicus* DSM 15454^T (JAGIOE000000000) was served as an outgroup. Bar, 0.1 substitutions per site

metabolism, respectively (Fig. S2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (Fig. S2) showed that approximately 74.1% genes involved in metabolism, including carbohydrate metabolism (319), amino acid metabolism (236), energy metabolism (142), and metabolism of cofactors and vitamins (148). The genome contains 231 genes encoding CAZymes, which were classified into auxiliary activities (AAs), carbohydrate esterases (CEs), glycoside hydrolases (GHs), and glycosyltransferases (GTs). Among them, genes encoding GHs enzymes occupy the largest proportion. This is consistent with a

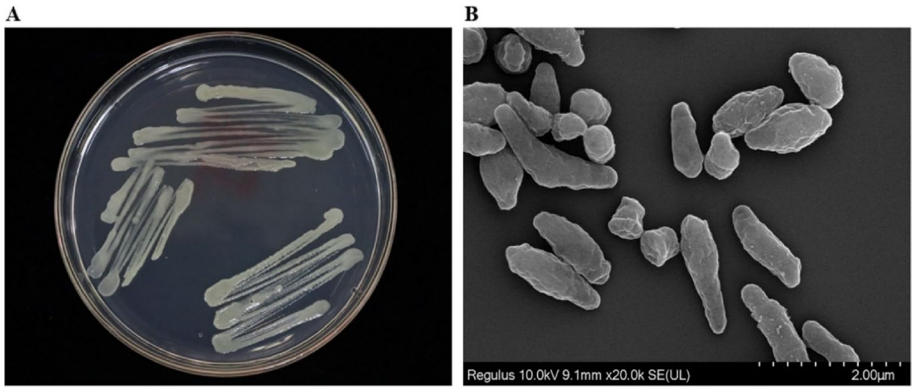

Fig. 2 Morphology of strain B2969^T. **A** Bacteria cultured on the modified ISP2 agar plate for 7 days. **B** The SEM micrograph of cells. Bar, 2 μm

previous study that detected a high number of GH families in several *Microbacterium* species [30].

Mangrove forests located in the interface between land and sea that appear to possess a remarkable capacity to retain heavy metals, enrich organic matter, and accumulate toxic pollutants [31, 32]. In view of this, mangrove microorganisms evolved the ability to tolerate high concentration of heavy metals, decompose organic matter, take part in cycling of nitrogen, sulfur, and phosphorous [32–34]. In the genome of strain B2969^T, genes coding for resistance mechanism to copper and arsenic were identified (Table S3). In addition, the genome contains a series of genes involved in nitrification and phosphate solubilization, which enable strain B2969^T to utilize nitrogen and phosphorus and thereby supporting plant growth (Table S4) [35]. *Microbacterium* species have been found to grow chemolithotrophically with thiosulfate as an energy source [36]. The Sox (*soxABXYZ* and *soxCDYZ*) pathways [37], which are known for sulfur oxidation in majority of sulfur-oxidizing bacteria lacking both *soxC* and *soxXYZ*, were detected in the genome of strain B2969^T (Table S5). It is suggested that the isolated strain might explore another way for thiosulfate oxidation/reduction.

Genome mining of biosynthetic gene clusters

The genome mining analysis of strain B2969^T using antiSMASH version 7.1.0 resulted in 8 biosynthesis gene clusters (BGCs) for putative secondary metabolites (Table S6). The type of BGCs included a siderophore, non-ribosomal polyketide synthetases (NRPSs), redox-cofactor, NAPAA, terpene, the modular type III polyketide synthases (T3PKSs), lanthipeptide-class-iv, and betalactone. BGCs encoding the biosynthesis of a siderophore and NAPAA have 100% similarities with published known desferrioxamines and ε-poly-L-lysine BGCs, respectively. Desferrioxamine is a well-known iron chelator and is considered an effective antitumor agent [38], while ε-poly-L-lysine possesses antimicrobial activity and is non-toxic toward human [39]. The gene cluster encoding redox-cofactor production in strain B2969^T shared 54.98%–95.10% amino acid identities to key genes *mftABCDEF* in mycofactocin biosynthesis (Table S7). The biosynthesis pathway of mycofactocin in strain B2969^T comprises three mycofactocin-coupled SDR family oxidoreductase proteins, showing slightly different from that in *Jiangella alkalipila* KCTC 1922 ^{T} and *Mycobacterium tuberculosis* H37Rv^T [40]. Mycofactocin is a member of ribosomally synthesized and post-translationally modified peptides that has been discovered in many actinomycetes. It is considered a class of peptide derived-redox cofactors, which is composed of members such as antioxidant pyrroloquinoline quinone (PQQ), antimicrobial bacteriocin, and topaquinone [41–43].

