Pseudomonas helleri sp. nov. and Pseudomonas weihenstephanensis sp. nov., isolated from raw cow’s milk


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Analysis of the microbiota of raw cow’s milk and semi-finished milk products yielded seven isolates assigned to the genus Pseudomonas that formed two individual groups in a phylogenetic analysis based on partial rpoD and 16S rRNA gene sequences. The two groups could be differentiated from each other and also from their closest relatives as well as from the type species Pseudomonas aeruginosa by phenotypic and chemotaxonomic characterization and average nucleotide identity (ANIb) values calculated from draft genome assemblies. ANIb values within the groups were higher than 97.3 %, whereas similarity values to the closest relatives were 85 % or less. The major cellular lipids of strains WS4917T and WS4993T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol; the major quinone was Q-9 in both strains, with small amounts of Q-8 in strain WS4917T. The DNA G+C contents of strains WS4917T and WS4993T were 58.08 and 57.30 mol%, respectively. Based on these data, strains WS4917T, WS4995 (=DSM 29141=LMG 28434), WS4999, WS5001 and WS5002 should be considered as representatives of a novel species of the genus Pseudomonas, for which the name Pseudomonas helleri sp. nov. is proposed. The type strain of Pseudomonas helleri is strain WS4917T (=DSM 29165T=LMG 28433T). Strains WS4993T and WS4994 (=DSM 29140=LMG 28438) should be recognized as representing a second novel species of the genus Pseudomonas, for which the name Pseudomonas weihenstephanensis sp. nov. is proposed. The type strain of Pseudomonas weihenstephanensis is strain WS4993T (=DSM 29166T=LMG 28437T).

The genus Pseudomonas Migula 1894 is known for its metabolic diversity, and members have been isolated from various environments and sources including animals, plants, soil and water (Moore et al., 2006). Some species are also known to be animal, human or plant pathogens (Kämpfer & Glaeser, 2012). At the time of writing, the genus Pseudomonas encompasses about 150 species with validly published names (Euzéby, 1997; Parte, 2014).

Abbreviations: ANI, average nucleotide identity; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and rpoD gene sequences of the strains WS4917T, WS4995, WS4999, WS5001, WS5002, WS4993T and WS4994 are KP738715, KP738716, KP738717, KP738718, KP738719, KP738720 and KP738721 (16S rRNA gene) and KP738722, KP738723, KP738724, KP738725, KP738726, KP738727 and KP738728 (rpoD), respectively. The accession numbers of the genome sequences of strains WS4917T, WS4995, WS4993T and WS4994, P. deceptionensis DSM 26521T, P. lundensis DSM 62521T, P. psychrophila DSM 17535T, P. taetrolens DSM 21104T, P. lini DSM 16768T, P. aeruginosa DSM 50071T and P. endophytica BSTT44T are JYLD00000000, JYLE00000000, JYKX00000000, JYKY00000000, JYKZ00000000, JYLA00000000, JYLB00000000, JYLC00000000 and LLWH00000000, respectively.

Six supplementary figures and two supplementary tables are available with the online Supplementary Material.

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Several species of *Pseudomonas* have been reported to be involved in food spoilage (Arslan et al., 2011; Ercolini et al., 2007; Franzetti & Scarpellini, 2007; Tryfinopoulou et al., 2002), and members of the genus are considered to be important milk spoilers due to their psychrotolerant nature and proteolytic activity (Hantsis-Zacharov & Halpern, 2007; Marchand et al., 2009b; Martins et al., 2006).

