

Mixta tenebrionis sp. nov., isolated from the gut of the plastic-eating mealworm *Tenebrio molitor* L.

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Abstract

A bacterial strain, BIT-26^T, was isolated from the gut of plastic-eating mealworm *Tenebrio molitor* L. The taxonomic position of this new isolate was investigated by using a polyphasic approach. Cells of the strain were Gram-stain-negative, facultatively anaerobic, motile rods with peritrichous flagella. The 16S rRNA gene sequence (1412 bp) of strain BIT-26^T showed the highest similarity (97.4%) to *Erwinia piriflorinigrans* CFBP 5888^T, followed by *Citrobacter sedlakii* NBRC 105722^T (97.3%), *Mixta calida* LMG 25383^T (97.3%), *Cronobacter muytjensii* ATCC 51329^T (97.2%) and *Mixta theicola* QC88-366^T (97.2%). The results of phylogenetic analyses, based on the 16S rRNA gene and concatenated sequences of four housekeeping genes (*atpD*, *gyrB*, *infB* and *rpoB*), placed strain BIT-26^T within the genus *Mixta* of the family *Erwiniaceae*. This affiliation was also supported by the chemotaxonomic data. Strain BIT-26^T had similar predominant fatty acids, including C_{12:0}, C_{14:0}, C_{16:0}, C_{17:0} cyclo and C_{19:0} cyclo ω8c, to species of the genus *Mixta*. *In silico* DNA–DNA hybridization and average nucleotide identity calculations plus physiological and biochemical tests allowed the genotypic and phenotypic differentiation of strain BIT-26^T from other species of the genus *Mixta* with validly published names. Therefore, strain BIT-26^T is considered to represent a novel species, for which the name *Mixta tenebrionis* sp. nov. is proposed. The type strain is BIT-26^T (=CGMCC 1.17041^T=KCTC 72449^T).

In 2016, the order ‘*Enterobacteriales*’, a large and diverse group of bacteria within the class *Gammaproteobacteria*, was divided into seven families: *Budviciaceae*, *Enterobacteriaceae*, *Erwiniaceae*, *Hafniaceae*, *Morganellaceae*, *Pectobacteriaceae*, *Thorselliaceae* and *Yersiniaceae* [1]. At the time of writing, the novel family *Erwiniaceae* contains 14 genera: *Benitsuchiphilus*, *Buchnera*, *Erwinia*, *Ishikawaella*, *Izhakiella*, *Mixta*, *Pantoea*, *Phaseolibacter*, *Purcellia*, *Rosenkranzia*, *Siccibacter*, *Stammerula*, *Tatumella* and *Wigglesworthia* [2]. Of these genera, *Mixta* was established in 2018 by reclassifying four species, *Mixta calida*, *Mixta gaviniae*, *Mixta intestinalis* and *Mixta theicola*, from the genus *Pantoea* [2]. Based on the results of multilocus sequence analysis (MLSA) and genome-based phylogenies, these four species of the genus *Mixta* form a monophyletic cluster, which was distinguished from all other genera within the *Erwiniaceae* [2]. Species of the genus *Mixta* have been isolated from a variety of environmental habitats. *M. calida* and *M. gaviniae* were first isolated from infant for-

mula and an infant formula production environment [3]. *M. calida* is the type species representing the genus *Mixta* [2]. The other species, *M. theicola* and *M. intestinalis*, were isolated as endophytes from surface-sterilized leaves of black tea and a faecal sample of a healthy human, respectively [4, 5].

Recently, we found that mealworm (*Tenebrio molitor* L.) can eat Styrofoam as its sole diet and degrade the plastic after passage through the gut [6]. Furthermore, it has been demonstrated that symbiotic gut bacteria play an essential role in plastic degradation, and a plastic-degrading bacterium (*Exiguobacterium* sp. strain YT2) has been isolated from the mealworm’s gut [7]. During the screening of the plastic-degrading gut bacteria associated with mealworms, strain BIT-26^T was isolated from the gut of a plastic-eating mealworm from Beijing, PR China. In this study, strain BIT-26^T was characterized on the basis of a taxonomic study using a polyphasic approach. We showed that the strain BIT-26^T represents a novel species of the genus *Mixta* and proposed

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; LB, Luria–Bertani; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NJ, neighbour-joining.

