INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Fiedler *et al.*, *Int. J. Syst. Evol. Microbiol.* 2022;72:005288

DOI 10.1099/ijsem.0.005288

MICROBIOLOGY SOCIETY

OPEN ACCESS

Pseudomonas rustica sp. nov., isolated from bulk tank raw milk at a German dairy farm

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Abstract

Here we present the description of a novel *Pseudomonas* species, designated *Pseudomonas rustica* sp. nov., which was isolated from raw milk samples obtained from Germany. Results of initial 16S rRNA gene sequence analysis assigned the strain into the genus *Pseudomonas* and showed *Pseudomonas helmanticensis*, *Pseudomonas neuropathica* and *Pseudomonas atagonensis* to be its closest relatives. Further studies including sequence analysis of the *rpoB* gene, multi-gene phylogenetic tree reconstruction, whole-genome sequence comparisons, cellular fatty acid analysis and chemotaxonomic characterization showed a clear separation from the known *Pseudomonas* species. Isolate MBT-4^T was closely related to *Pseudomonas helmanticensis*, '*Pseudomonas crudilactis*' and *Pseudomonas neuropathica* with average nucleotide identities based on BLAST values of 88.8, 88.8 and 88.6%, respectively. Therefore, the strain can be classified into the *Pseudomonas koreensis* subgroup of the *Pseudomonas fluorescens* group. The G+C content of strain MBT-4^T was 58.9 mol%. The strain was catalase- and oxidase-positive, while the β-galactosidase reaction was negative. Growth occurred between 4 and 30 °C and at pH values from pH 6.0 to 8.0. In conclusion, strain MBT-4^T belongs to a novel species, for which the name *Pseudomonas rustica* sp. nov. is proposed. The type strain is MBT-4^T (=DSM 112348^T=LMG 32241^T) and strain MBT-17 is also a representative of this species.

The genus *Pseudomonas* contains over 253 validly described species according to the List of Prokaryotic names with Standing in Nomenclature [1]. At the time of writing, *Pseudomonas* is one of the most diverse genera within the Gram-negative bacteria. Due to their genetic diversity and metabolic adaptability, representatives of the genus *Pseudomonas* are ubiquitous and occupy a wide variety of habitats, such as soil, water, plants and animal tissue [2]. *Pseudomonas* species often cause spoilage of refrigerated foods due to their psychrotolerant properties and production of exoenzymes, which degrade food compounds such as peptides and fat [3]. Pseudomonads are also found together with micrococci, corynebacteria, streptococci, microbacteria, lactobacilli, aerobic spore-formers, enterobacteria, aeromonads and flavobacteria in the microbiota of raw milk in total numbers of 10^3 to 10^5 c.f.u. ml^{-1} [4]. However, the entry routes of these micro-organisms are manifold and can be traced back primarily to the udder, the milking personnel, litter, feed, the air in the barn, the milking equipment and the milk collection tank [5]. Due to the permanent cooling of the milk during the whole processing line, the microbiota of raw milk shifts mainly to psychrophilic organisms, especially *Pseudomonas* species [6]. In milk and dairy products, several novel *Pseudomonas* species have been identified, mainly from the *Pseudomonas fluorescens* lineage (*fluorescens* and *fragi* subgroup) [7–11]. Several studies have shown that the psychrophilic genus *Pseudomonas* is one of the most prevalent and abundant genera in raw milk [6, 12]. Due to the heat treatment used in dairy plants, *Pseudomonas* species are eliminated, but their exoenzymes, such as lipases and proteinases, are very heat-resistant and can therefore lead to quality loss in terms of extended shelf life [6]. Within a Germany-wide investigation of microbiological raw

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Keywords: *Pseudomonas*; spoilage; food; *P. koreensis* subgroup; MALDI-TOF MS.
Abbreviations: ANIb, average nucleotide identity based on BLAST; dDDH, digital DNA–DNA hybridization; MALDI-TOF MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; TSA, tryptic soy agar; TSB, tryptic soy broth.
The GenBank accession number for the 16S rRNA gene sequences of strain MBT-4^T (*Pseudomonas rustica*) is MW936631. Additionally, the whole genome sequences were deposited at the DDBJ/EMBL/GenBank under the accession number JAGYHF000000000 for MBT-4^T (BioProject: PRJNA656947; Biosample: SAMN18880036).
‡These authors contributed equally to this work
Two supplementary tables are available with the online version of this article.