A putative type III PKS gene *ctg4_41* in strain B2969^T shared 64.96% amino acid sequence identity to a putative naringenin-chalcone synthase from *M. oleivorans*. Naringenin-chalcone synthase encoding gene that involved in the precursor formation of numerous antibiotics and antitumor agents has been frequently detected in fungi and plants [44]. In recent years, naringenin-chalcone synthase and its related product have been reported from actinomycetes, such as *Streptomyces* and *Saccharopolyspora* [45, 46], but little is known in *Microbacterium*. Although bacterial type III PKSs typically shared less than 50% amino acid identity, members from different families can produce the same secondary metabolites [47]. The phylogenetic analysis based on the type III PKSs revealed a close relationship between Ctg4_41 and the type III PKSs CepA (Fig. 4). Since CepA was found to be related to the production of alkylresorcinols or alkylpyrones in *Candidatus* Entotheonella serta TSWA1 [48], we expected strain B2969^T to produce the same or similar compounds. The bioinformatic analysis predicted gene

Table 1 Differential phenotypic characteristics between strain B2969^T and its phylogenetically closest relatives. Strains: 1, strain B2969^T; 2. *M. arborescens* DSM 20754^T; 3. *M. ginsengisoli* DSM 18659^T. All data for *M. ginsengisoli* DSM 18659^T are from Park MJ et al. [22]. All strains produced acids from D-xylose, D-glucose, D-fructose, D-mannose, D-mannitol, esculin, D-cellobiose, D-maltose, and D-trehalose, and do not produced acids from meso-erythritol, D-arabinose, L-xylose, D-adonitol, dulcitol, inositol, D-sorbitol, methyl *α*-D-glucopyranoside, inulin, D-fucose, L-fucose, D-arabitol, L-arabitol, and 2-ketogluconate. All strains were positive for acid phosphatase and negative for alkaline phosphatase, lipase (C14), *β*-glucoronidase, *α*-manosidase, and *α*-fucosidase. +, Positive; −, negative; w, weakly positive; Tr, fatty acids that accounted for < 1.0% of the total in each strain

Characteristic	1	2	3
Colony color	yellow	orange	yellow
Growth at 37℃	+	-	-
NaCl tolerance for growth	1–3%	1–5%	1–4%
pH range for growth	5–11	5–11	5–9
Acid production from:			
glycerol	-	w	+
L-Arabinose	+	+	-
D-Ribose	w	w	-
Methyl *β*-D-xylopyranoside	+	+	-
D-Galactose	-	+	-
L-Sorbose	-	+	-
L-Rhamnose	-	+	+
Methyl *α*-D-mannopyranoside	-	+	-
N-Acetyl-D-Glucosamine	w	+	-
Amygdalin	w	+	-
Arbutin	w	+	-
Salicin	w	+	-
D-Lactose	-	+	-
D-Melibiose	w	-	-
D-Melezitose	-	+	-
D-Raffinose	+	+	-
Starch	-	+	-
Glycogen	w	+	-
Gentiobiose	-	+	-
D-Turanose	-	+	+
D -Lyxose	-	+	-
D-Tagatose	-	+	-
Potassium gluconate	-	+	-
5-Ketogluconate	+	+	-
Enzymic activities			
Esterase (C4)	+	-	+
Esterase lipase (C8)	+	-	+
Leucine arylamidase	+	-	+
Valine arylamidase	-	+	+
Cystine arylamidase	-	+	-
Trypsin	-	+	+
α-chymotrypsin	+	-	-
Naphthol-AS-BI-phosphohydrolase	+	+	-
α-Galactosidase	w	-	-
β-Galactosidase	w	-	+
α-Glucosidase	+	-	+
β-Glucosidase	+	-	+
N-acetyl-*β*-glucosamidase	+	-	+
Major fatty acids			
anteiso-$C_{15:0}$	42.2%	-	32.8%
iso-$C_{16:0}$	16.1%	-	19.5%
anteiso-$C_{17:0}$	11.1%	63.4%	40.4%
iso-$C_{17:0}$	10.4%	Tr	1.3%
$C_{16:0}$	Tr	16.4%	1.8%