In a study investigating the microbiota of raw milk for its composition and enzymic spoilage potential, 20 raw-milk samples (von Neubeck et al., 2015) and 20 samples of semi-finished milk products (data not shown) were analysed. *Pseudomonas* turned out to be the most prevalent and abundant genus, and a large number of potentially novel species of the genus *Pseudomonas* were detected. Seven isolates (WS4917, WS4993, WS4999, WS5001, WS5002, WS4993 and WS4994) from seven different samples were assigned to the genus *Pseudomonas* but could not be identified to the species level. The phylogeny of the isolates was determined by 16S rRNA gene sequence and partial *rpoD* nucleotide sequence analysis, since the 16S rRNA gene cannot be used alone to discriminate related species of the genus *Pseudomonas* (Mulet et al., 2009, 2010). DNA extraction for PCR and PCR conditions have been described elsewhere (von Neubeck et al., 2015). As a modification, the annealing temperature for partial *rpoD* nucleotide sequences was increased to 51 °C to avoid unspecific primer binding. Primers used for PCR were 16S_27F (5′-AGAGTTTGATCCTGGCTCA-3′) and 16S_1492r (5′-CGGCTACCTTGTTACGAC-3′), leading to almost-complete 16S rRNA gene sequences, and *PsEG30F* (5′-ATGYGAATGCACAARCG-3′) and *PsEG790R* (5′-CGGTTGATKTCCTTGA-3′), resulting in partial *rpoD* gene sequences of ~750 bp (Mule et al., 2009). Nucleotide sequencing was performed at LGC Genomics GmbH (Berlin, Germany) using primer *PsEG30F* for the partial *rpoD* gene and primers 16S_926F (5′-CGGCTAAATCCCTTGAGTGTTC-3′) and 16S_519F (5′-CAGGCAGCGGCCTTATAC-3′) for the 16S rRNA gene. The *rpoD* gene sequences were analysed for similarity to nucleotide sequences of type strains for further analyses were chosen by combining the results of *rpoD* and 16S rRNA gene sequence analysis (Fig. 1) and almost-complete 16S rRNA gene sequences (Fig. 2). Reference strains for further analyses were chosen by combining the results of *rpoD* and 16S rRNA gene sequence similarity and the phylogenetic position of the seven isolates. Strains used as references in this study were *P. deceptionensis* DSM 26521T (Carrión et al., 2011), *P. endophytica* BSTT44T (Ramirez-Bahena et al., 2015), *P. fragi* DSM 3456T (Skerman et al., 1980), *P. lundensis* DSM 6252T (Molin et al., 1986), *P. psychrophila* DSM 17535T (Yumoto et al., 2001) and *P. taetronius* DSM 21104T (Skerman et al., 1980) as well as the type strain of the type species, *Pseudomonas aeruginosa* DSM 50071T (Skerman et al., 1980).

In addition to the single-gene sequence analysis, whole-genome sequencing was performed. Genomic DNA was isolated from all reference strains (except *P. fragi* DSM...
Pseudomonas taetrolens IAM 1653T (D84027)
Pseudomonas lundensis ATCC 49968T (AB021395)
Pseudomonas weihenstephanensis WS4993T (KP738720)
Pseudomonas weihenstephanensis WS4994 (KP738721)
Pseudomonas deceptioensis M1T (GU936597)
Pseudomonas fragi IFO 3458T (AB021413)
Pseudomonas psychrophila E-3T (AB041885)
Pseudomonas endophytica BSTT44T (LN624760)
Pseudomonas helleri WS4999 (KP738717)
Pseudomonas helleri WS5001 (KP738718)
Pseudomonas helleri WS5002 (KP738719)
Pseudomonas helleri WS4917T (KP738715)
Pseudomonas helleri WS4995 (KP738716)
Pseudomonas chlororaphis subsp. aureofaciens DSM 6698T (AY509898)
Pseudomonas chlororaphis subsp. aurantiaca NCIB 10068T (DQ682655)
Pseudomonas chlororaphis subsp. chlororaphis DSM 50083T (Z76673)
Pseudomonas kilonensis 520-20T (AJ292426)
Pseudomonas corrugata ATCC 29736T (D84012)
Pseudomonas thivervalensis CFBP 11126T (AF100323)
Pseudomonas brassicacearum subsp. brassicacearum DBK11T (AF100321)
Pseudomonas mediterranea CFBP 5447T (AF386080)
Pseudomonas lini CFBP 5737T (AY035996)
Pseudomonas arsenicoxydans VC-1T (FN645213)
Pseudomonas migulae CIP 105470T (AF074383)
Pseudomonas meridiana CMS 38T (AJ537602)
Pseudomonas mucidolens IAM 12406T (D84017)
Pseudomonas brennerii CML 97-39T (AF268968)
Pseudomonas proteolytica CMS 64T (AJ537603)
Pseudomonas gessardii CIP 105469T (AF074384)
Pseudomonas frederiksbergensis JA28T (AJ249382)
Pseudomonas mandelli CIP 105273T (AF058826)
Pseudomonas protegens CHAO1 (AJ278812)
Pseudomonas saponiphila DSM 9751T (FM082864)
Pseudomonas asplenii ATCC 23835T (AB021397)
Pseudomonas fuscovaginae ICMP 5940T (FJ483519)
Pseudomonas agarici LMG 2112T (Z76652)
Pseudomonas moraviensis CCM 7280T (AY970952)
Pseudomonas koreensis Ps 9-14T (AF468452)
Pseudomonas jessenii CIP 105274T (AF068259)
Pseudomonas reinekei MT1T (AM293565)
Pseudomonas baetica a390T (FM201274)
Pseudomonas mohnii IpA-2T (AM293567)
Pseudomonas umsongensis Ps 3-10T (AF468450)
Pseudomonas moorei RW10T (AM293566)
Pseudomonas vancouverensis Dha-51T (AJ011507)
Pseudomonas pohangensis H3-R18T (DQ339144)
Pseudomonas aeruginosa DSM 50071T (HE978271)
Acinetobacter baumannii DSM 3007T (X81660)

