**Microbacterium helvum** sp. nov., a novel actinobacterium isolated from cow dung

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**Abstract**

A Gram-positive, aerobic, non-motile, non-spore-forming, short rod-shaped strain, NEAU-LLC\(^{\mathrm{T}}\), was isolated from cow dung in Shangzhi City, Heilongjiang Province, Northeast China and identified by a polyphasic taxonomic study. Colonies were light yellow, round, with entire margin. Strain NEAU-LLC\(^{\mathrm{T}}\) was grown at 15–45 °C and pH 6.0–10.0. NaCl concentration ranged from 0 to 5% (W/V). The 16S rRNA gene sequence of NEAU-LLC\(^{\mathrm{T}}\) showed the high similarities with *Microbacterium kyungheense* JCM 18735\(^{\mathrm{T}}\) (98.5%), *Microbacterium trichothecenolyticum* JCM 1358\(^{\mathrm{T}}\) (98.3%) and *Microbacterium jejuense* JCM 18734\(^{\mathrm{T}}\) (98.2%). The whole-cell sugars were glucose, rhamnose and ribose. The menaquinones contained MK-12 and MK-13. Ornithine, glutamic acid, lysine and a small amount of alanine and glycine were the amino acids in the hydrolyzed products of the cell wall. The major fatty acids were iso-C\(_{16:0}\), iso-C\(_{18:0}\), anteiso-C\(_{15:0}\) and anteiso-C\(_{17:0}\). The main polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. The genome of NEAU-LLC\(^{\mathrm{T}}\) was 4,369,375 bp and G+C content is 70.28 mol%. A combination of DNA–DNA hybridization result and some phenotypic characteristics demonstrated that strain NEAU-LLC\(^{\mathrm{T}}\) could be distinguished from its closely related strains. Therefore, the strain NEAU-LLC\(^{\mathrm{T}}\) was considered to represent a novel species, which was named *Microbacterium helvum* sp. (Type strain NEAU-LLC\(^{\mathrm{T}}\) = CCTCC AA 2018026\(^{\mathrm{T}}\) = JCM 32661\(^{\mathrm{T}}\)).

**Keywords** Genome · *Microbacterium helvum* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

**Abbreviations**

- ANI: Average nucleotide identity
- CCTCC: China center for type culture collection
- dDDH: Digital DNA:DNA hybridization
- DPG: Diphosphatidylglycerol
- GC–MS: Gas chromatography–mass spectrometer
- GY: Glucose yeast extract
- ISCC-NBS: Inter-society color council-national bureau of standards
- ISP: International streptomyces project
- JCM: Japan collection of microorganisms
- MEGA: Molecular evolutionary genetics analysis
- PG: Phosphatidylglycerol
- SSA: Sodium succinate–asparagine agar
- SSC: Saline-sodium citrate
- UGL: Unidentified glycolipid

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**Introduction**

*Microbacterium* genus was first proposed by Orla-Jensen (1919). Subsequently, the genus was reclassified by Collins et al. (1983) and then Takeuchi and Hatano (1998) revised the description of the genus and transferred *Aureobacterium* into *Microbacterium*. The authors suggested that although the amino acids in the peptidoglycan of the two genera are
different (lysine in members of the genus *Microbacterium* and ornithine in members of *Aureobacterium*), the intermixed phenotype of the two genera had similar physiological and biochemical properties. Krishnamurthi et al. (2012), Alves et al. (2014) and Fidalgo et al. (2016) emended the description of the genus *Microbacterium*. *Microbacterium* are typically Gram-positive, non-spore-forming and rod-shaped (Collins et al. 1983, Takeuchi and Hatano 1998). *Microbacterium* have been isolated from a variety of environmental sources, including soil (Kageyama et al. 2006), water (Torkko et al. 2000), human blood (Clermont et al. 2009), marine environments (Kageyama et al. 2007), biofilms (Kim et al. 2005), plants (Li et al. 2015), faeces (Chen et al. 2016), sediment (Mawlankar et al. 2015) and other environments. Some strains of *Microbacterium* genus have the ability to degrade crude oil (Schippers et al. 2005) and are halotolerant (Yang et al. 2018) and UV radiation-resistant (Zhang et al. 2010). At the time of writing, the genus containing 122 species effectively released (LPSN, http://www.bacterio.cict.fr/index.html). In this paper, the phenotype and genotype characteristics of a novel were analyzed and its taxonomic place determined by polyphase taxonomic analysis.