The GenBank/EMBL/DBJ accession number for the genome, 16S rRNA, *atpD*, *gyrB*, *infB* and *rpoB* gene sequences of strain BIT-26^T are VHQI00000000, MK722097, MN089590, MN089593, MN089596 and MN089599, respectively.

Four supplementary figures are available with the online version of this article.

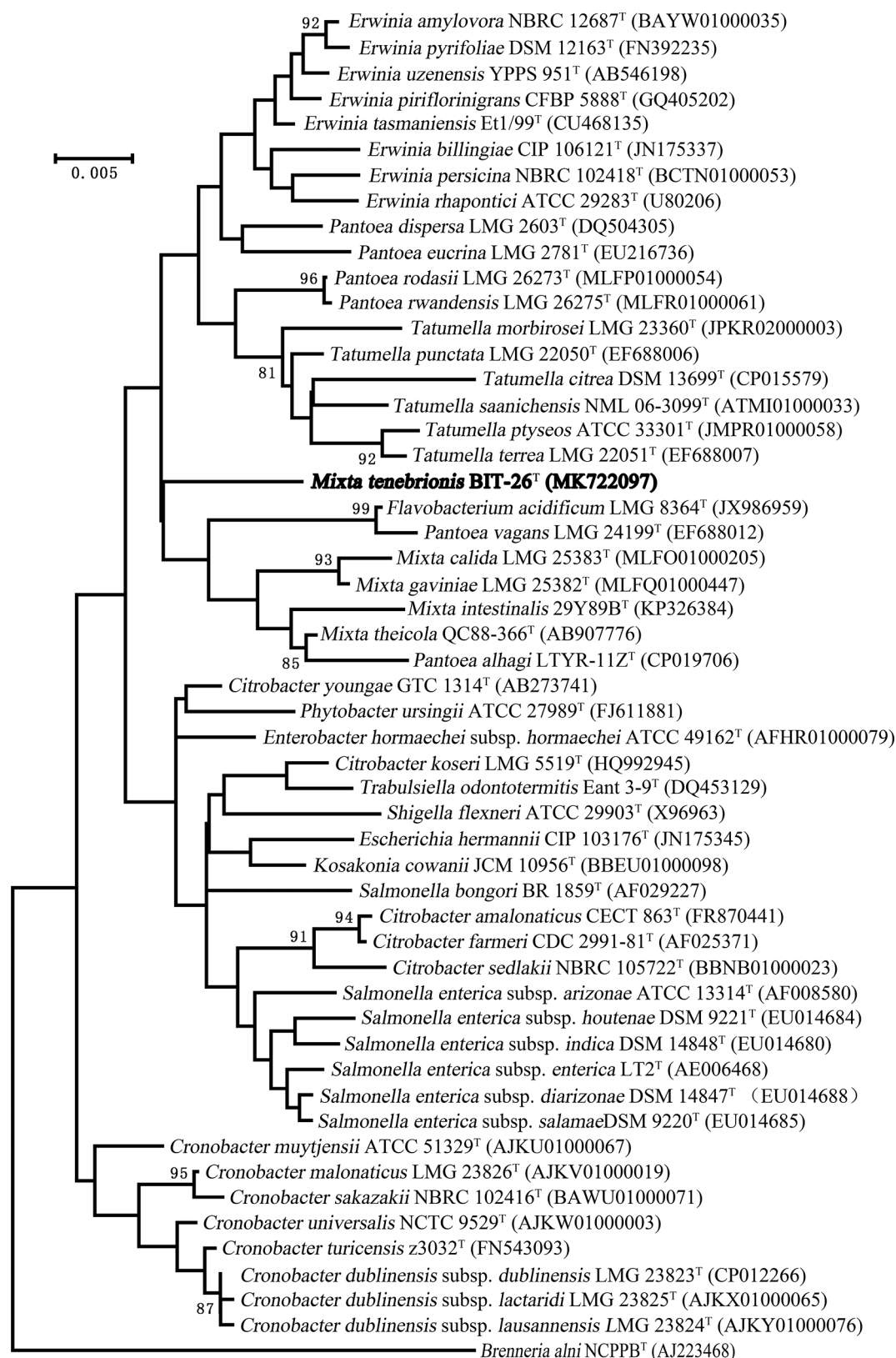


Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences of strain BIT-26^T and its closely related type species. Bootstrap values based on 1000 replicates are expressed as percentages. Asterisks indicate species names that have not previously been validly published. The 16S rRNA gene sequence of *Brenneria alni* NCPPB^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