M. von Neubeck and others
Fig. 2. Maximum-likelihood phylogenetic tree based on almost-complete 16S rRNA gene sequences (1409 nucleotide positions in the final dataset). The tree shows the phylogenetic position of the five strains of *P. helleri* sp. nov. (*WS4917*<sup>T</sup>, *WS4995*, *WS4999*, *WS5001* and *WS5002*), the two strains of *P. weihenstephanensis* sp. nov. (*WS4993*<sup>T</sup> and *WS4994*<sup>T</sup>) and closely related species. Bootstrap values (>50 %) based on 1000 replications are depicted as percentages at branch nodes. *Acinetobacter baumannii* DSM 30007<sup>T</sup> was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

3456<sup>T</sup>, as the genome of the strain *P. fragi* B25<sup>T</sup> is already available under accession number AHZX00000000) and strains *WS4917*<sup>T</sup>, *WS4995*, *WS4993*<sup>T</sup> and *WS4994* grown on tryptic soy agar (TSA; Roth) supplemented with 1 % glucose (referred to as TSG) for 24 h at 22 °C using the Qiagen QIAamp DNA Mini kit according to the manufacturer’s instructions for bacteria from plate cultures. Sequencing of genomic DNA was performed using an Illumina MiSeq platform and the Illumina TruSeq DNA PCR-Free Sample Preparation kit. After quality control and filtering, sequence data were assembled using SPAdes. Draft genome assemblies were used for calculating the average nucleotide identity (ANI). ANIb was calculated on the basis of pairwise comparison of draft genome assemblies with the script ANI.pl (written by Jiapeng Chen, School of Energy and Environment, City University of Hong Kong) available at https://github.com/chjp/ANI that uses the algorithm described by Richter & Rossello-Móra (2009). The species demarcation of 94–96 % ANIb (Richter & Rossello-Móra, 2009) was used as a benchmark. Analysis of ANIb values confirmed the results of rpoD gene analysis. Strains *WS4917*<sup>T</sup> and *WS4995* of species group 1 showed an ANIb value of 97.3 %, and strains *WS4993*<sup>T</sup> and *WS4994* of species group 2 had an ANIb value of 99.2 % (Table 1). The ANIb value of strains *WS4917*<sup>T</sup> and *WS4995* to strains *WS4993*<sup>T</sup> and *WS4994* was lower than 82 %, confirming that the two species groups represent two distinct species. ANIb values of strains *WS4917*<sup>T</sup>, *WS4995*, *WS4993*<sup>T</sup> and *WS4994* to all reference strains were lower than 85 %, and the two species groups therefore represent two novel species distinct from their closest relatives. Genomes have been annotated by the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2014). Genome size, DNA G+C content, number of genes and proteins listed in Table 2 were obtained from the annotation and assembly reports.

The DNA G+C contents of strains *WS4917*<sup>T</sup>, *WS4995*, *WS4993*<sup>T</sup> and *WS4994* in the range 57–58 mol% are comparable to those of other species of the genus *Pseudomonas* (Moore et al., 2006).

In addition, the phylogenetic relationships of the two novel species of the genus *Pseudomonas* to their closest relatives were determined by multilocus sequence analysis (MLSA). Following the widely used method of Mulet et al. (2010), partial sequences of the 16S rRNA, gyrB, rpoD and rpoB genes were concatenated, aligned and used to calculate a neighbour-joining phylogenetic tree (Fig. S1, available in the online Supplementary Material). The corresponding phylogenetic tree based on concatenated deduced partial amino acid sequences of Gyrb, RpoD and RpoB is shown in Fig. S2. The sequences of all four genes of each reference strain, except the 16S rRNA gene sequences of the type strains of *P. fragi* (strain IFO 3458<sup>T</sup>; GenBank accession no. AB021413) and *P. lini* (strain CFBP 5737<sup>T</sup>; AY035996), were retrieved from the NCBI database. Each strain was characterized by the presence of all key genes and proteins listed in Table 2. The phylogenetic tree constructed with the corresponding phylogenetic tree based on concatenated deduced partial amino acid sequences of Gyrb, RpoD and RpoB is shown in Fig. S2. The sequences of all four genes of each reference strain, except the 16S rRNA gene sequences of the type strains of *P. fragi* (strain IFO 3458<sup>T</sup>; GenBank accession no. AB021413) and *P. lini* (strain CFBP 5737<sup>T</sup>; AY035996), were retrieved from the NCBI database.