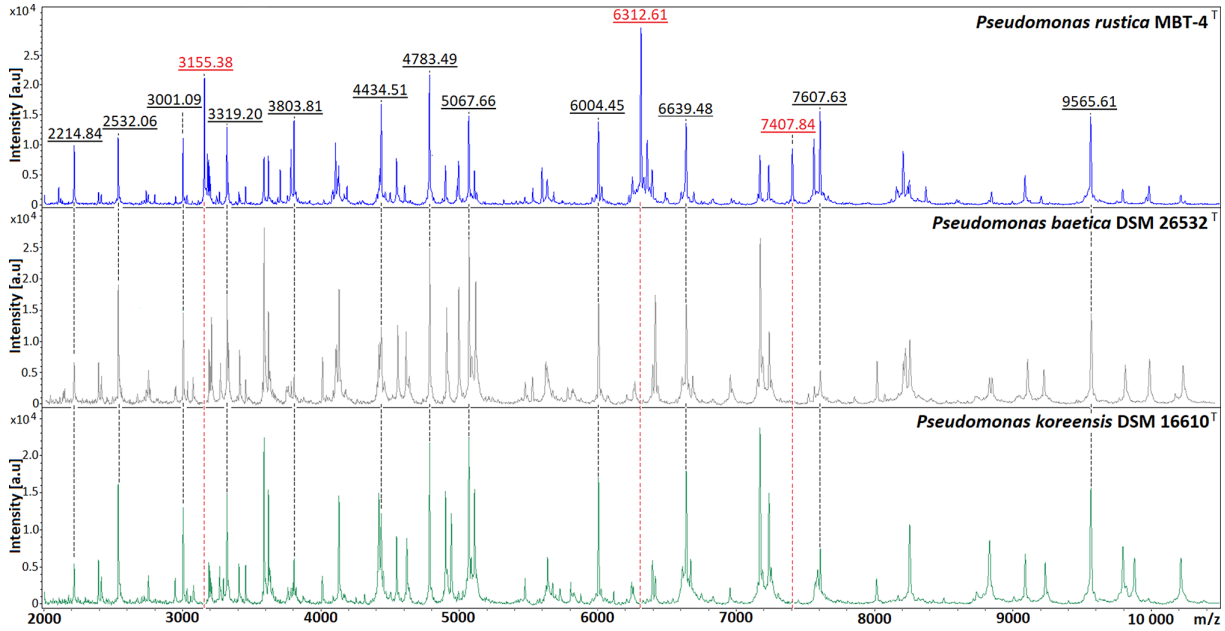

Fig. 1. MALDI-TOF MS spectra of strain MBT-4 ^{T}, *Pseudomonas baetica* DSM 26532^{T} and *Pseudomonas koreensis* DSM 16610^{T}. Spectra were processed by multipolygon baseline subtraction and smoothing using a Savitzky–Golay filter with a 25 Da frame size. The three primary peak mass (m/z) characteristics of strain MBT-4 ^{T} are marked in red.

milk quality [13], approximately 815 strains, primary belonging to the genus *Pseudomonas*, were isolated and identified by the use of matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS).

Strain MBT-4^{T} was selected for further identification and characterization using standard genotypic, phenotypic and chemotaxonomic characterization methods. After allocation of this strain to a presumptive new species and submission of its reference spectra (Fig. 1) to an in-house MALDI-TOF database, the MALDI-TOF MS spectra of the 815 isolates were re-identified. Subsequently, one more strain (MBT-17) could also be assigned presumptively to the new species. These two strains (MBT-4^{T} and MBT-17) were obtained from two raw milk samples from different sampling sites and both were isolated between 2019 and 2020 in northern Germany.

Here we describe a novel *Pseudomonas* species which was identified by MALDI-TOF MS and whole genome sequencing.

Milk-associated isolates were identified by MALDI-TOF MS analyses based on their species-specific protein fingerprints in the mass range of 2–20 kDa and in the positive mode. For this purpose, single colonies were extracted by the formic acid method as previously described [13]. Subsequently, 1 µl extract was transferred in duplicate to a steel target and, after drying, coated with 1 µl HCCA matrix containing 10 mg l^{-1} α-cyano-4-hydroxycinnamic acid dissolved in acetonitrile (50%), water (47.5%) and trifluoroacetic acid (2.5%). Detection of mass spectra was performed with a Microflex LT MALDI-TOF MS (Bruker Daltonics) using a nitrogen laser (337 nm) and a pulse rate of 60.0 Hz. A protein spectrum was generated by accumulating a maximum of 240 satisfactory laser shots in 40-shot increments from different grid points of the target. In accordance with previously described standard methods [13], the milk-associated isolates were identified by matching the mass spectra against different databases (Bruker BDAL database MBT Compass Library, revision F, version 9, 8468 entries; Bruker Library for filamentous fungi, 364 entries; Bruker SR Library, 104 entries; in-house database, 289 milk- and meat-associated isolates). The databases represent 336 *Pseudomonas*-related reference spectra encompassing 104 different species of this genus. Protein spectra of milk-associated isolates that showed low similarity to database entries were considered as potential novel species and further analysed by whole genome sequencing.

MALDI-TOF MS analysis of the strain MBT-4^{T} extract resulted in confident genus and probable species identification with a score value of 2.3. The protein spectrum that exhibited the most pronounced similarity was attributable to *Pseudomonas koreensis* DSM 16610^{T} (Fig. 1). Nevertheless, the MS fingerprint of strain MBT-4^{T} is clearly different from *P. koreensis* and *P. baetica*, whose sequencing data suggest a close relationship to *P. rustica*.

GENOMIC COMPARISON

Based on the MALDI-TOF results, the proposed novel species type strain MBT-4^{T} was prepared for whole genome sequencing. Additionally, strain MBT-17 was chosen for sequencing, because the MALDI-TOF spectra indicate that this isolate is also a

Table 1. Characteristics of the draft genomes of strain MBT-4^T and MBT-17

Strain	Assembly size (bp)	G+C content (mol%)	No. of contigs	N50 (bp)	Sequencing depth (-fold)	Coding DNA sequences (CDS)
MBT-4^T	5 861 604	58.9	46	446 374	45.4	5294
MBT-17	6 470 456	58.9	50	437 585	83.8	5763

representative of the new species showing a score ≥2.63 to strain MBT-4^T. Genomic DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. Library preparation was performed with the Illumina TruSeq DNA Nano Kit and DNA was sequenced on an Illumina MiSeq platform as described previously [13]. The draft genomes were assembled using Unicycler version 0.4.8 [14] and annotation was done by the NCBI Prokaryotic Genome Annotation Pipeline version 4.11 [15]. The statistical parameters for the draft genome quality are shown in Table 1.

The assemblies were deposited at DDBJ/EMBL/GenBank under the accession numbers JAGYHF000000000 for MBT-4^T and JAGYHE000000000 for MBT-17 (BioProject: PRJNA656947; Biosample: SAMN18880036 and SAMN18880037). The 16S rRNA gene sequence of strain MBT-4^T (*Pseudomonas rustica* sp. nov.) was deposited at DDBJ/EMBL/GenBank under the accession number MW936631. All raw genome sequence data have been deposited in the Short Read Archive at NCBI under accession numbers SRR14453977 for MBT-4^T and SRR14453976 for MBT-17.