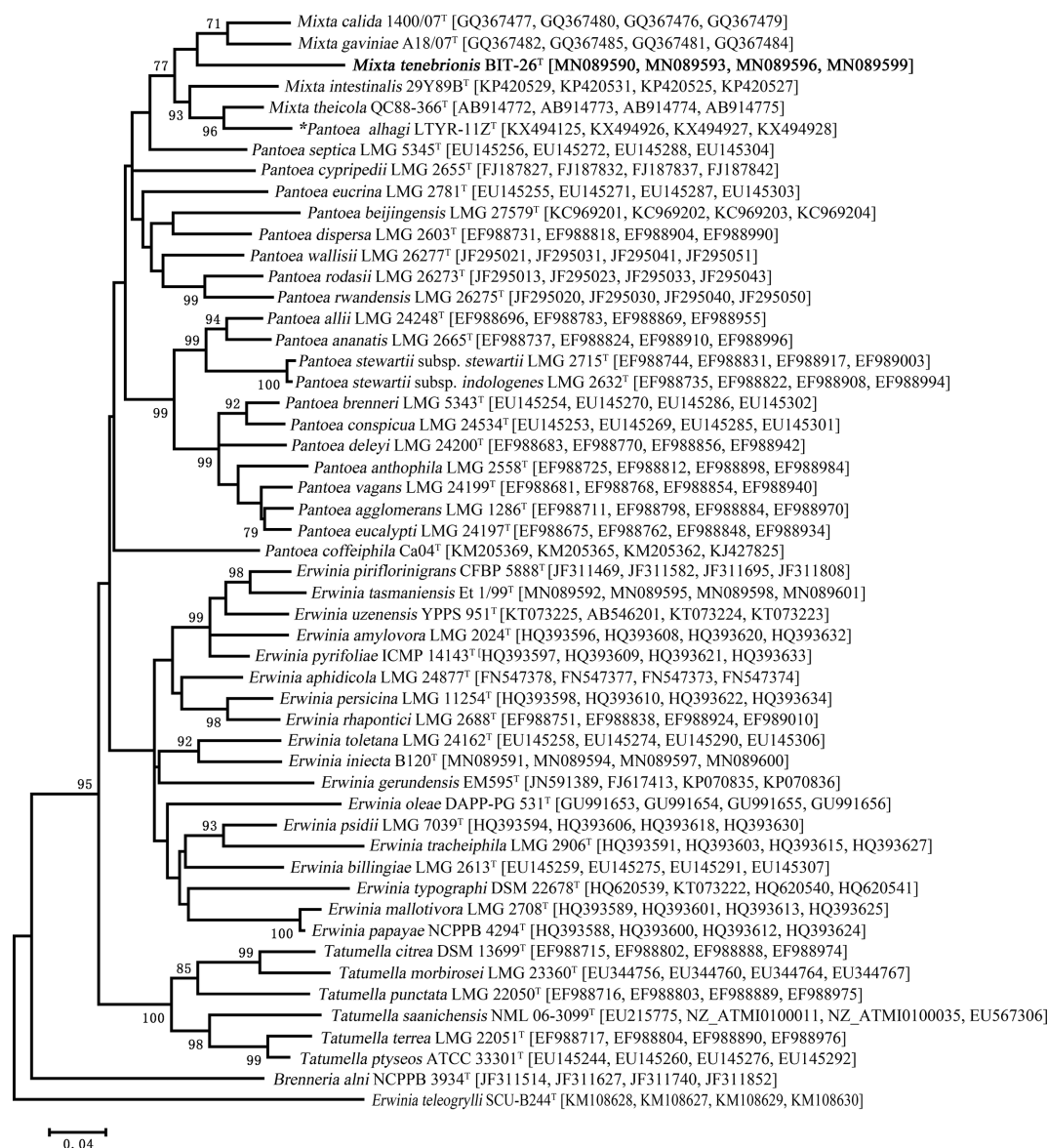


Fig. 2. Maximum-likelihood tree based on concatenated protein sequences of the *atpD*, *gyrB*, *infB* and *rpoB* genes of strain BIT-26^T and recognized species of the genus *Mixta*, *Pantoea*, *Erwinia* and *Tatumella*. Similar protein sequences of *Brenneria alni* NCPPB^T were used as an outgroup. Asterisks indicate species names that have not previously been validly published. Bootstrap values based on 1000 replicates are expressed as percentages. Bar, 0.04 substitutions per amino acid position.

to establish for this strain the species name *Mixta tenebrionis* sp. nov.

ISOLATION AND ECOLOGY

Strain BIT-26^T was isolated from the gut of a plastic-eating mealworm *Tenebrio molitor* L. during the investigation of the role of the gut microbial community in the breakdown of Styrofoam [6]. Mealworms were sampled from Daxing Insect Breeding Plant, Beijing, PR China (N 39° 41' 53.31", E 116° 18' 57.73"). The mealworms were fed with Styrofoam as their sole diet in a climate chamber (RQH-250, Jinghong) under controlled conditions (25±1 °C, 80±2% humidity and a 16:8 light/dark photoperiod). After 2 weeks,