Table 1. ANIb between all sequenced strains of *P. helleri* sp. nov. and *P. weihenstephanensis* sp. nov. and reference type strains

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Table 2. Characteristics that differentiate *P. helleri* sp. nov. WS4917<sup>T</sup> and *P. weihenstephanensis* sp. nov. WS4993<sup>T</sup>, the type strains of the most closely related species and the type strain of the type species, *P. aeruginosa*

| Strains: 1, *P. helleri* sp. nov. WS4917<sup>T</sup>; 2, *P. weihenstephanensis* sp. nov. WS4993<sup>T</sup>; 3, *P. endophytica* BSTT<sup>T</sup>4; 4, *P. deceptionensis* DSM 26521<sup>T</sup>; 5, *P. fragi* DSM 3456<sup>T</sup>; 6, *P. lundensis* DSM 6252<sup>T</sup>; 7, *P. psychrophila* DSM 17535<sup>T</sup>; 8, *P. taetrolens* DSM 21104<sup>T</sup>; 9, *P. lini* DSM 16768<sup>T</sup>; 10, *P. aeruginosa* DSM 50071<sup>T</sup>. All data are from this study unless indicated. All strains are positive for utilization of D-glucose, D-fructose, D-ribose, glycerol and potassium gluconate and negative for reduction of nitrate and utilization of amygdalin, arbutin, D-adonitol, cellobiose, D-fucose, lactose, maltose, melezitose, D-tagatose, turanose, dulcitol, erythritol, gentiobiose, glycogen, L-arabitol, L-rhamnose, L-sorbose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xylopyranoside, salicin and xylitol. +, Positive; —, negative; W, weakly positive.

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<td>Growth on cetrimide agar</td>
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<td>5.33</td>
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<td>59.4‡</td>
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*aStrain WS4995 showed a negative phenotype; WS4917<sup>T</sup> and the three other tested strains were positive.

†Three of the five tested strains (including WS4917<sup>T</sup>) grew at up to 31 °C; WS4995 and one other strain grew at up to 33 °C.

‡Obtained from the published genome sequence of *P. fragi* B25<sup>T</sup> (accession no. AHZX01000000).

§Numbers were taken from the master records of the whole-genome shotgun sequencing project unless indicated.

‖Number of proteins obtained from the Genome Assembly and Annotation report is given (accession no. AHZX01000000).

whole-genome sequences. The same was true for strains WS4917<sup>T</sup>, WS4995, WS4993<sup>T</sup> and WS4994, representing the two novel species of the genus *Pseudomonas* in the MLSA. The strains of the two novel species formed two clearly separated branches within the phylogenetic neighbour-joining trees (Figs S1 and S2), confirming their distinctness as members of novel species. The proportion of nucleotide sites differing between the concatenated partial gene
sequences (p-distance) was calculated using MEGA 5.2.2 (Tamura et al., 2011) and is given as a percentage of sequence similarity in Table S1. Intraspess species similarity between the two strains of the two novel species was higher than 99.5 %, while the highest sequence similarity to the most closely related type strain was 96 % or less. Based on the benchmark of 97 % MLSA (16S rRNA, gyrB, rpoD and rpoB) sequence similarity proposed by Mulet et al. (2010), strains WS4917\(^{T}\) and WS4995 and strains WS4993\(^{T}\) and WS4994 are members of two independent species of the genus Pseudomonas.