**Materials and methods**

**Isolation and maintenance of the organism**

Strain NEAU-LLCT\(^T\) was isolated from cow dung collected from Shangzhi, Heilongjiang Province, Northeast China (45°12′E, 127°57′N). The cow dung samples were ground into powder and then suspended in sterile distilled water followed by a standard serial dilution technique. The diluted cow dung suspension was spread on a plate of sodium succinate–asparagine agar medium (SSA, which contained g

\[\begin{align*}
\text{K}_{2} \text{HPO}_{4} & : 0.6, \text{MgSO}_{4} \cdot 7 \text{H}_{2} \text{O} & : 0.1, \text{CaCl}_{2} \cdot 2 \text{H}_{2} \text{O} & : 0.2, \text{KCl} \cdot 0.3, \text{FeSO}_{4} \cdot 7 \text{H}_{2} \text{O} & : 0.001, \text{agar} & : 20)\end{align*}\]

supplemented with cycloheximide (50 mg l\(^{-1}\)) and nalidixic acid (20 mg l\(^{-1}\)). After 14 days of aerobic incubation at 28 °C, colonies were transferred and purified on International *Streptomyces* Project (ISP) 3 medium (Shirling and Gottlieb 1966) and maintained as glycerol suspensions (20%, v/v) at – 80 °C.

**Phenotypic characteristics**

Morphological characteristics were observed by light microscopy (Nikon ECLIPSE E200) and transmission electron microscope (model JEM1010; JEOL) using cultures grown on ISP 2 medium at 28 °C for 3 days. Color determination was done with ISCC-NBS colour charts Standard Samples No 2106 (Kelly 1964). Hydrolysis of Tweens (20, 40 and 80) and production of urease were tested as described by Smibert and Krieg (1994). Other physiological and biochemical properties were tested with API 20NE and API ZYM strips (bioMérieux) and acid production was tested using the API 50 CH system (bioMérieux) according to the manufacturers’ instructions, using cells grown on ISP 2 medium for 3 days at 28 °C. The utilization of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, coagulation and peptonization of milk, liquefaction of gelatin and production of H\(_2\)S were examined as described previously (Gordon et al. 1974; Williams et al. 1989; Yokota et al. 1993a, b). Growth at different temperatures (10, 15, 18, 20, 25, 28, 32, 35, 38, 40, 42 and 45 °C) was determined on glucose–yeast extract (GY) medium (Jia et al. 2013) after incubation for 14 days. The pH range for growth (pH 4–12, at intervals of 1 pH units) was tested in GY broth. The buffer systems were: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH\(_2\)PO\(_4\)/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO\(_3\)/0.1 M Na\(_2\)CO\(_3\); pH 11.0–12.0, 0.2 M KH\(_2\)PO\(_4\)/0.1 M NaOH (Cao et al. 2020; Zhao et al. 2019). NaCl tolerance was determined in GY broth supplemented with 0–10% NaCl (w/v, with an interval of 1% w/v) at 28 °C for 14 days on a rotary shaker. Growth under anaerobic conditions was tested in ISP2 in Hungate tubes filled with oxygen-free N\(_2\) at 28 °C (Ruan et al. 2014).

**Chemotaxonomic characterization**

Biomass for chemotaxonomic studies was prepared by growing the strain in GY broth in shake flasks at 28 °C for 7 days. Cells were harvested by centrifugation, washed with distilled water twice and then freeze-dried. The preparation of cell wall peptidoglycan in the cell wall was performed in accordance with the work by Komagata and Suzuki (1987). Cell wall amino acids were identified by TLC (Hasegawa et al. 1983) and High-Speed Amino Acid Analyzer (Hitachi LA8080, Japan). The whole-cell sugars were tested according to the procedures developed by Lechevalier and Lechevalier (1980). The phospholipids in cell were examined by two-dimensional TLC and identified using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985) and analyzed by a HPLC–UV method (Wu et al. 1989) using an Agilent Extend-C18 Column (150 × 4.6 mm, i.d. 5 µm), monitored at 270 nm. The mobile phase was acetonitrile/propyl alcohol (60:40, v/v). The strain NEAU-LLCT\(^T\) was incubated in ISP2 broth at 28 °C in shaking flasks for 7 days to determine the fatty acid composition of cells. Fatty acid methyl esters were extracted from the biomass.
as described by Gao et al. (2014) and analyzed by GC–MS using the method of Xiang et al. (2011).