a group of 30 mealworms fed with Styrofoam was collected. In order to dislodge the bacteria on the body surface, the worms were sterilized by immersion in 75% ethanol for 1 min and then rinsed twice with sterile salt water (NaCl 0.85%, w/v, pH 7.2). Their guts were drawn out and pooled in a 10 ml centrifuge tube containing 5 ml sterile salt water. After being homogenized on a vortex mixer for 5 min, the gut tissues were carefully removed with a pipette, while the liquid was spread by the standard dilution-plating technique on D-fructose medium (containing 6.67 g D-fructose, 0.1 g yeast extract, 6.0 g K₂HPO₄, 4.0 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 0.02 g CaCl₂, 10.0 mg FeCl₃, 2.0 mg Na₂MoO₄·2H₂O, 2.1 mg MnSO₄, 2.8 mg H₃BO₃,

Table 1. Differential features between strain BIT-26^T and its closest relatives in the genus *Mixta*

Strains: 1, BIT-26^T; 2, *Mixta calida* A18/07^T; 3, *Mixta gaviniae* LMG 1400/07^T; 4, *Mixta intestinalis* 29Y89B^T; 5, *Mixta theicola* QC88-366^T. Data for strains 2–3, 4 and 5 from Popp et al. [3], Prakash et al. [5] and Kato Tanaka et al. [4], respectively. +, Positive reaction; –, negative reaction; w, weakly positive reaction; NA, no data available