(*ctg9_128*) encoding lanthionine synthetase LanC family protein (51.06% similarity) in a putative lanthipeptide gene cluster. Lanthionine synthetases represent remarkable biocatalysts yielding conformationally constrained peptides with various biological activities [49]. Furthermore, the putative BGCs related to NRPS and betalactone did not show significantly similarity with known clusters in MIBiG database, proposing that they might be associated with the production of novel compounds.

Chemical composition of crude extract analysis

The combination of LC–MS/MS and GNPS-based molecular networking analyses identified 10 potentially cytotoxic compounds in the crude extracts of strain B2969^T (Table S9). Consistent with the predicting result obtained by the antiSMASH analysis, the ion corresponding to desferrioxamine B ($[M+H]^+$, m/z 561.3947) was also detected in the extracts. Molecular ions at m/z 197.1274, 211.1429, 245.1272, and 261.1222 $[M+H]^+$ were identified as diketopiperazines cyclo(Val-Pro), cyclo(Pro-Leu), cyclo(Phe-Pro), and cyclo(Pro-Tyr), respectively, which had been proved to possess different levels of cytotoxicity [50–52]. The ion at m/z 298.0951 $[M+H]^+$ was identified as 5'-methylthioadenosine, a sulfur-containing nucleoside present in prokaryotes, yeast, and higher eukaryotes, which showed cytotoxic against various cancer cell lines [53]. It is noteworthy that some $[M+H]^+$ ion peaks were annotated as previously known cytotoxic natural products in prokaryotes, such as 8-hydroxyquinoline (m/z 146.0593), bonactin (m/z 401.2517), mefenamic acid (m/z 242.1165), and norharman (m/z 169.0751) [54–57] (Fig. 5). However, these compounds have not been isolated from *Microbacterium* species and it was not known which molecules conferred the anti-NPC ability of the strain. Thus, further studies are needed to determine the structure of these secondary