**DNA preparation, amplification and determination of 16S rRNA gene sequence**

Extraction of chromosomal DNA and PCR amplification of the 16S rRNA gene sequence were carried out according to the procedure developed by Kim et al. (2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced using an Applied Biosystems DNA sequencer (model 3730XL). The almost full-length 16S rRNA gene sequence of strain NEAU-LLCT (1514 bp) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using Clustal W algorithm. Phylogenetic trees were generated with the neighbour-joining (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) algorithms using Molecular Evolutionary Genetic Analysis (MEGA) software version MEGA7.0 (Kumar et al. 2016). The stability of the topology of the phylogenetic tree was assessed using the bootstrap method with 1000 replicates (Felsenstein 1985). The distance matrix was generated using Kimura’s two-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Pairwise alignment analysis of 16S rRNA gene sequence similarities between strains were calculated on the EzBioCloud server (Yoon et al. 2017a).

**DNA base composition and DNA–DNA hybridization**

Digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values were employed to further genomically distinguish strain NEAU-LLCT from *M. kyungheense* JCM 18735T and *M. trichothecenolyticum* JCM 1358T (Meier-Kolthoff et al. 2013; Yoon et al. 2017b). In the present study, ANI and dDDH values were determined from the genomes of these three strains using the orthoANIu algorithm from EzBioCloud (Yoon et al. 2017a, 2017b) and the genome-to-genome distance calculator (GGDC 2.0) at http://ggdc.dsmz.de. Because of the lack of the genome sequence of strain *M. jejuense* JCM 18734T, DNA–DNA relatedness test between strain NEAU-LLCT and *M. jejuense* JCM 18734T was carried out by the thermal renaturation method described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983), using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostated 6×6 multiecell changer and a temperature controller with in situ temperature probe (Varian). The concentration and purity of DNA samples were determined by measuring the optical density at 260, 280 and 230 nm. The DNA samples used for hybridization were diluted to OD260 around 1.0 using 0.1×SSC (saline sodium citrate buffer (Thomas et al. 2000), then sheared using a JY92-II ultrasonic cell disruptor (ultrasonic time 3 s, interval time 4 s, 90 times). The DNA renaturation rates were detected in 2×SSC at 70 °C. The experiments were performed with three replications and the DNA–DNA relatedness value was expressed as mean of the three values.

**Results and discussion**

**Phylogenetic analysis**

Identification using the EzBioCloud server revealed that strain NEAU-LLCT belongs to the genus *Microbacterium* and shared high sequence similarities with *M. kyungheense* JCM 18735T (98.5%), *M. trichothecenolyticum* JCM 1358T (98.3%) and *M. jejuense* JCM 18734T (98.2%). The phylogenetic tree based on the 16S rRNA gene sequences indicated that strain NEAU-LLCT formed a cluster with *M. trichothecenolyticum* JCM 1358T (98.3%) and *M. jejuense* JCM 18734T (98.2%) in the neighbor-joining tree (Fig. 1), this relationship also recovered by the maximum likelihood (Fig. S3). Thus, based on the phylogenetic relationship, 16S rRNA sequences similarities, the isolate was mostly related to *M. kyungheense* JCM 18735T, *M. trichothecenolyticum* JCM 1358T and *M. jejuense* JCM 18734T (Fig. 2).

**Phenotypic characteristics**

Morphological observation of NEAU-LLCT strain cultured on ISP 2 medium revealed that the strain has typical characteristics of members of the genus *Microbacterium*. Strain NEAU-LLCT had characteristics shared by all members of the genus *Microbacterium* (Fidalgo et al. 2016). Detailed physiological and biochemical properties, enzyme activity (API 20NE, API ZYM) and the production of acid (API 50CH) are presented in the species description and the differential characteristics of strain NEAU-LLCT and three closely related species of the genus *Microbacterium* are summarized in Table 1. For example, the NEAU-LLCT strain could grow at pH 6.0, while the closely related strain could not. The tolerance of NEAU-LLCT to NaCl was up to 5%, lower than that of *M. kyungheense* JCM 18735T and *M. jejuense* JCM 18734T, but higher than *M. trichothecenolyticum* JCM 1358T. Other phenotypic differences include the hydrolysis of urea and Tweens (20, 40 and 80) and patterns of carbon and nitrogen utilization. The negative characteristics of nitrogen assimilation tests, the enzyme activities (API 20 NE, API ZYM) and the production of acid (API 50CH) for NEAU-LLCT are summarized in Table S3.
Chemotaxonomic characterization

Whole-cell sugars contained glucose, rhamnose and ribose. The peptidoglycan hydrolysates of strain NEAU-LLCT contained glutamic acid, lysine, ornithine, a small amount of alanine and glycine. Through a partial hydrolysis and determination of the amino acid linkage of the fragmented products, the peptidoglycan type was deduced to be most likely B2β type. This must be clearly stated in the manuscript.