In order to document morphological features, cells were grown routinely on TSG at 25 °C for 24 h unless otherwise mentioned. Physiological characteristics were examined in two completely independent repetitions. Oxidase activity was determined using Bactident Oxidase strips (Merck) according to the manufacturer’s instructions and the catalase reaction was tested with 3 % (v/v) \( \text{H}_2\text{O}_2\). Determination of the Gram type was performed by using the non-staining KOH method (Powers, 1995; Ryu, 1940). Cell shape of strains WS4917\(^{T}\) and WS4993\(^{T}\) (Figs S3 and S4) and motility were observed using the bright-field of a fluorescence microscope (BX51; Olympus) after growth at 25 °C without shaking for 24 h in trypticase soy broth [TSB; containing (l\(^{-1}\)) 17.0 g peptone from casein, 3.0 g peptone from soymeal, 2.5 g \( \text{KH}_2\text{PO}_4\), 2.5 g glucose, 5.0 g \( \text{NaCl}\), pH 7.3 ± 0.2]. Growth on cetrimide agar (Merck), R2A agar (Merck) and Columbia blood agar supplemented with 5 % sheep blood (Oxoid) was determined at 25 °C for 24 h. In order to test for \( \beta\)-galactosidase activity, 40 ml X-Gal solution (20 mg ml\(^{-1}\); Carlin Roth) and 40 ml IPTG (100 mM; Applichem) were spread with a sterile spatula on LB agar plates. These plates were incubated for 24 h at 25 °C. Hydrolysis of starch was studied after 48 h at 25 °C by flooding the plates (l\(^{-1}\)) 3.0 g beef extract, 10.0 g soluble starch, 12.0 g agar, pH 7.5 ± 0.2) with Lugol’s iodine for the visualization of possible clearing zones. Pyocyanin and fluorescein production were investigated using King agar A and B, respectively (King et al., 1954). Growth under anaerobic conditions was evaluated on TSG using an anaerobic jar with the addition of Anaerocult A (Merck) as anaerobic catalyst as described by Rieser et al. (2013). Hydrolysis of skimmed milk and tributyrin was tested at 6 and 30 °C as described previously (von Neubeck et al., 2015). Tubes with 5 ml nutrient gelatin (l\(^{-1}\)) 3.0 g beef extract, 5.0 g peptone, 120.0 g gelatin) were used to screen for gelatin hydrolysis; tubes were incubated at 25 °C and analysed after 48 h and 14 days for turbidity and liquefaction (tilting the tubes after incubation at 8 °C for 30 min). Production of \( \text{H}_2\text{S}\) and formation of indole were checked using semi-solid SIM medium (Merck) and Kovacs’ reagent (Merck). Reduction of nitrate and nitrite was tested using reagents NIT 1 and NIT 2 supplied with the API 20NE strips (bioMérieux) and zinc. Cultures were grown at 25 °C for 24 h in tubes without shaking containing 5 ml TSB supplemented with 0.1 % (w/v) \( \text{KNO}_3\) and 0.17 % (w/v) agar for semi-anaerobic conditions. Tests were performed in 1.5 ml reaction tubes after transfer of 1 ml culture.

Assimilation of different carbohydrates as single carbon and energy sources was assessed in microtitre plates. Carbohydrates were dissolved in water and filter-sterilized using twice the concentrations given in the API 50CH strips manual in mg per well per 100 μl. Bacterial strains were pre-grown overnight at 25 °C on TSG and one loop of cell material was suspended to an OD\(_{600}\) of ~0.4 in double-strength suspension medium. The medium contained (l\(^{-1}\)) 200 ml M9 salts [10 x : 4 ml MgSO\(_4\) solution (1 M), 0.2 ml CaCl\(_2\) (1 M) and 10 ml trace elements stock solution, containing (l\(^{-1}\)) 232 mg H\(_3\)BO\(_3\), 174 mg ZnSO\(_4\) \( \cdot \) 7H\(_2\)O, 116 mg FeSO\(_4\)\( \cdot (\text{NH}_4)\)\(_2\)SO\(_4\) \( \cdot \) 6H\(_2\)O, 81 mg CoCl\(_2\) \( \cdot \) 6H\(_2\)O, 22 mg (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\) \( \cdot \) 4H\(_2\)O, 8 mg CuSO\(_4\), 5H\(_2\)O and 6 mg MnSO\(_4\) \( \cdot \) H\(_2\)O). Tests were performed in 96-well microtitre plates; in each well, 100 μl suspended cells and 100 μl of the respective carbohydrate solution were combined aseptically. Plates were sealed with Breathe-Easy foil (Diversified Biotech) and incubated at 25 °C with shaking at 550 r.p.m. for 3 days. Turbidity of the medium as proof of assimilation was evaluated at day 3 and after 7 days of additional incubation at 25 °C without shaking. Growth under varying conditions was assessed in TSB. Growth was tested in the presence of 0–8 % (w/v) NaCl (at intervals of 1 %) and at pH 4–10 (at intervals of 0.5 pH unit) by supplementing TSB with appropriate buffer systems (pH 4–5.5, 0.1 M citric acid/0.1 M sodium citrate; pH 6–8, 0.1 M K\(_2\)HPO\(_4\)/0.1 M NaOH; pH 8.5–10, 0.1 M NaHCO\(_3\)/0.1 M Na\(_2\)CO\(_3\)) according to Xu et al. (2005). After autoclaving, the pH of the medium was adjusted by the addition of 1 M NaOH or HCl before sterile filtration (pore size 0.22 μm) of the medium. Analyses were performed at 25 °C for 3 days by shaking at medium intensity in the Bioscreen C instrument and measuring the OD\(_{600}\) every 20 min. Each well contained 200 μl of the selected medium and was inoculated at a dilution of 1 : 1000 with an overnight culture of cells grown in unmodified TSB at 25 °C for 24 h. Periphery pH and NaCl growth conditions obtained during the Bioscreen approach were also studied in 5 ml tubes at 25 °C for 1 week. Growth at 4–42 °C was tested in tubes containing 7 ml TSB with shaking at 130 r.p.m. for 8 days. All strains were precultured overnight at room temperature (22 °C) on TSG before suspending one loop of cells in 1 ml TSB as inoculation culture. The 7 ml test tubes were inoculated with 10 μl of the inoculation culture. The optimal growth temperature was tested using the Bioscreen C instrument and TSB under the same conditions as used for testing growth at different NaCl concentrations and pH. The range for optimal growth was determined by the length of the lag phase until the cultures achieved an increase in OD\(_{600}\) of 0.2.