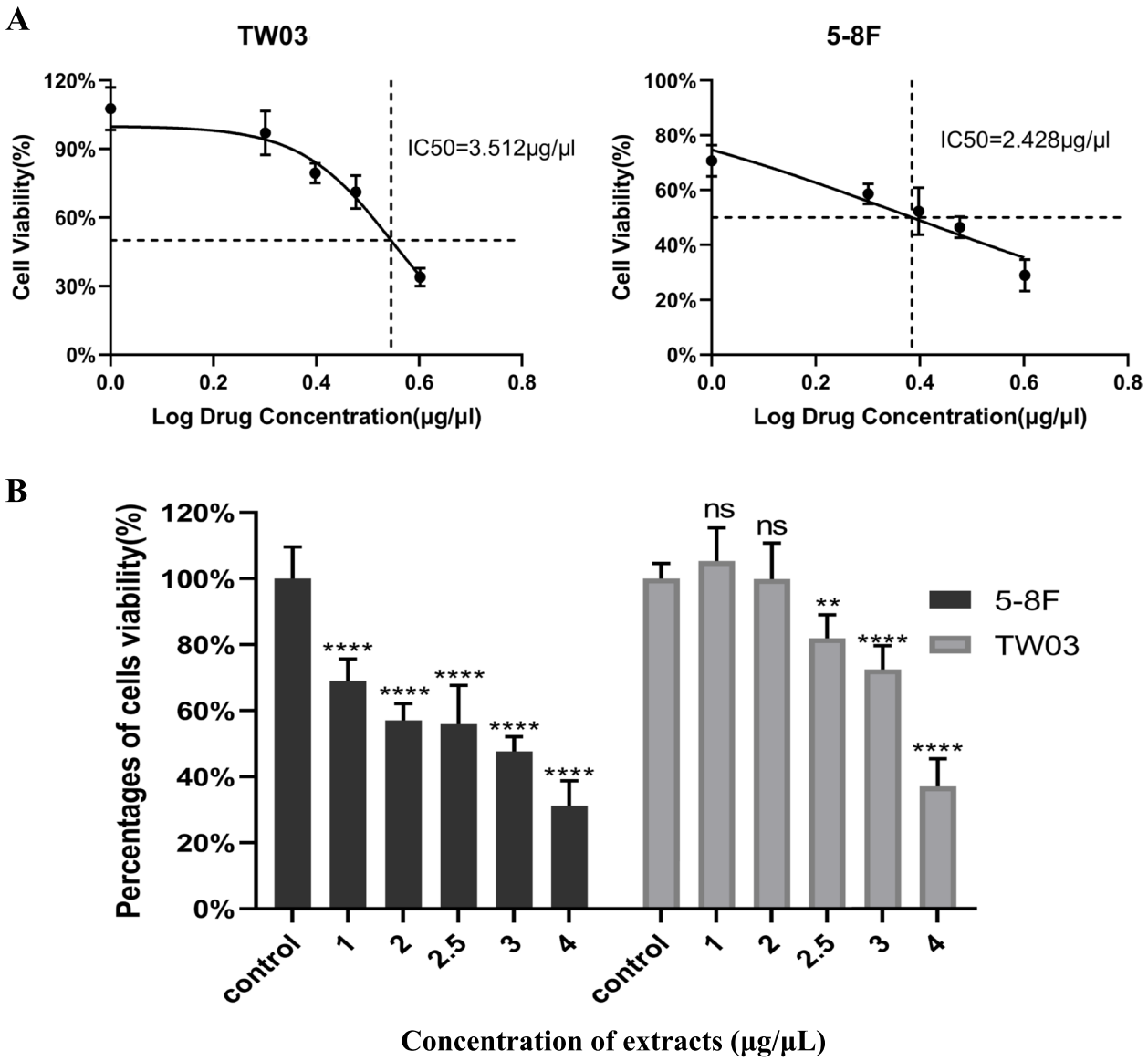

Fig. 3 The cytotoxic activity of ethyl acetate extracts of strain B2969.^{T} on the cell viability of TW03 and 5-8F. **A** The IC_{50} value of extracts; **B** The different concentration of extracts on the viability of TW03 and 5-8F cells. ns, $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

metabolites and their cytotoxic activity against NPC cell lines.

Conclusion

The strain B2969^{T} isolated from mangrove sediments was classified into the genus *Microbacterium* as a novel type strain on the basis of the polyphasic approach. The genome analysis of the strain B2969^{T} highlighted its significance in terms of heavy metal resistance, nitrogen fixation, and phosphorus solubilization. The crude extracts of strain B2969^{T} exhibited low activity against NPC cell lines. Both genomic analysis and metabolite profiling support that strain B2969^{T} is able to produce compounds with diverse structures and can be considered as a promising source for exploring bioactive compounds.

Description of Microbacterium alkaliflavum sp. nov.

Microbacterium alkaliflavum (al.ka.li.fla'vum. N.L. n. *alkali*, alkali; L. masc. adj. *flavus*, yellow; N.L. neut. adj. *alkaliflavum*, growing in the alkaline conditions and yellow).

Cells are Gram-stain-positive, non-spore-forming, and rod-shaped (0.4–0.6 μm in width and 1.5–2 μm in length). Growth was detected on modified ISP2 agar,