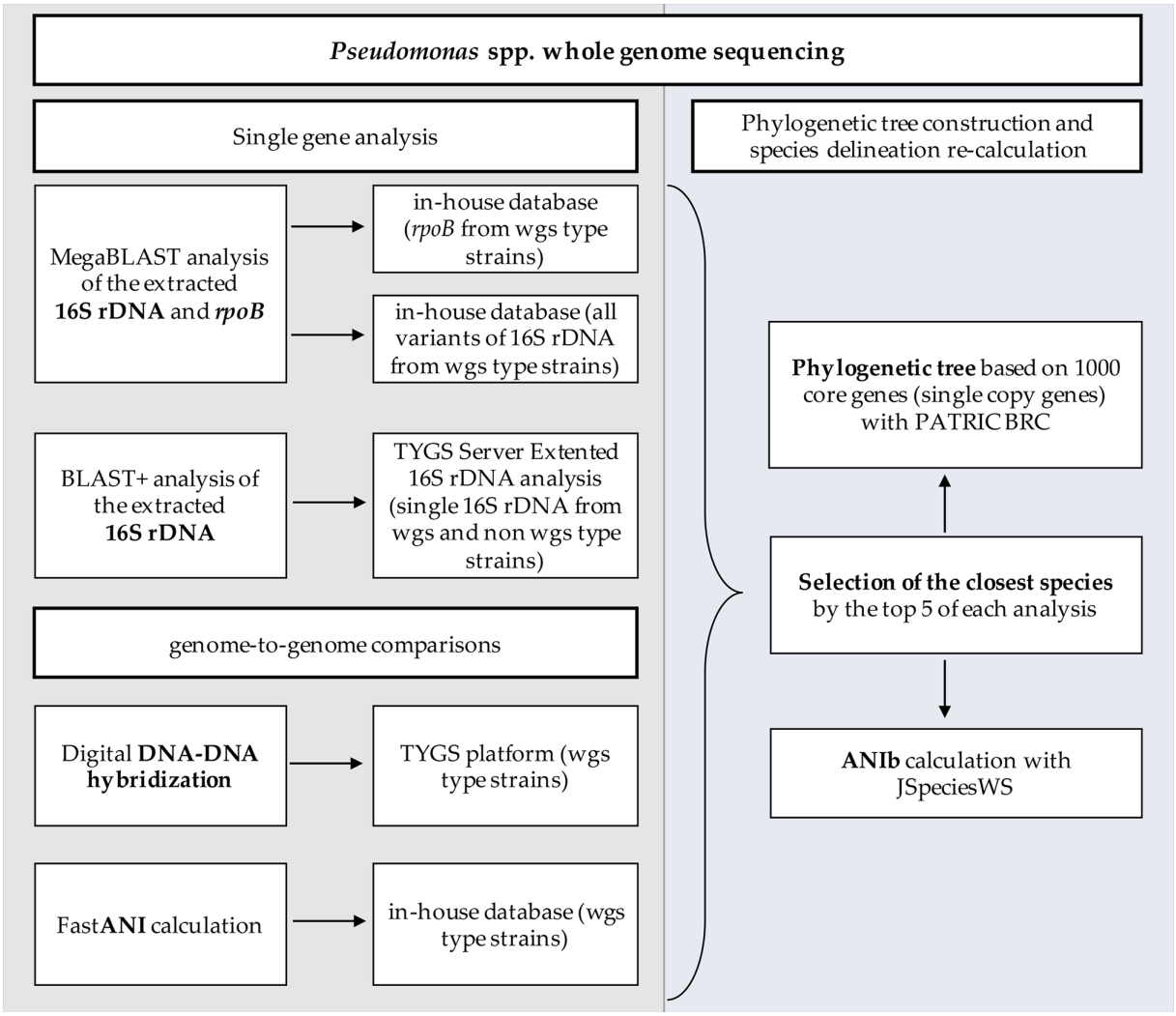

Fig. 2. Flow chart of the bioinformatic workflow for species description used in this study.