The growth characteristics of the novel strains resembled those observed for the reference strains. All strains were
psychrotolerant and grew at 4 °C, which is a common feature for species of *Pseudomonas*. The maximum growth temperature was also similar for most strains, with the exception of *P. aeruginosa* DSM 50071T, which grew at up to 42 °C. Also, the NaCl concentrations and pH tolerance were comparable for all strains, with the exception of *P. lini* DSM 16768T, which tolerated only 3 % NaCl. More differences were observed in the assimilation of carbohydrates and other physiological properties. The strains of the novel species could be differentiated from the most closely related type strains by at least four traits. The differential phenotypic characteristics are listed in Table 2.

During the survey to determine phenotypic characteristics of the novel strains, it was observed that not all strains of the same species displayed the same characteristics in all tests. Production of yellow to reddish extracellular pigments on nearly all assimilated carbon sources was observed for one (WS5001) of the five tested isolates of species group 1. In addition, colonies of WS5001 growing on plate count agar supplemented with 1 % skimmed milk powder for more than 5 days at room temperature (22 °C) revealed a quite hard, shrivelled and wrinkled surface, while the other four strains displayed a smooth colony surface. The maximum growth temperature for the isolates of species group 1 was also variable; three isolates (WS4917T, WS4999 and WS5001) grew at up to 31 °C, while the other isolates (WS4995 and WS5002) grew at up to 33 °C. In addition, strains WS4917T, WS4999, WS5001 and WS5002 were positive for the assimilation of D-arabitol, D-mannitol and trehalose, while strain WS4995 showed negative results. The phenotypic data for the reference strains used are also not fully congruent with data obtained in other studies (Carrión et al., 2011; Ramírez-Bahena et al., 2015). For example, the ability to produce a diffusible fluorescent pigment during growth on King B agar, for which *P. endophytica* LMG 28456T, *P. fragi* DSM 3456T, *P. lundensis* DSM 6252T and *P. psychrophila* DSM 17535T tested negative during our survey, were reported as positive in the study of Ramírez-Bahena et al. (2015). However, *P. psychrophila* DSM 17535T was reported as negative previously by Carrión et al. (2011). Another example of a discrepancy is the assimilation of gentiobiose by *P. deentionis* DSM 26521T, *P. psychrophila* DSM 17535T, *P. taetrolens* DSM 21104T and *P. fragi* DSM 3456T, which was negative in our study but was reported as positive in the study of Carrión et al. (2011). These differences can probably be attributed to different test conditions, particularly for the assimilation of carbohydrates. As the API 50 CHB/E medium used in many studies contains yeast extract and tryptone, this might have supported growth on substrates that test negative when a minimal medium is used, as in this study. Nevertheless, the use of the same test conditions for all strains ensures the high comparability of results in this study and allows an analysis of differentiating features.

One focus of the project, given where the novel strains were isolated, was their proteolytic activity in milk; this activity was therefore tested for all isolates as well as for all reference strains using skimmed milk agar. All strains tested negative at an incubation temperature of 4 °C. However, strain WS4993T was also analysed in liquid ESL milk medium [10 % (v/v) ESL milk, 90 % TSB] and showed high proteolytic activity (C. Glück, E. Rentschler, M. Krewinkel, M. Merz, M. von Neubeck, M. Wenning, S. Scherer, M. Stoeckel, J. Hinrichs, T. Stessler and L. Fischer, unpublished). The peptidase of strain WS4993T was purified and identified by mass spectrometry as the well-known AprA metallopeptidase common for members of the genus *Pseudomonas* (Koka & Weimer, 2000; McCarthy et al., 2004). Analyses of the genome sequences obtained in this study showed that all strains except those of species group 1 and its closest relative *P. endophytica* BSTT44T, as well as *P. psychrophila* DSM 17535T and *P. taetrolens* DSM 21104T, contain the apr operon. Peptidase production has been shown before for *P. lundensis* and *P. fragi* (Baur et al., 2015; Marchand et al., 2009a) as well as *P. aeruginosa* (Baur et al., 2015). It is therefore likely that all species containing the operon are able to produce this metallopeptidase and to degrade milk proteins.