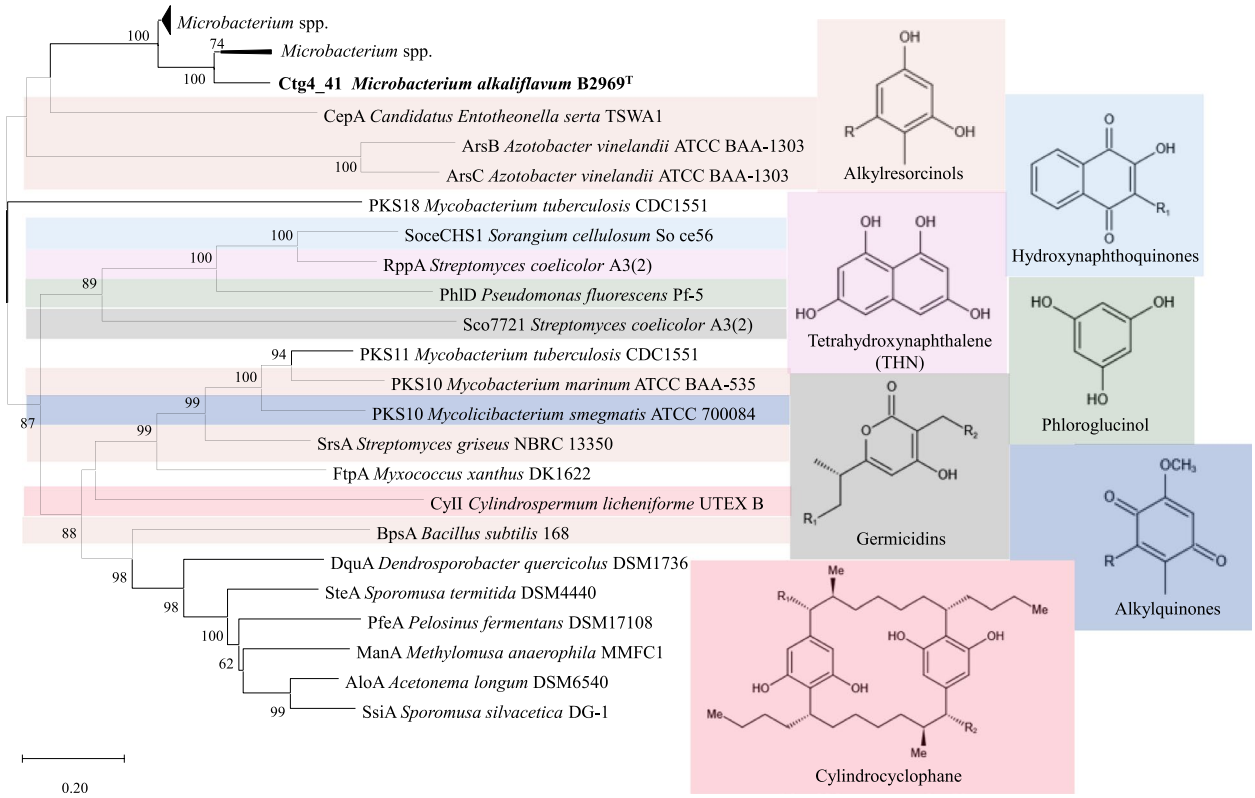

Fig. 4 The phylogenetic tree based on type III polyketide synthases of strain B2969^T and characterized enzymes. Accession numbers of characterized enzymes can be found in Table S8. Colored blocks highlight distinct families of type III PKSs and their related products

R2A agar, and marine agar 2216, but not on Am2ab and modified ISP7 agar. The temperature range for growth is from 28 to 50 °C (optimum 28 °C), pH range is from 5 to 11.0 (optimum pH 7-8), and NaCl concentration range is from 1 to 3.0% (optimum 1%). In the API ZYM test strip, the strain is positive for hydrolysis esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, and N-acetyl-glucosamine, but it negatives for the hydrolysis of alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, β-glucoronidase, α-manosidase, and α-fucosidase. In the API 50CH test system, strain B2969^T is found to be positive for L-arabinose, D-xylose, methyl *β*-D-xylopyranoside, D-glucose, D-fructose, D-mannose, D-mannitol, esculin, D-cellobiose, D-sacharose, D-raffinose, and 5-ketogluconate, but it negatives for glycerol, meso-erythritol, D-arabinose, L-xylose, D-adonitol, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-lactose, inulin, D-melezitose, starch, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, and 2-ketogluconate.

The type strain B2969^T (=MCCC 1K099113^T=JCM 36707 ^{T}) was isolated from mangrove sediments and has a genome size of 5,169,222 bp with a GC content of 69.92%. The GenBank accession numbers of genome and 16S rRNA gene sequence are JBIQWL000000000 and PQ144666, respectively.