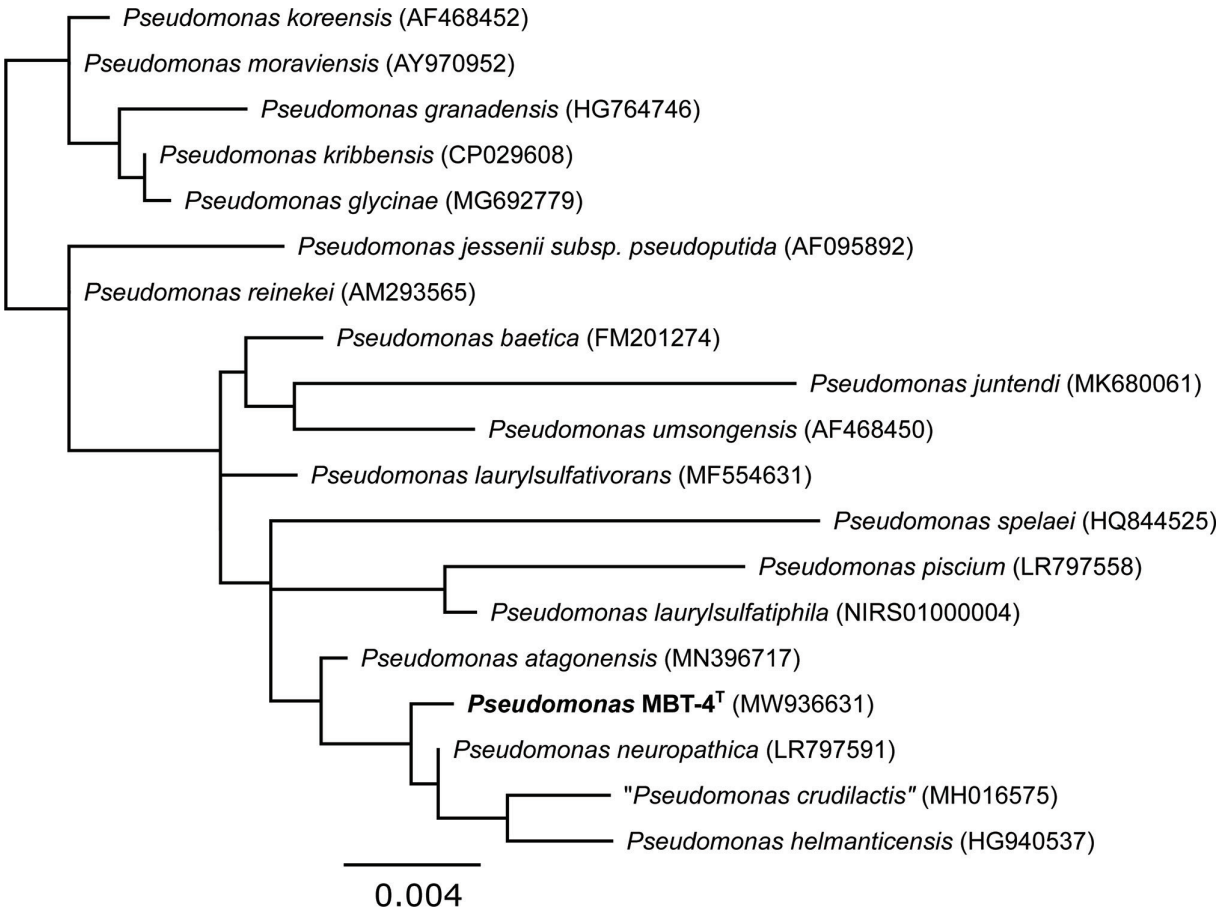

Fig. 3. 16S rRNA gene phylogenetic tree calculated by extended 16S rRNA analysis on the TYGS platform. The calculations were based on the maximum-likelihood method inferred under the GTR+GAMMA model and rooted by midpoint-rooting. The branches are scaled in terms of the expected number of substitutions per site.

We used a polyphasic approach for species delineation. First, we determined the most similar species (type strains) based on 16S rRNA, *rpoB*, digital DNA–DNA hybridization (dDDH) and FastANI calculations. Subsequently, the top five most similar species were included to recalculate values of average nucleotide identity based on BLAST (ANIb). This second calculation served to confirm the initial species delimitation by dDDH and FastANI analyses. The used software, tools and resources were described in detail by Gieschler *et al.* [13]. Digital whole genome comparisons such as average nucleotide identity (ANI) and dDDH are the current gold standards for novel species descriptions [16, 17]. With an ANI value <95–96% and/or a dDDH value <70%, the analysed strain represents a novel species [16]. An overview of the bioinformatic analyses performed in this study for species classification is shown in Fig. 2.

Table 2. Most closely related *Pseudomonas* species as results from whole genome comparisons

***Pseudomonas* MBT-4^T genome-to-genome comparisons**				
Rank (highest similarity)	**TYGS platform**		**FastANI (in-house database)**	
	Species and digital DNA–DNA hybridization (d4, in %)		**Species and average nucleotide identity (in %)**	
1	*Pseudomonas baetica* LMG 25716^T	35.9	*Pseudomonas helmanticensis* strain BIGb0525	90.5
2	*Pseudomonas atagonensis* LMG 31496^T	35.7	*'Pseudomonas crudilactis'* DSM 109949^T	90.4
3	*Pseudomonas koreensis* DSM 16610^T	35.1	*Pseudomonas neuropathica* CCUG 74875^T	90.2
4	*Pseudomonas granadensis* DSM 28040^T	33.9	*Pseudomonas atagonensis* LMG 31496^T	89.3
5	*Pseudomonas moraviensis* DSM 16007^T	33.7	*Pseudomonas baetica* LMG 25716^T	89.3

Table 3. Average nucleotide identity based on BLAST (ANIb) values (%) of strains MBT-4^T and MBT-17 compared to their most related *Pseudomonas* species and in brackets the percentage of sequences that could be aligned between the genomes

Species	MBT-4^T	MBT-17
Pseudomonas atagonensis LMG 31496^T	87.57 (75.32)	87.18 (74.12)
Pseudomonas baetica LMG 25716^T	87.46 (73.47)	87.08 (72.58)
'Pseudomonas crudilactis' DSM 109949^T	88.79 (77.07)	82.89 (64.15)
Pseudomonas granadensis LMG 27940^T	86.53 (71.83)	86.16 (70.08)
Pseudomonas helmanticensis strain BIGb0525	88.84 (76.98)	88.41 (76.14)
Pseudomonas koreensis LMG 21318^T	87.06 (74.35)	86.64 (73.24)
Pseudomonas laurylsulfativorans DSM 105098^T	83.17 (66.08)	82.69 (63.31)
Pseudomonas moraviensis LMG 24280^T	86.40 (71.45)	86.26 (71.77)
Pseudomonas neuropathica CCUG 74875^T	88.59 (76.50)	83.05 (66.60)
***Pseudomonas rustica* MBT-4^T**	*	97.21 (90.29)
Pseudomonas rustica MBT-17	**96.61** (83.03)	*

Single gene analysis

Because of the limited informative value of Sanger-sequenced 16S rRNA and partial *rpoB* genes for species classification [18, 19], we waived initial Sanger sequencing of these genes. However, for species assignment of isolates whose genomes are not yet available, an analysis of the complete *rpoB* gene and 16S phylogenies has been found useful. To take this into account, these sequences were extracted from the genome assemblies and were aligned with an updated in-house database (last update 16 April 2021) as described previously [13]. The sequences were analysed for similarity (pairwise identity) to the corresponding gene of the *Pseudomonas* species type strains using a local megaBLAST algorithm within the software Geneious 9.1 (www.geneious.com). In addition, we performed an extended 16S rRNA gene analysis with TYGS (Type (Strain) Genome server), as this database also contains 16S rRNA gene sequences of type strains that have not yet been completely genome-sequenced [20]. Identical rRNA and *rpoB* sequences were found for MBT-17, the further representative of MBT-4^T.

On the basis of single gene analysis, the most closely related type strains to MBT-4 ^{T} are shown in Table S1 (available in the online version of this article). Highest pairwise similarities were found between MBT-4^T and *P. helmanticensis* with 99.9% (16S rRNA gene, in-house database), *P. neuropathica* with 99.9% (16S rRNA gene, TYGS platform) and *P. helmanticensis* with 98.6% (*rpoB*, in house database) and there were only minor differences between the three analyses. All species belong to the *P. fluorescens* group and can be classified in the *P. koreensis* subgroup [19].