Fatty acid analyses were carried out for all reference strains and the proposed type strains WS4917T and WS4993T by the Identification Service of the DSMZ (Braunschweig, Germany) by modifying the methods of Miller (1982) and Kuykendall et al. (1988). Complete fatty acid profiles for the proposed type strains and reference strains are listed in Table 2. The predominant fatty acids of WS4917T were C16 : 0 (32.4 %), summed feature C16 : 1 07c/iso-C15 : 0 2-OH (21.1 %), C17 : 0 cyclo (17.0 %), C18 : 1 07c (7.4 %) and C19 : 0 cyclo 08c (4.2 %). Those of WS4993T were summed feature C16 : 1 07c/iso-C15 : 0 2-OH (37.3 %), C16 : 0 (30.9 %) and C18 : 1 07c (12.2 %). These results are in agreement with those obtained for other species of the genus *Pseudomonas* (Moore et al., 2006). Comparing the profiles of the reference strains with profiles determined in other publications (Carrión et al., 2011; Ramírez-Bahena et al., 2015) reveals some differences. In particular, the amounts of C17 : 0 cyclo varied between this study and the data obtained by Ramírez-Bahena et al. (2015). Carrión et al. (2011), however, obtained similar values to ours for C17 : 0 cyclo. However, as the fatty acid composition of cells is greatly influenced by cultivation and test conditions, discrepancies between different studies are likely to occur.

Analysis of respiratory quinones and polar lipids (see Figs S5 and S6) was carried out by the Identification Service of the DSMZ. Biomass for these analyses was produced by growing strains WS4917T and WS4993T at 25 °C for 24 h in TSB. Subsequently, the cultures were centrifuged and washed with sterile Ringer’s solution (1/4-strength) before freeze drying. Extraction and analysis were carried out as described elsewhere (Bligh & Dyer, 1959; Tindall, 1990a, b; Tindall et al., 2007). The major polar lipids of
WS4917ᵀ and WS4993ᵀ were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, and the major respiratory quinone was Q-9 for both strains. These results are consistent with previously published data for species of the genus *Pseudomonas* (Moore et al., 2006). In addition, small amounts of respiratory quinone Q-8 were found in strain WS4917ᵀ.

Based on the genetic, phylogenetic, chemotaxonomic, physiological and biochemical data presented here, it is clear that the two groups of strains represent two novel species of the genus *Pseudomonas*, for which the names *Pseudomonas helleri* sp. nov. (strains WS4917ᵀ, WS4995, WS4999, WS5001 and WS5002) and *Pseudomonas weihenstephanensis* sp. nov. (strains WS4993ᵀ and WS4994) are proposed.

**Description of *Pseudomonas helleri* sp. nov.**

*Pseudomonas helleri* (hel’le.ri. N.L. gen. masc. n. *helleri* named in honour of Knut Heller, former microbiologist of the Max-Rubner-Institute in Kiel, Germany, with great merit in food microbiology).

Cells are Gram-negative, motile, non-spore-forming, fluorescent on King B agar, rod-shaped (∼3.1 μm long and ∼0.75 μm wide) and catalase- and oxidase-positive. Beige, smooth and round colonies, 0.5–1.0 mm diameter, are formed after incubation at 25 °C for 24 h on TSG. Growth occurs at 4–31 °C (highest growth temperature varies from 31 to 33 °C; growth at temperatures lower than 4 °C has not been tested). Optimal growth temperature is between 25 and 29 °C. Grows at pH 5–8 and in the presence of up to 6 % (w/v) NaCl. Positive for the utilization of D-arabinose, D-glucose, D-galactose, D-fructose, D-mannose, D-ribose, D-xylose, glycerol, L-fucose and potassium gluconate and negative for the utilization of amygdalin, arbutin, D-adonitol, cellobiose, D-fucose, lactose, L-lyxose, maltose, melibiose, melezitose, raffinose, D-sorbitol, sucrose, D-tagatose, turanose, dulcitol, erythritol, gentiobiose, glycogen, L-arabinose, L-arabinose, L-arabinose, L-arabinose, L-arabinose, L-sorbose, L-xylose, methyl β-D-glucoside, methyl β-D-mannoside, methyl β-D-xylopyranoside, N-acetylglucosamine, salicin and xylitol. Assimilation of D-arabitol, D-mannitol and trehalose is variable.