Methods

Strain isolation

Strains B2969^T was isolated from mangrove sediments collected at the Beibu Gulf (21°53′15″N, 108°31′25″E) in Guangxi Zhuang Autonomous Region. Soil sample (1 g) was added to 100 mL and 1000 mL sterilized water to prepare soil dilutions (10^{-2} and 10^{-3} g/mL). Afterward, 100 μL of the diluent was plated on the modified International *Streptomyces* Project 2 (ISP2, yeast extract 0.2% w/v, malt extract 0.2% w/v, D-(+)-glucose anhydrous 0.2% w/v, agar 1.5% w/v, water 1L) medium using the standard dilution plating method. After incubating at 28 °C for 7 days, individual colonies with distinct morphology were selected and purified. Pure cultures were stored for long-term at −80 °C in 20% (v/v) glycerol. The strain B2969^T was deposited in the Marine Culture Collection of China (MCCC) and Japan Collection

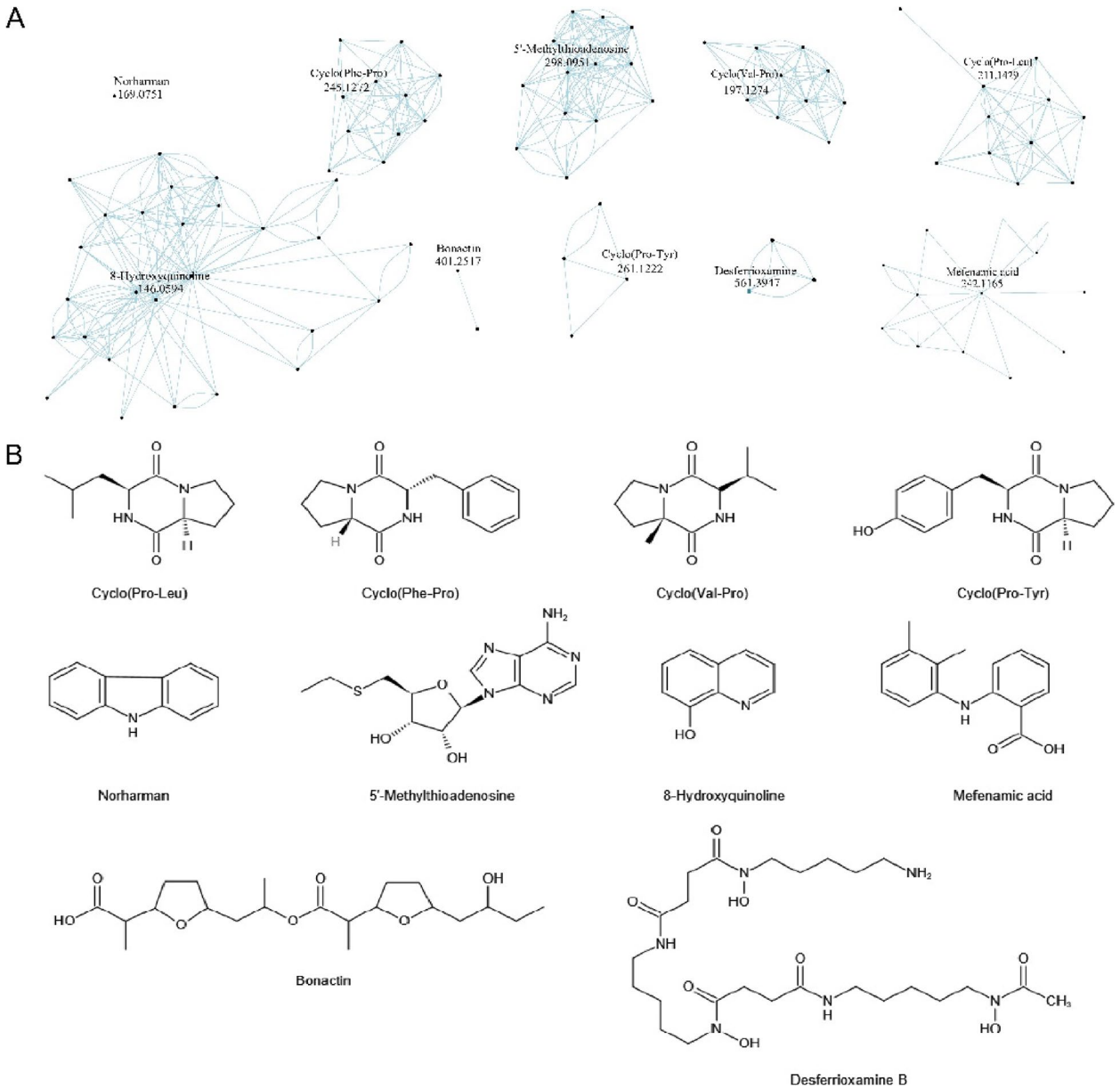

Fig. 5 Molecular network analysis of the metabolites in the crude extracts of strain B2969^T (**A**) and the chemical structure of identified compounds (**B**). The blue nodes represented compounds identified by LC-HRMS and antiSMASH analysis, and black nodes represented compounds only identified by LC-HRMS

of Microorganisms (JCM). Clinical trial number: not applicable.