Characteristic	1	2	3	4	5
Colony pigmentation	Yellow	White	White	Cream	White
Range for growth:					
Temperature (°C)	12–41	10–44	7–41	15–45	10–40
pH	4.5–9.0	NA	NA	4.0–9.0	5.0–9.0
NaCl (% w/v)	0–7.5	NA	NA	0–10.0	<9.0
Voges–Proskauer test	+	+	+	+	–
Indole production	–	–	–	–	–
Citrate utilization	–	+	+	NA	+
Enzyme activity:					
Gelatinase	–	–	–	–	–
Urease	–	–	–	–	–
Arginine dihydrolase	–	–	–	NA	–
Lysine decarboxylase	–	–	–	NA	–
Ornithine decarboxylase	+	–	–	NA	–
Acid production from:					
D-Sorbitol	–	+	–	–	–
Amygdalin	–	–	–	–	+
Salicin	+	+	+	–	+
Cellobiose	–	+	+	NA	+
Lactose	+	+	+	+	–
Sucrose	–	+	+	–	–
Raffinose	–	+	+	NA	–
D-Arabitol	–	–	–	NA	+
D-Fucose	w	–	–	+	+
Gluconate	w	+	+	NA	NA
2-Keto-gluconate	w	+	+	NA	NA
5-Keto-gluconate	w	+	–	NA	NA
DNA G+C content (mol%)	56.1	57.4	58.4	59.1	57.2

0.04 mg Cu(NO₃)₂·3H₂O, 0.24 mg ZnSO₄·7H₂O and 20 g agar per litre of distilled water, pH 6.8). After 5 days of incubation at 28 °C in air atmosphere, individual colonies were randomly picked and streaked repeatedly on Luria-Bertani (LB) agar to obtain pure cultures. The isolate, designated as strain BIT-26^T, was routinely cultured on LB medium for additional taxonomic experiments and preserved as both LB medium slants at 4 °C and suspensions with 15 % (v/v) glycerol at –80 °C.

16S RNA GENE PHYLOGENY AND MLSA

For 16S rRNA gene sequencing and phylogenetic analysis, genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were carried out according to the previously described method [5]. The closest phylogenetic neighbours and the corresponding similarity were determined by aligning the obtained 16S rRNA sequence against the bacterial type species recorded in the EzBioCloud database [8]. Sequence alignment of 16S rRNA gene sequence of the strain

Table 2. Predominant fatty acid compositions of strain BIT-26^T and its closest relatives in the genus *Mixta*

Strains: 1, BIT-26^T; 2, *Mixta calida* A18/07^T; 3, *Mixta gaviniae* LMG 1400/07^T; 4, *Mixta intestinalis* 29Y89B^T; 5, *Mixta theicola* QC88-366^T. Data for strains 2–4 from Prakash et al. [5]; data for strain 5 from Chen et al. [22]. All data is expressed as percentages of peak areas. –, Not detected.

Fatty acid	1	2	3	4	5
C _{12:0}	2.16	2.5	4.1	3.9	5.9
C _{14:0}	6.88	8.2	5.7	5.3	8.4
C _{16:0}	33.08	35.7	29.8	29.4	32.5
C _{17:0} cyclo	10.30	19.5	13.8	18.2	10.8
C _{18:0}	0.23	0.5	0.4	0.6	1.2
C _{19:0} cyclo ω8c	1.18	4.3	1.4	4.2	–
C _{19:0} iso	–	–	–	–	2.5
Summed feature 2*	8.0	10.0	9.8	9.0	9.0
Summed feature 3*	18.9	5.3	16.6	8.0	15.4
Summed feature 8*	18.3	11.3	17.3	18.0	14.3

*Summed features are combinations of fatty acids that cannot be separated by the MIDI system. Summed feature 2 comprises any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain length 10.9525; summed feature 3 comprises C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 8 comprises C_{18:1}ω7c and/or C_{18:1}ω6c.

BIT-26^T and the closely related type species was performed by using CLUSTAL X 2.0 software [9]. Aligned sequences were used to reconstruct the neighbour-joining (NJ) [10] and maximum-likelihood (ML) [11] phylogenetic trees using the program MEGA 6.0 [12], with bootstrap values based on 1000 replications.