Growth is detected on R2A agar and cetrimide agar. No blue pigment is formed on King A agar. No β-galactosidase activity is detected. Shows weak lipolysis on tributyrin agar at 25 °C after 10 days, but is negative for proteolysis using skimmed milk agar at both 25 and 4 °C. Highly proteolytic in liquid 10 % milk medium at 6 °C. Haemolysis cannot be detected. Negative for hydrolysis of starch and gelatin. H₂S is not produced. Negative for indole formation and for the reduction of nitrate and nitrite. No growth is observed under anaerobic conditions. The predominant fatty acids of the type strain are C₁₆:₀, summed feature C₁₆:₀10MeCISO-C₁₅:₀ 2-ΟΗ, C₁₇:₀ cyclo, C₁₈:₁0MeCISO-C₁₉:₀ cyclo, C₁₉:₁0MeCISO-C₁₉:₀ cyclo, the polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and the major respiratory quinone is Q-9, with small amounts of Q-8.

The type strain is WS4917ᵀ (=DSM 29165ᵀ=LMG 28433ᵀ), isolated from raw cow’s milk. The DNA G+C content of the type strain is 58.1 mol% and the genome size is 5.67 Mb. Three additional strains of the species, WS4995 (=DSM 29141=LMG 28434), WS4999 and WS5002, were isolated from three different samples of raw cow’s milk. One additional strain (WS5001) was isolated from an industrial semi-finished product (skimmed milk concentrate).

**Description of *Pseudomonas weihenstephanensis* sp. nov.**

*Pseudomonas weihenstephanensis* (wei.hen.ste.pha.nen’sis. N.L. fem. adj. *weihenstephanensis* pertaining to Freising/Weihenstephan in southern Germany, where the type strain was isolated).

Beige, smooth and round colonies, 0.5–1.0 mm in diameter, are formed after incubation at 25 °C for 24 h on TSG. Cells are rod-shaped (∼2.1 μm long and ∼0.75 μm wide), non-spore-forming, fluorescent on King B agar, motile, catalase- and oxidase-positive and Gram-negative. Grows at 4–33 °C (growth at temperatures lower than 4 °C has not been tested); optimal growth temperature is between 23 and 27 °C. Grows at pH 5–8 and in the presence of up to 5 % (w/v) NaCl. Positive for utilization of D-glucose, D-fructose, D-mannose, D-ribose, trehalose, D-xylose, glycerol, inositol and potassium gluconate and negative for utilization of amygdalin, arbutin, D-adonitol, D-arabinose, D-arabitol, cellobiose, D-fucose, D-galactose, lactose, D-lyxose, maltose, D-mannitol, melibiose, melezitose, raffinose, D-sorbitol, sucrose, D-tagatose, turanose, dulcitol, erythritol, gentiobiose, glycogen, L-arabinose, L-arabinose, L-arabinose, L-fucose, L-sorbose, L-xylose, methyl β-D-glucoside, methyl β-D-mannoside, methyl β-D-xylopyranoside, N-acetylglucosamine, salicin and xylitol. Grows on R2A agar and cetrimide agar. No blue pigment is formed on King A agar. No β-galactosidase activity is detected. Shows weak lipolysis on tributyrin agar at 25 °C after 10 days, but is negative for proteolysis using skimmed milk agar at both 25 and 4 °C. Highly proteolytic in liquid 10 % milk medium at 6 °C. Haemolysis cannot be detected. Negative for hydrolysis of starch and gelatin. H₂S is not produced. Negative for indole formation and for the reduction of nitrate and nitrite. No growth is observed under anaerobic conditions. The predominant fatty acids of the type strain are summed feature C₁₆:₁0MeCISO-C₁₅:₀ 2-ΟΗ, C₁₆:₀ and C₁₈:₁0MeCISO-C₁₉:₀ cyclo, the polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and the respiratory quinone is Q-9.

The type strain is WS4993ᵀ (=DSM 29166ᵀ=LMG 28437ᵀ), isolated from raw cow’s milk. The DNA G+C content of the type strain is 57.3 mol% and the genome size is 4.79 Mb. WS4994 (=DSM 29140=LMG 28438), an additional strain of the species, was isolated from another raw cow’s milk sample.
Acknowledgements

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