The differences between the two 16S rRNA gene analyses are mainly due to the differences between the genome entries in the databases and the heterogeneity of the 16S rRNA genes in some type strain genomes. For example, the recently published species "*P. crudilactis"* [21], *P. anatoliensis* [22] and *P. gozinkensis* [23] were not yet included in the database at the time of the extended 16S analysis at the TYGS platform. Furthermore, sometimes a different variant of the 16S rRNA gene is deposited as a reference in the TYGS than in our in-house database determined as the one of the intragenomic copies with the highest similarity to MBT-4^T. Compared to the *rpoB* analysis, the 16S rRNA analysis usually had a much lower predictive power for *Pseudomonas* [13, 17], but for strain MBT-4^T the predicted *Pseudomonas* species are highly similar (Table S1).

Initially, *P. helmanticensis* was not included in our in-house database, because the genome of the type strain (OHA11) had not been sequenced before. Based on the analyses performed by the TYGS platform, we have included the available genome of *P. helmanticensis* strain BIGb0525 (non-type strain) in the in-house database. Because of these possible discrepancies with respect to sources, the advantage of parallel analysis across different databases and genes becomes clear. The traditional 16S rRNA gene based phylogenetic tree was calculated by the extended maximum-likelihood/maximum-parsimony 16S rRNA analysis method on the TYGS platform [20] and is shown in Fig. 3.

Genome-to-genome comparisons

Digital whole genome comparisons are the current gold standard to check if a strain belongs to a known species [16]. For this reason, we determined the dDDH values to the related species directly by two individual applications on the basis of dDDH and ANI analysis against all completely sequenced type strains. If all genomes of type strains were fully sequenced, there would no longer be a need for single gene analyses for the description of new *Pseudomonas* species [17]. The software, tools and resources were described in detail by Gieschler *et al.* [13].

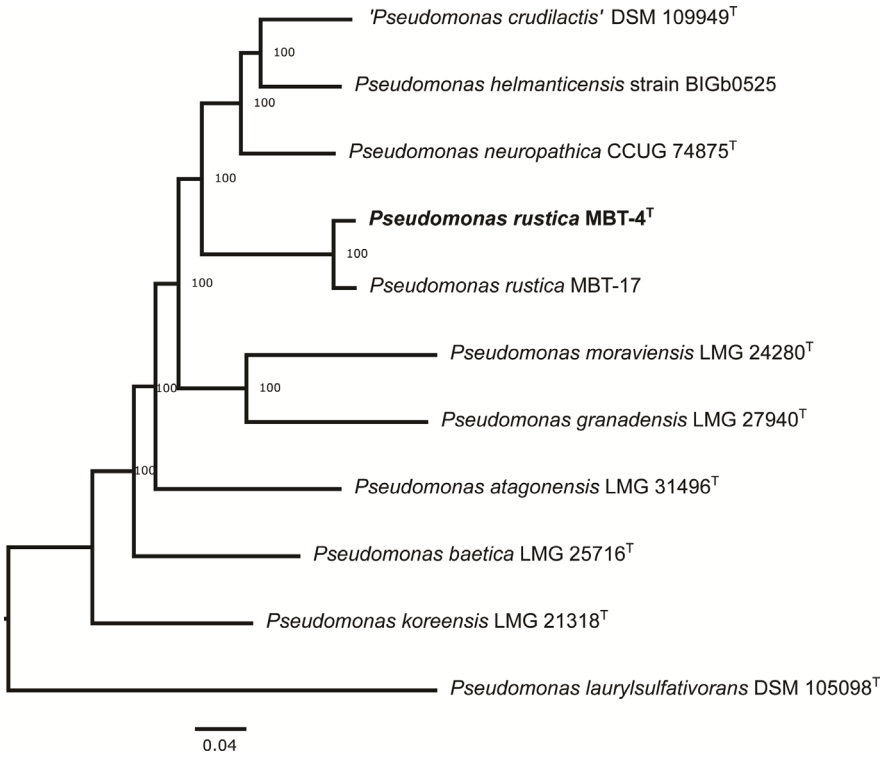

Fig. 4. Phylogenetic tree (Codon Tree method selects 1000 single-copy genes (PATRIC PGFams) and analyses aligned proteins and coding DNA using the program RAxML version 8.2.11). All species shown belong to the *Pseudomonas koreensis* subgroup, except *Pseudomonas laurylsulfativorans* which belongs to the *Pseudomonas jessenii* subgroup. The bar indicates sequence divergence. Bootstrap values are shown at branch points.

On the basis of genome-to-genome comparisons, the most closely related type strains to MBT-4^T are shown in Table 2. The results of the two methods (dDDH and FastANI) differed slightly (Table S2). Strain MBT-4^T showed a maximal dDDH value of 35.9% to the closest related species *P. baetica* LMG 25716, which does not match the highest FastANI result of 90.5% with *P. helmanticensis*. This demonstrates that the isolate is a member of a hitherto unknown *Pseudomonas* species.

As a combining final analysis, the top five most similar species from the 16S rRNA, *rpoB* and genome-to-genome (dDDH, FastANI) analyses were included to recalculate the ANIb values by using JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/#home) [24]. For strain MBT-4^T, *P. atagonensis* LMG 31496^T, *P. baetica* LMG 25716^T, "*P. crudilactis"* DSM 109949^T, *P. granadensis* LMG 27940^T, *P. helmanticensis* BIGb0525, *P. koreensis* LMG 21318^T, *P. laurylsulfativorans* DSM 105098^T, *P. moraviensis* LMG 24280^T and *P. neuropathica* CCUG 74875^T were used (Table 3).