Phylogenetic analysis

The genomic DNA of strain B2969^T was isolated using TIANamp Genomic DNA kit (TIANGEN). The 16S rRNA gene was amplified using a universal primer pair 27F and 1492R. The 16S rRNA gene sequences were obtained according to the methods described by Hu et al. [58] and then compared with publicly available sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) and EzBioCloud online pairwise sequence alignment tool [59]. Phylogenetic trees were set up using the neighbour-joining (NJ), maximum-likelihood (ML), and minimum-evolution (ME) methods in MEGA X software [60]. The topologies of all trees were determined using the bootstrap resampling method based on 1000 replications [61]. A phylogenomic tree based on the concatenated alignments of 92 bacterial core genes from genomes of strain B2969^T and its closely related type

strains was reconstructed with the UBCG (Up-to-date Bacterial Core Gene) [62].

Genome sequencing and comparative genomics

The whole genome of strain B2969^T was performed using Hiseq 2500-PE150 platform and Illumina NovaSeq at Majorbio Bio-pharm Technology Co., Ltd (Shanghai, PR China). The assembly of good-quality paired reads were performed using the SOAP denovo software (v2.04) [63]. The obtained genome sequences were annotated by the NCBI Prokaryotic Genome Annotation Pipeline. The predicted genomic coding sequences were annotated through the comparison of protein sequences in six databases (Non-Redundant Protein Sequence database, Pfam database, Gene Ontology database, Clusters of Orthologous Groups database, Swiss-Prot database, and Kyoto Encyclopedia of Genes and Genomes database). The putative genes encoding for the Carbohydrate-active enzyme (CAZyme) were identified and annotated using dbCAN3 server with HMMER search against the dbCAN CAZyme database and default parameters [64]. Values of average nucleotide identity (ANI) and the digital DNA-DNA hybridization (dDDH) were determined based on genome sequences using the ANI calculator tool from the EzBioCloud [65] and the Genome-to-Genome Distance calculator [66], respectively. The average amino acid identity (AAI) was determined using EzAAI (1.2.2), and the percentage of conserved proteins (POCP) was calculated according to Qin [67].

Genome mining

Genome mining for biosynthetic gene clusters (BGCs) of secondary metabolites was carried out using the antiSMASH bacterial version 7.0 with default parameters [68]. The function of putative biosynthetic genes was further analyzed with an online BLAST program (http://blast.ncbi.nlm.nih.gov/). The identity and similarity values between biosynthetic pathway-related proteins and their homolog proteins were determined using an online Protein BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi? PROGRAM = blastp).

The amino acid sequence of putative type III polyketide synthase (PKS) in strain B2969^T was used as a template for a BLAST-p search against available bacterial genomes to identify type III PKSs. Amino acid sequences of all type III PKS proteins were aligned using the MUSCLE algorithm in MEGA (7.0) [69]. A phylogenetic tree was constructed from these sequences using the neighbor-joining method with 1000 bootstrap replicates in MEGA (7.0). For sequences that were previously characterized, their related products were identified through a literature search (Table S8).

Phenotypic and chemotaxonomic properties

To determine cultural characteristics of strain B2969^T, the strain was cultured at 28 °C on different media, included modified ISP 2 agar, marine agar 2216 (BD Difco), and Reasoner' 2A (BD Difco) agar, as well as frequently used actinomycetes media, Am2ab agar [70] and modified ISP 7 agar [27]. Cell morphology was observed by a scanning electron microscope (SEM, Hitachi S-3400 N II) using cultures of strain B2969^T grown on modified ISP2 medium at 28 °C for 7 days. The strain *M. arborescens* DSM 20754^T was used in this study as reference strain for phenotypic and chemotaxonomic analysis. The tolerance to salinity was tested in modified ISP2 medium supplemented with different concentrations of the NaCl (1–5%, w/v, at interval of 1 unit). Growth at different temperatures (4, 20, 28, 30, 37, and 50 °C) and at various pH ranges (pH 4.0–12.0, at intervals of 1.0 pH unit) were examined in commercial R2A medium. The pH of mediums was adjusted using the buffer systems as described by Hu et al. [58]. Acid production and enzyme activities were analyzed using commercial bioMérieux API 50CH and API ZYM reagent strips according to the guidance of manufacturer. Cellular fatty acids were determined for strains grown on trypticase soy agar for 3 days. The fatty acid methyl esters of cells grown on modified ISP2 agar for 7 days at 28 °C were prepared according to the protocol of the Sherlock Microbial Identification System (MIDI) and then analyzed by gas chromatography (6890; Hewlett Packard) using the Microbial Identification System [71].