The 16S rRNA gene sequence (1412 bp) of strain BIT-26^T showed highest similarity (97.4%) to *Erwinia piriflorinigrans* CFBP 5888^T, followed by *Citrobacter sedlakii* NBRC 105722^T (97.3%), *Mixta calida* LMG 25383^T (97.3%), *Cronobacter muytjensii* ATCC 51329^T (97.2%) and *Mixta theicola* QC88-366^T (97.2%). The ML phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) showed that strain BIT-26^T grouped in a wide cluster including related species from the genera *Mixta*, *Pantoea*, *Erwinia* and *Tatumella* within the family *Erwinaceae*, but not the cluster containing the related species from the genera *Citrobacter*, *Cronobacter* and *Salmonella* within the family *Enterobacteriaceae*. The consistent clustering pattern could also be observed in the NJ phylogenetic tree (Fig. S1, available in the online version of this article). It was obvious that strain BIT-26^T represented a novel species in the family *Erwinaceae*. However, it was difficult to determine the accurate allocation of strain BIT-26^T to the genus level based on 16S rRNA gene sequences, because the genera *Mixta*, *Pantoea*, *Erwinia* and *Tatumella* within the family *Erwinaceae* are not monophyletic when using 16S rRNA gene sequences.

MLSA of concatenated protein sequences of four housekeeping genes (*atpD*, *gyrB*, *infB* and *rpoB*) has been applied as a useful method for the classification of the genera and species within the family *Erwinaceae* [2–5, 13]. Thus, to refine the taxonomic position of strains BIT-26^T, we carried out MLSA for the strain BIT-26^T. The partial sequences of the *atpD*, *gyrB*, *infB* and *rpoB* genes of strain BIT-26^T were amplified,

sequenced and concatenated following the protocol of Brady et al. [13]. Multiple alignments of all concatenated datasets for the four housekeeping genes of strain BIT-26^T and the type species in the genera *Mixta* (five species), *Pantoea* (20 species), *Erwinia* (19 species) and *Tatumella* (six species) were also performed by using CLUSTAL X 2.0 software [9]. Aligned protein sequences were used to reconstruct the NJ and ML phylogenetic trees as described for the 16S rRNA gene.

As seen in the MLSA-derived ML phylogenetic tree (Fig. 2), the three genera, *Mixta*, *Erwinia*, *Pantoea* and *Tatumella* can be subdivided into monophyletic clusters, as described in previous reports [2–5]. Strain BIT-26^T clustered with all type species of the genus *Mixta*, but simultaneously formed a separate subclade. The similar clustering pattern could also be observed in the MLSA-derived NJ phylogenetic tree (Fig. S2). Therefore, our MLSA results suggested that strain BIT-26^T was a member of the genus *Mixta* close to *M. calida* and *M. gaviniae*, but representing a distinct species.

GENOME FEATURES

To further confirm that strain BIT-26^T was a novel species of the genus *Mixta*, the genome sequencing of the strain BIT-26^T was carried out. Subsequently, the *in silico* DNA–DNA hybridization (DDH) tests and the average nucleotide identity (ANI) calculation between the genome of strain BIT-26^T and that of the closest related type species were performed. The next-generation shotgun-sequencing of the genome of strain BIT-26^T was performed using the Illumina HiSeq 2000 platform. The raw sequence data was quality assessed, trimmed and assembled using SOAPdenovo version 2.04 [14]. Open reading frames, rRNA and tRNA were predicted and annotated using the Glimmer 3.02, Barrnap 0.4.2 and

tRNAscan-SE version 1.3.1 [15–17]. The *in silico* DDH tests between strain BIT-26^T and the closest related species from the genus *Mixta*, *Pantoea*, *Erwinia* and *Tatumella* were performed using the Genome-to-Genome Distance Calculator (GGDC) web server version 2.0 with the recommended settings [18]. The ANI calculation was carried out by using the online ANI calculator in the EzBioCloud [19].