Strain MBT-4^T showed a maximal ANIb value of 88.8% (Table 3) to the closest related species *P. helmanticensis* strain BIGb0525, "*P. crudilactis"* DSM 109949 and *P. neuropathica* CCUG 74875, which did not match the highest dDDH result of 35.9% for *P. baetica* LMG 25716. With an ANI value <95% and a dDDH value <70%, strain MBT-4^T is a member of a hitherto unknown *Pseudomonas* species, which we described here as *Pseudomonas rustica* sp. nov. Strain MBT-17 is a further representative of *P. rustica* MBT-4^T with an ANIb value of 96.6%. The whole genome sequencing confirmed the initial species assignment carried out originally via MALDI-TOF identification.

PHYLOGENY

Reference strains for phylogenetic comparisons were selected based on their highest similarity values of *rpoB*, 16S rRNA and dDDH, as shown in Table 3. For the phylogenetic classification we used a core gene based phylogenetic tree (Fig. 4) due to the higher discrimination capacity when compared to multilocus sequence analysis [17]. For this, a phylogenetic tree-building service was used based on 1000 core genes (single copy genes) to generate the tree at PATRIC BRC [25], as described earlier [13].

In the maximum-likelihood phylogenetic tree (Fig. 4), strains MBT-4^T and MBT-17 clustered next to *P. neuropathica* CCUG 74875, *P. helmanticensis* BIGb0525 and *P. crudilactis* DSM 109949 as closest related species and next to the monophyletic clade of *P. granadensis* LMG 27940^T and *P. moraviensis* LMG 24280^T. This shows that strains MBT-4^T and MBT-17 were located within

Table 4. Differential phenotypic characteristics of strain MBT-4^T and representatives of the most closely related *Pseudomonas* species

Strains: 1, MBT-4^T; 2, *P. neuropathica* P155^T; 3, *P. helmanticensis* OHA11^T; 4, "*P. crudilactis*" UCMA 17988^T; 5, *P. granadensis* F-278,770^T; 6, *P. moraviensis* 1B4^T; 7, *P. baetica* a390^T; 8, *P. atagonensis* PS14^T; 9, *P. koreensis* Ps9-14^T. ND, Not detected/not reported; +, positive, –, negative; W, weak; V, variable.

Feature‡	1	2[a]	3[b]	4[c]	5[d]	6[e]	7[f]	8[g]	9[h]
G+C content (mol%)	58.9	59.2	58.1	59.1	60.2	60.3	58.8	59.6	60.5
Flagellation	Lophotrichous	Lophotrichous	Polar, two	Lophotrichous	Polar, two	Polar, two	ND	ND	Polar, multiple
Oxidase	+	+	+	+	+	+	+	+	+
Arginine dihydrolase activity*	+	+	+	+	+	–	+	ND	+
Nitrate reduction*	–	–	–	–	–	–	–	–	–
Hydrolysis of gelatin*	+	+	–	–	+	–	+	+	–
Fluorescent pigment on King B	–	+	+	–	–	+	+	+	–
β-Galactosidase	–	–	–	–	–	–	–	ND	ND
D-Arabinose†	–	+	–	ND	ND	–	ND	ND	ND
D-Ribose†	+	ND	–	ND	ND	+	V	ND	+
Melibiose†	+	ND	–	+	ND	–	–	ND	–
L-Arabinose†	+	+	+	+	+	+	+	ND	+
D-Mannitol†	+	ND	+	ND	+	+	+	+	+
Inosine	ND	+	+	+	–	+	+	W	–
Cellobiose†	–	ND	–	+	–	+	–	ND	+
Trehalose†	–	+	–	–	+	+	–	ND	–
Sucrose†	–	–	–	–	ND	–	+	ND	ND
D-Fucose†	+	W	+	+	W	–	+	W	–
N-Acetylglucosamine†	–	+	+	+	+	+	+	+	+
2-Ketogluconate†	–	ND	+	–	ND	+	ND	ND	ND
5-Ketogluconate†	–	ND	–	–	ND	–	ND	ND	ND
Temperature range for growth (°C)	4–30	4–37	4–30	4–30	4–37	4–37	4–30	5–32	4–37

*Result from API 20E test.
†Result from API 50CH test; Data from MBT-4^T were obtained in this study.
‡Data from: *a*, [27]; *b*, [32]; *c*, [21]; *d*, [33]; *e*, [34]; *f*, [35]; *g*, [26]; *h*, [36].

Table 5. Cellular fatty acid composition characteristics of strain MBT-4^T and representatives of the most closely related *Pseudomonas* species

Strains: 1, MBT-4^T; 2, *P. neuropathica* P155^T; 3, *P. helmanticensis* OHA11^T; 4, "*P. crudilactis*" UCMA 17988^T; 5, *P. granadensis* F-278,770^T; 6, *P. moraviensis* 1B4^T; 7, *P. baetica* a390^T; 8, *P. atagonensis* PS14^T; 9, *P. koreensis* Ps9-14^T. ND, not detected/not reported; TR trace (<1%).

Fatty acid*	1	2[a]	3[b]	4[c]	5[d]	6[e]	7[f]	8[g]	9[h]
$C_{10:0}$	0.2	ND	0.1	0.1	ND	ND	0.1	ND	ND
$C_{10:0}$ 3-OH	4.2	4.2	3.4	4.0	3.2	2.6	3.4	3.2	2.2
$C_{12:0}$	1.6	1.7	1.7	1.4	1.5	2.1	1.7	1.6	1.6
$C_{12:0}$ 2-OH	5.3	5.4	5.5	5.2	4.7	4.9	5.5	5.3	5
$C_{12:0}$ 3-OH	4.3	4.7	3.2	4.2	2.5	4.1	3.2	4.5	4
$C_{14:0}$	0.4	TR	0.5	0.6	ND	0.4	0.5	ND	0.7
$C_{16:0}$	30.8	38.4	29.4	33.7	32	29	29.4	32.8	33
Cyclo-$C_{17:0}$	4.5	11.6	3.2	2.8	6.9	2.4	3.2	11.5	2
$C_{18:0}$	0.5	TR	0.3	0.4	ND	0.5	0.3	TR	0.7
$C_{19:0}$ ω8*c*	ND	ND	ND	ND	ND	0.2	ND	ND	ND
Summed feature 3†	33.1	24.6	39.5	36.2	36	36	39.5	27.2	37
Summed feature 8†	14.2	7.7	12.2	11.3	12	17	12.2	10.7	13

*Data from MBT-4^T were obtained in this study. Data for other type strains were obtained from references *a*, [27]; *b*, [32]; *c*, [21]; *d*, [33]; *e*, [34]; *f*, [35]; *g*, [26]; *h*, [36].

†Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed feature 3 consists of $C_{16:1}\omega7c/C_{16:1}\omega6c$; summed feature 8 consists of $C_{18:1}\omega7c/C_{18:1}\omega6c$.

the *P. koreensis* subgroup of the *P. fluorescens* lineage [17, 19]. Results of the single gene and genome-to-genome analyses also support this classification.

Recently, the *P. koreensis* subgroup was extended by the inclusion of *P. atagonensis* [26], *P. neuropathica* [27], "*P. crudilactis*" [21], *P. anatoliensis* and *P. iridis* [22], which were isolated from soil, fish and raw milk. This suggests that representatives of this subgroup are generally distributed and highly adaptable to different environmental factors.

PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Additionally, strain MBT-4^T was characterized phenotypically as described by Gieschler *et al.* [13]. In brief, all physiological analyses were performed in three independent repetitions. Cells were routinely cultivated on tryptic soy agar (TSA) plates at 30 °C for 48 h or in tryptic soy broth (TSB; Merck) at 30 °C overnight. Optimal growth conditions and growth limits were determined in TSB with NaCl concentrations from 0–8% (steps of 1%), pH values from pH 4 to 10 (intervals of 0.5) and at different temperatures between 4 and 42 °C. Determination of the tolerated NaCl concentration, temperature limits and upper and lower pH values were performed in 10 ml of the corresponding media, and turbidity was tested after 1 week at 30 °C or at the defined temperature, respectively.

Assimilation of different carbohydrates as single carbon and energy sources was assessed in microtiter plates. Forty-seven different sugars were dissolved in water and filter-sterilized with concentrations twice as high as given in the API 50CH strips manual (bioMérieux). Each strain was cultivated overnight at 30 °C in TSB and a cell suspension with an OD600 value of ~0.4 was produced in API 50 CHB/E medium. Afterwards, 100 µl cell suspension was mixed aseptically with 100 µl sugar solution in each well. The plates were sealed with Breathe-Easy sealing membrane (Diversified Biotech) and incubated (shaking at 500 r.p.m. for 7 days at room temperature). The presence of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase and gelatinase, as well as the production of H_2S and the Voges–Proskauer reaction, were tested following the manufacturer's instructions of the API 20E test kit (bioMérieux). The results were recorded after 48 h incubation at 30 °C.

Additionally, strain MBT-4^T was prepared for transmission and scanning electron microscopy. In the case of transmission electron microscopy, the samples were prepared as described by Soerensen *et al.* [28] after 48 h growth in TSB at 30 °C. The only modification made was that 0.1% (v/v) glutaraldehyde was used for fixation and 0.5% (w/v) uranyl acetate for negative staining.

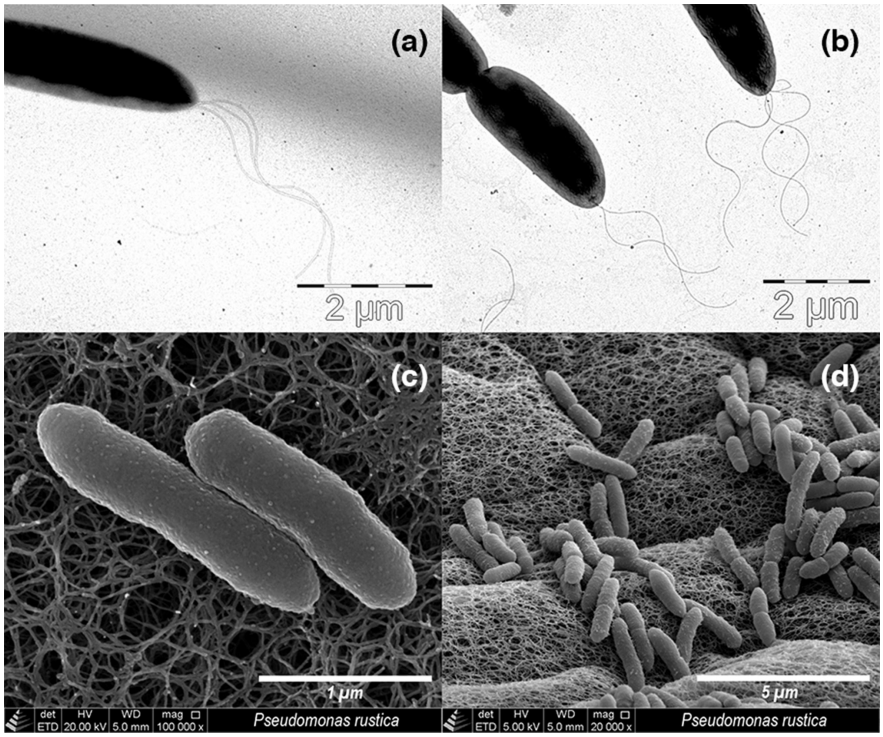

Fig. 5. (a, b) Transmission electronic microscopy images of cells of strain MBT-4^T showing a lophotrichous flagellation. More than 80% of the cells showed truncated or no flagellation. (c, d) Scanning electron microscopy images of two cells of strain MBT-4^T showing the typical rod-shaped appearance. The net-like mesh in the background is the drying-residue of the nutrient agar.

Subsequently, samples were analysed using a Tecnai 10 transmission electron microscope (FEI Thermo Fisher Scientific), at an accelerating voltage of 80 kV. Digital photos were taken with a Megaview G2 CCD camera (Emsis).