The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol and unidentified glycolipids (Fig. S2). The menaquinones were MK-12 (17.9%) and MK-13 (82.1%). The cellular fatty acids of strain NEAU-LLCT were anteiso-C_{15:0} (26.3%), anteiso-C_{17:0} (25.6%), C_{16:0} (4.3%), iso-C_{14:0} (1.6%), iso-C_{16:0} (28.3%), iso-C_{17:0} (1.5%), iso-C_{18:0} (12.1%) and iso-C_{19:0} (0.3%) (Table S1). Detailed fatty acid profiles of strain NEAU-LLCT and the closely related type strains are given in Table S1. All these chemotaxonomic data showed that strain NEAU-LLCT should be assigned to the genus Microbacterium. In addition to the above the phenotypic characteristics and chemotaxonomic characteristics, genotype characteristics of the strain NEAU-LLCT and its closely related strains were also different (Table 1).

Molecular characteristics

To determine whether strain NEAU-LLCT could be considered to represent a new species, DNA–DNA hybridization by the thermal renaturation was carried out with M. jejuense JCM 18734^T (M23411) was used as an outgroup. Asterisks indicate branches also recovered in the maximum-likelihood tree; Bar, 0.0100 substitutions are shown at branch points. Arthrobacter globiformis DSM 20124^T (M23411) was used as an outgroup. Asterisks indicate branches also recovered in the maximum-likelihood tree; Bar, 0.0100 substitutions

![Diagram](https://example.com/diagram.png)
The digital DNA–DNA hybridization levels between NEAU-LLC^T and *M. kyunghee* JCM 18735^T, *M. trichothecenolyticum* JCM 1358^T were 37.1 ± 2.5% and 25.7 ± 2.5%, respectively. These values are below the 70% threshold recommended by Wayne et al. (1987) for assigning strains to the same genomic species. Similarly, the ANI values of NEAU-LLC^T and the two reference strains were 86.36% and 82.41%, respectively, which were lower than the 95%-96% threshold defined by prokaryotic species (Richter and Rossello-Mora 2009; Chun and Rainey 2014). Detailed genomic information and other general features of genome sequences are shown in Table S2.

In conclusion, it is evident from phenotype and genotype data that NEAU-LLC^T strain represents a novel species in the genus *Microbacterium*, for which the name *Microbacterium helvum* is proposed.

**Description of *Microbacterium helvum* sp. nov.**

*Microbacterium helvum* (hel’vum. L. neut. adj. helvum, honey-yellow)

Short rods, about 0.4–1.3 μm in length and 0.4–0.8 μm width. Gram-stain positive, showing aerobic respiratory metabolism. Spores are not observed. Colonies are smooth, circular and light yellow after 3 days at 28 °C on ISP 2 agar. Growth at 15 °C to 45 °C (optimum 28 °C) and in the range of pH 6 to 10 (optimum pH 7.0). Tolerate up to 5% (w/v) NaCl in the culture medium. Positive for hydrolysis of aesculin and starch, production of urease, but negative for coagulation and peptonization of milk, decomposition of cellulose, hydrolysis of Tween 20, 40 and 80, liquefaction of gelatin, production of H₂S and reduction of nitrate. Utilizes d-fructose, d-galactose, d-glucose, d-maltose, d-mannitol, d-mannose, d-ribose, d-sorbitol, dulcitol, d-xylene, inositol, l-arabinose, lactose, l-rhamnose and sucrose as sole carbon sources, creatine, l-asparagine, l-aspartic acid, l-glutamine, l-serine and l-tyrosine as sole nitrogen sources. In API 20NE test strips, positive for hydrolysis adipic acid, aesculin, β-galactosidase, d-glucose, d-maltose, d-mannitol, d-mannose, d-ribose, N-acetyl-glucosamine and urease. In API ZYM tests, positive result in tests for acid phosphatase. Cystine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, lipase (C14), naphthol-AS-BI-phosphohydrolase and valine arylamidase. The phospholipid profile contains diphosphatidylglycerol, phosphatidylglycerol and unidentified glycolipids. Whole-cell sugars are galactose, glucose and ribose. The peptidoglycan of strain NEAU-LLC^T contained glutamic acid, lysine, ornithine, a small amount of alanine and glycine. The menaquinones are MK-12 and MK-13 and the major cellular fatty acids are anteiso-C₁₅:₀, anteiso-C₁₇:₀, iso-C₁₆:₀ and iso-C₁₈:₀. The DNA G+C content of the type strain is 70.28 mol %.