A total of 0.999 Gb raw DNA sequence was generated with an average sequence length of 150 bp, which could be assembled into 73 scaffolds with an N50 value of 382 938 bp. The size of the assembled BIT-26^T draft genome was at least 4 655 478 bp with a G+C content of 56.1%. In total, 4414 genes, two rRNA and 78 tRNA were predicted in the assembled draft genome. The results of *in silico* DDH tests (or ANI calculation) revealed that the highest genomes similarity was 29.2% (84.8%) between strain BIT-26^T and *M. gaviniae*, followed by 29.1% (84.8%) between strain BIT-26^T and *M. calida*, 26.6% (82.1%) between strain BIT-26^T and *M. theicola*. The most similar genomes of the type species in the genus *Erwinia*, *Pantoea* and *Tatumella*, were *Erwinia amylovora* with a similarity value of 22.8% (78.0%), *Pantoea eucrina* with 22.0% (78.4%) and *Tatumella citrea* with 19.9% (72.6%), respectively. The DDH values (or ANI values) between strain BIT-26^T and other type species in the genus *Mixta* were lower than the generally accepted species-level boundary of 70% (or <95–96% ANI) [20]. Therefore, we can confirm that strain BIT-26^T represents a novel species in the genus *Mixta*.

PHYSIOLOGY AND CHEMOTAXONOMY

The properties of the isolate were investigated after 48 h growth on LB agar at 30 °C. The Gram staining was performed with cells grown on the LB agar as described by Gerhardt *et al.* [21]. Growth under anaerobic atmosphere (N₂ : CO₂ : H₂; 80:10:10, v/v) was determined after incubation in an anaerobic chamber on LB agar. Cell motility was examined by observing the spread of diffuse growth in test tubes containing semi-solid LB medium with 0.75% agar. Cell morphology and flagella were observed using SEM (SU8010, Hitachi) and TEM (JEM-1400, JEOL) after 12 h incubation on LB agar at 30 °C, respectively. The software linked to SEM or TEM was used to measure the size and length of bacteria. Growth at different temperatures (4, 10, 20, 28, 30, 35, 37, 41 and 44 °C) in LB medium and at various pH values (pH 4.0–11.0, prepared with appropriate biological buffers at intervals of 0.5 unit) in LB medium at 30 °C were determined after 48 h incubation. Salt tolerance was determined after 48 h incubation in LB medium supplemented with 0–15% (w/v) NaCl (at 1.5% intervals). Catalase activity was determined by the observation of bubble production in a 3% (v/v) hydrogen peroxide solution, while oxidase activity was examined using 1% (w/v) tetramethyl-*p*-phenylenediamine. Acid production from carbohydrates were examined with the API 50CH system (bioMérieux), while other biochemical and enzymatic tests were performed with the API 20E system (bioMérieux) according to the manufacturer's instructions.

Colonies of strain BIT-26^T on LB agar were moist, yellow, convex and circular in diameter of 1.0–2.5 mm. Strain BIT-26^T was Gram-stain-negative, facultatively anaerobic, rod-shaped and approx. 0.4–0.6×1.3–2.2 μm in size (Fig. S3). Cells were observed to be motile by using peritrichous flagella (Fig. S4). Strain BIT-26^T was able to grow at 12–41 °C (optimum, 20–40 °C), at pH 4.5–9.0 (pH 6.0–7.5) and in the presence of 0–7.5% (w/v) NaCl (1–1.5%). The phenotypic characteristics of strain BIT-26^T generally corresponded to the current description of the genus *Mixta* [2]. Differential phenotypic characteristics between strain BIT-26^T and its closest related species of the genus *Mixta* are summarized in Table 1. Strain BIT-26^T could be separated from its closest phylogenetic neighbours, *M. calida* and *M. gaviniae*, because it was able to produce ornithine decarboxylase, but unable to utilize citrate and to produce acids from cellobiose, sucrose and raffinose. Strain BIT-26^T could be distinguished from *M. intestinalis* by its ability to produce acids from salicin. Additionally, strain BIT-26^T differed from *M. theicola* in that it was positive for the Voges–Proskauer test and able to produce ornithine decarboxylase and acids from lactose, but unable to utilize citrate or to produce acids from amygdalin, cellobiose and D-arabitol. Other physiological and biochemical characteristics of strain BIT-26^T are summarized in the species description.