Cytotoxic activity assay

Strains B2969^T was cultured in a 500-mL Erlenmeyer flask containing 200 mL of modified ISP2 broth medium. The precipitated cell pellets were removed from the fermentation cultures (1 L) by centrifugation (8000 rpm, 10 min) after 7 days of growth at 28 °C, 180 rpm. The supernatants were extracted three times with ethyl acetate (1:1 v/v). The extracted solution was evaporated to yield a reddish-brown residue. NPC cell lines TW03 and 5-8F were offered by Professor Maria Li Lung (The University of Hong Kong, China) and selected for testing. All cells grown in DMEM (C11995500BT, Gibco, USA) supplemented with 10% FBS (10099141C, Gibco, USA) and 1% Penicillin–Streptomycin (P1400, Solarbio, China). For cytotoxic activity assay, cells were cultured in 96-well plates (5×103 cells/well) overnight (5% CO_2, 37℃). Then, cells were treated with 100 μL of extracted solution (1 mg/mL in 0.1% DMSO). Cells were treated with an equal volume of DMSO were served as control. After treatment for

24 h, 10 μL of CCK-8 reagent (CK04, Dojindo Laboratories, Japan) was added to each well, and all plates were incubated at 37℃ in an CO_2 incubator for 1.5 h. All treatments were conducted in triplicate. The treated and control absorbance value was recorded at 450 nm in a Multiskan GO plate reader (Thermo, USA).

LC–MS analysis of crude extracts

Mass spectra (Fig. S3) were recorded on a LCMS-IT-TOF system (Shimadzu, Kyoto, Japan). The scan range was from 50 to 2000 m/z. The mass number calibration (ion trap and TOF analyzer) was completed using a solution of trifluoroacetic acid (TFA) and sodium hydrate. Ion accumulation time was set to 10 ms and the detector voltage was fixed at 1.6 kV. The ESI source conditions were set as follow: spray voltage, 4.5 kV (positive) or − 3.5 kV (negative); nebulizer gas flow rate, 1.5 L/min; drying gas, 100 kpa; heat block temperature, 200℃; CDL temperature, 200℃; IT Area Vacuum, 1.0×10^{-2} Pa; TOF Area Vacuum, 5×10^{-4} Pa.

Molecular networking analysis

Mass spectrometry data were subjected to MS-Convert software to convert the files to mzXML format and were analyzed using molecular networking in Global Natural Products Social Molecular Networking (GNPS) server [72]. A molecular network for strain B2969^T extracts was constructed from the positive ion mode using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). Molecular networks were imported and visualized using Cytoscape 3.10.2 software (https://cytoscape.org/).

Abbreviations

NPC	Nasopharyngeal carcinoma
ANI	Average Nucleotide Identity
dDDH	Digital DNA-DNA hybridization
CAZymes	Carbohydrate-active enzymes
KEGG	Kyoto encyclopedia of genes and genomes
Sox	Sulfur-oxidation
DMSO	Dimethyl sulfoxide

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

H.W.J. conducted the strain isolation, bioinformatic, and metabolic profiling analyses. D. L. X. performed the cytotoxic assay and data analysis. H. Y. Y. cultures cell lines for experiment. W. X. C. and Q. J. L. collected the mangrove sediments samples. Z. H. J. and Z. X. revised the manuscript. P. X. L., Z. X. Y., and C. J. M. designed the experiment and wrote the manuscript.

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Data availability

The datasets generated and analyzed during the current study are available at the National Center for Biotechnology Information (NCBI). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain B2969 is PQ144666, and the genome sequence is JBIQWL000000000.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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