The type strain is NEAU-LLC^T (= CCTCC AA 2018026^T = JCM 32661^T), isolated from cow dung.
Table 1  Differential phenotypic properties between strain NEAU-LLCT and the type strain of the most closely related species of the genus Microbacterium

| Characteristics                              | 1   | 2   | 3   | 4   |
|----------------------------------------------|-----|-----|-----|-----|
| Growth pH                                    | 6–10| 7–11| 7–10| 7–10|
| Tolerance of NaCl (% w/v)                    | 5   | 6   | 2   | 7   |
| Hydrolysis of:                               |     |     |     |     |
| Urea                                         | +   | –   | –   | –   |
| Tweens 20                                    | –   | –   | +   | –   |
| Tweens 40                                    | –   | –   | +   | +   |
| Tweens 80                                    | –   | –   | –   | +   |
| Production of urease                         | +   | +   | –   | –   |
| Production of H2S                            | –   | –   | +   | +   |
| Utilize as sole carbon source:               |     |     |     |     |
| Lactose                                      | +   | –   | –   | +   |
| α-Fructose                                   | +   | +   | +   | +   |
| Inositol                                     | +   | –   | +   | –   |
| Sucrose                                      | +   | –   | +   | –   |
| Raffinose                                    | +   | –   | +   | –   |
| Utilize as sole nitrogen source:             |     |     |     |     |
| Glycine                                      | –   | +   | –   | +   |
| L-Proline                                    | +   | +   | –   | +   |
| L-Threonine                                  | w   | +   | –   | –   |
| L-Arginine                                   | –   | +   | –   | +   |
| Enzyme activity (API20NE):                   |     |     |     |     |
| Arginine dihydrolase                         | –   | +   | –   | +   |
| Urease                                       | +   | +   | –   | –   |
| Malate                                       | +   | –   | +   | –   |
| Adipic acid                                  | +   | –   | –   | –   |
| Glucose fermentation                         | –   | +   | +   | +   |
| Malic acid                                   | –   | +   | +   | +   |
| Potassium gluconate                          | +   | –   | –   | –   |
| Enzyme activity (API ZYM):                   |     |     |     |     |
| Alkaline phosphatase                         | –   | +   | –   | +   |
| α-Chymotrypsin                               | –   | +   | +   | +   |
| Cystine arylamidase                          | +   | +   | –   | +   |
| Esterase (C4)                                | +   | +   | –   | +   |
| Esterase lipase (C8)                         | +   | –   | –   | –   |
| α-Galactosidase                              | +   | –   | –   | +   |
| β-Galactosidase                              | –   | –   | +   | w   |
| α-Glucosidase                                | +   | –   | w   | w   |
| β-Glucosidase                                | –   | –   | w   | w   |
| Valine arylamidase                           | +   | –   | –   | –   |
| Acid production from (API 50 CH):            |     |     |     |     |
| L-Arabinose                                  | +   | –   | –   | +   |
| Inositol                                     | –   | –   | +   | –   |
| N-Acetylglucosamine                          | +   | –   | –   | +   |
| Amygdalin                                    | –   | –   | –   | +   |
| Lactose                                      | –   | +   | –   | –   |
| D-Melibiose                                  | +   | –   | –   | +   |
| Sucrose                                      | +   | –   | +   | –   |
| Trehalose                                    | +   | –   | –   | –   |
| Melezitose                                   | +   | –   | –   | –   |
| Raffinose                                    | +   | –   | +   | –   |
| Glycogen                                     | –   | +   | +   | +   |
| Gentiose                                     | –   | –   | –   | –   |
| Whole-cell sugars                            |     |     |     |     |
| Rha, Glu, Rib                                | Rha, Rib, Glu |   |   |   | |
collected from Shangzhi, Heilongjiang Province, northeast China.

The GenBank accession number for the 16S rRNA gene sequence and the draft genome sequence of the type strain are MW009703 and JACXZS000000000, respectively.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02311-9.

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Author contributions XL performed the laboratory experiments, analyzed the data and drafted the manuscript. LZ contributed to the polyphasic taxonomy. JZ participated to the discussions of each section of experiments. WX and XW designed the experiments and revised the manuscript.

Declarations
Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants and/or animals performed by any of the authors. The formal consent is not required in this study.

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Table 1 (continued) Strains: 1, NEAU-LLC T; 2, M. kyungheese JCM 18735T; 3, M. trichotheconolyticum JCM 1358T and 4, M. jejuense JCM 18734T. +, positive, –, negative, w weakly positive. All tests were performed under similar conditions except Whole-cell sugars, data for which was taken from Kook et al. (2014) and Yokota et al. (1993a, b).
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