For determination of the whole-cell fatty acid composition, strain BIT-26^T was incubated on LB medium for 48 h at 30 °C. The fatty acids were extracted and analysed according to the recommendations of the commercial identification system (Microbial Identification System, MIDI), and the whole fatty acid composition was determined using gas chromatography with Agilent 6890 N apparatus.

The whole-cell fatty acid profiles of strain BIT-26^T and its closest related species in the genus *Mixta* are shown in Table 2. Strain BIT-26^T shared the similarly predominant fatty acids, including C_{12:0}, C_{14:0}, C_{16:0}, C_{17:0} cyclo and C_{19:0} cyclo ω8c, with other closely related species of the genus *Mixta*. However, strain BIT-26^T could be distinguished from these related species by its smaller ratio of C_{12:0} and C_{17:0} cyclo and its larger ratio of summed feature 8. The similarities and differences in the fatty acid profiles between strain BIT-26^T and its closest related species of the genus *Mixta* further supported that strain BIT-26^T belonged to the genus *Mixta* and represented a novel species.

In summary, on the basis of phylogenetic results, genome relatedness, unique phenotypic characteristics and results of chemotaxonomic analyses, we suggest that strain BIT-26^T represents a novel species of the genus *Mixta*, for which the name *Mixta tenebrionis* sp. nov. is proposed.

DESCRIPTION OF *MIXTA TENEBRIONIS* SP. NOV.

Mixta tenebrionis (te.ne.bri.o'nis. N.L. gen. n. *tenebrionis* of the mealworm *Tenebrio*, the host from where the type strain was isolated).

Cells are Gram-stain-negative, facultatively anaerobic, motile rods (approx. $0.4\text{--}0.6 \times 1.3\text{--}2.2\ \mu\text{m}$) with peritrichous flagella. Colonies on LB agar are circular, smooth, yellow and 1–2 mm in diameter within 48 h at 30 °C. Growth occurs at 12–41 °C (optimum, 20–40 °C), at pH 4.5–9.0 (pH 6.0–7.5) and in the presence of 0–7.5% (w/v) NaCl (1–1.5%). Catalase activity, the Voges–Proskauer reaction, β -galactosidase and ornithine decarboxylase are positive, whereas oxidase activity, arginine dihydrolase, lysine decarboxylase, tryptophan deaminase, gelatinase, urease, citrate utilization, production of H_2S and indole production are negative. In the API 50CH system, acids are produced from L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, inositol, mannitol, N-acetylglucosamine, arbutin, aesculin, salicoid, maltose, lactose and trehalose; weakly produced from glycerinum, D-arabinose, melibiose, geranyl, D-fucose, gluconate, 2-keto-gluconate and 5-keto-gluconate; and are not produced from erythrose, L-xylose, adonitol, methyl β -D-xyloside, sorbose, dulcitol, sorbitol, methyl α -D-mannosidase, methyl α -D-glucoside, amygdalin, cellobiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol and L-arabitol. The predominant fatty acids are $\text{C}_{12:0}$, $\text{C}_{14:0}$, $\text{C}_{16:0}$, $\text{C}_{17:0}$ cyclo, $\text{C}_{19:0}$ cyclo $\omega 8c$, summed feature 2 (comprising any combination of $\text{C}_{12:0}$ aldehyde, an unknown fatty acid of equivalent chain length 10.9525), summed feature 3 (comprising $\text{C}_{16:1}$ $\omega 7c$ and/or $\text{C}_{16:1}$ $\omega 6c$) and summed feature 8 (comprising $\text{C}_{18:1}$ $\omega 7c$ and/or $\text{C}_{18:1}$ $\omega 6c$).

The type strain, BIT-26^T (=CGMCC 1.17041^T=KCTC 72449^T), was isolated from the gut of the plastic-eating mealworm *Tenebrio molitor* L. in Beijing, PR China. The DNA G+C content of the type strain is 56.08%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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