For the morphological characterization with scanning electron microscopy, the strain was grown on TSA plates for 2 days at 30 °C. Chemical fixation, dehydration and critical point drying of the samples were carried out as described previously [29]. Imaging was performed with a Quanta 250 FEG field emission scanning electron microscope (FEI) equipped with an Everhart–Thornley detector under high vacuum conditions and with an acceleration voltage of 10 kV. The cell lengths and diameters of a number of 150 bacteria cells were measured using the ObjectJ plug-in of ImageJ (version 1.52p).

Phenotypic characteristics of the novel species are reported below in the species description and the differences with respect to the closest species of the genus *Pseudomonas* are recorded in Table 3. The phenotypic characteristics of strain MBT-4^T support its classification within the genus *Pseudomonas*. The growth characteristics of strain MBT-4^T were comparable to the type strains of the closest related species (see Table 4), especially with regard to the growth behaviour at different salt concentrations and pH values and as well as their growth temperatures.

Further differences between the strains were observed in the assimilation of carbohydrates and other physiological properties. Strain MBT-4^T could be differentiated from the most closely related type strains by a combination of the ability/inability to assimilate cellobiose, trehalose, melibiose, D-fucose, *N*-acetylglucosamine, 2-ketogluconate and 5-ketogluconate and by the presence/absence of the enzyme for gelatin hydrolysis. Strain MBT-4^T produced no pyocyanin when grown on King A agar and no fluorescent pigment on King B.

Fatty acid analyses were performed by the DSMZ (German Collection of Microorganisms and Cell Cultures). Cellular fatty acids were analysed after conversion into fatty acid methyl esters by saponification, methylation and extraction using minor modifications of the methods of Miller [30] and Kuykendall *et al.* [31]. The fatty acid methyl esters of the potential new species were obtained from cells grown on TSA at 28 °C for 24 h.

The major fatty acids of strain MBT-4^T were summed feature 3 ($C_{16:1}$ ω7*c* $C_{16:1}$ ω6*c*; 33.1%), $C_{16:0}$ (30.8%), summed feature 8 ($C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c*; 14.2%), $C_{12:0}$ 2-OH (5.3%), cyclo-$C_{17:0}$ (4.5%), $C_{12:0}$ 3-OH (4.3%), $C_{10:0}$ 3-OH (4.2%) and $C_{12:0}$ (1.6%).

These results agree with those obtained for the closest relatives [21, 32] and also for other species of the genus *Pseudomonas* [33–35]. When comparing the profiles of the reference strains *P. neuropathica* and *P. atagonensis* with the profile determined for

P. rustica there are only small differences. In particular, the amounts of summed feature 3 ($C_{16:1}$ ω7*c*), $C_{16:0}$ and summed feature 8 ($C_{18:1}$ ω7*c*) and cyclo-$C_{17:0}$ (see Table 5). Nevertheless, the fatty acid composition of bacteria cells is especially influenced by test conditions, cultivation media and temperature, which could explain the discrepancies between different studies.

Based on phylogenetic analysis, phenotypic characteristics and fatty acid composition, strain MBT-4^T is considered to represent a novel species, for which the name *Pseudomonas rustica* sp. nov. is proposed.

DESCRIPTION OF *PSEUDOMONAS RUSTICA* SP. NOV.

Pseudomonas rustica (rus'ti.ca. L. fem. adj. *rustica*, rural, peasant, referring to the source of isolation of the species)

The colonies of this species are smooth, beige, shiny and round with a diameter of 1–2 mm after incubation at 30 °C for 24 h on TSA. The rod-shaped cells (see Fig. 5) have a length of 1.2 µm–2.4 µm (mean 1.7 µm) and a width of 0.4 µm–0.6 µm (mean 0.5 µm) and are Gram-negative, catalase-positive, oxidase-positive and mobile. Strain MBT-4^T shows no *β*-haemolysis when cultivated on Columbia agar supplemented with 5% sheep blood. Grows on Reasoner's 2A agar and on cetrimide agar, but only weakly under anaerobic conditions. Pigments are not produced on King A and King B agar. Growth is detected from 4 to 30 °C (growth at temperatures lower than 4 °C has not been tested), with an optimal growth temperature between 20 and 25 °C. pH values between pH 6.0 and 8.0 are tolerated (optimal pH 6.5–7.5) as well as salt concentrations of up to 4.0% (w/v; optimum, 0.5–1.0%). Strain MBT-4^T shows proteolysis on skimmed milk agar, lipolysis on tributyrin agar at both 20 and 30 °C, but no starch hydrolysis. Positive reactions are observed for arginine dihydrolase, citrate utilization, gelatin hydrolysis, cytochrome-oxidase and denitrification, but negative reactions for *β*-galactosidase, lysine decarboxylase, ornithine decarboxylase (2/3), H_2S production, urease, tryptophan deaminase, indole production, Voges–Proskauer reaction and for nitrate reduction. Positive reactions for the utilization of glycerin, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, melibiose, D-gentiobiose, D-fucose and D-arabitol were detected. Acid production of D-lyxose and D-tagatose is variable. Strain MBT-4^T does not produce acid from erythritol, L-xylose, D-arabinose, D-adonitol, methyl *β*-D-xylopyranose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, lactose, cellobiose, maltose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, L-fucose, L-arabitol, potassium-gluconate, 2-ketogluconate and 5-ketogluconate. The predominant fatty acids of the type strain are summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*), $C_{16:0}$, summed feature 8 ($C_{18:1}$ ω7*c*/$C_{18:1}$ ω6*c*), $C_{12:0}$, $C_{12:0}$ 3-OH, $C_{12:0}$ 2-OH and $C_{10:0}$ 3-OH.

The type strain is MBT-4^T (DSM 112348^T=LMG 32241^T), which was isolated from raw milk. The genome has a DNA G+C content of 58.9 mol% and a draft genome size of 5.86 Mb with 46 contigs and 5294 coding DNA sequences (CDS).

Funding information
This work was supported by an IGF Project AiF 20027 N of the FEI via AiF within the programme for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a resolution of the German Parliament.

Acknowledgements
We thank Imke Wahl, Katja Magnussen, Adrian Prager, Angela Back and Simone Brümmer for their skillful technical assistance.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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