Possible misidentification of species in the *Pseudomonas fluorescens* lineage as *Burkholderia pseudomallei* and *Francisella tularensis*, and emended descriptions of *Pseudomonas brenneri*, *Pseudomonas gessardii* and *Pseudomonas proteolytica*

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Bacteria were isolated from an industrial water circuit in the Netherlands. These strains were identified using API 20 NE as possible, or likely, *Burkholderia pseudomallei*. With VITEK 2 some of these strains scored ‘low discrimination’ for *Francisella tularensis*, amongst others. A total of twenty-six strains were assigned to the species *Pseudomonas brenneri*, *Pseudomonas gessardii* or *Pseudomonas proteolytica*. Because of the possibility of misidentification of these environmental species as medical- and public-health relevant *B. pseudomallei* and *F. tularensis*, an emended description, based on tests results more customarily used in clinical laboratories, was suitable. For this reason, the strains in this study, including the type strains DSM 15294T, DSM 17152T and DSM 15321T, were subjected to a polyphasic identification procedure. This procedure consisted of multiple phenotypic tests, fatty acid analysis, 16S rDNA sequence analysis, matrix-assisted laser desorption ionization time-of-flight mass spectronomy and various species-specific molecular tests. Based on the results of the polyphasic procedures, the species descriptions of *P. brenneri*, *P. gessardii* and *P. proteolytica* have been emended.

Over the course of several months, bacteria were isolated from an industrial, closed water circuit, and initially identified as *Burkholderia pseudomallei* using API 20 NE (BioMerieux). *B. pseudomallei* can be found in water, soil and various animals, but they also have major implications for medical practice and public health.

Containment of *B. pseudomallei* requires Biosafety Level 3, and it is categorized by the Centers for Disease Control and Prevention (CDC) as a Bioterroristic Agent Category B and a Tier 1 agent. These are agents considered to pose a large risk for deliberate misuse (CDC, 2001). Because of these major implications for practice and public health, the suspected strains were sent to the Centre for Infectious Disease Control (Clb) of the Dutch National Institute of Public Health and the Environment (RIVM) for confirmation.

At the Clb, a polyphasic procedure resulted in the identification of the strains as either *Pseudomonas brenneri* or *Pseudomonas gessardii*. This polyphasic procedure included phenotypic tests, fatty acid analysis, 16S rDNA sequence analysis and various species-dependent molecular tests.

The outcome of these identifications prompted further research into related strains present in the in-house strain collection, including two strains isolated from human samples (Table S1, available in the online Supplementary Material).
Emended descriptions of Pseudomonas brenneri, gessardii and proteolytica

Material. These latter strains were identified as Pseudomonas proteolytica during this study.

P. brenneri and P. gessardii are both species that belong to the Pseudomonas fluorescens cluster, based on 16S rDNA sequences, as described by Moore (1996). Mulet et al. (2010) subdivided this P. fluorescens cluster into nine subgroups and one single strain based on multi locus sequence typing (MLST) of four genes. One of these subgroups is the P. gessardii subgroup, to which P. brenneri, P. gessardii and P. proteolytica belong. P. Brenneri and P. gessardii were first isolated from mineral water (Verhille et al., 1999; Baida et al., 2001). P. proteolytica was isolated from surface water in Antarctica (Reddy et al., 2004).

Because P. gessardii, P. brenneri and P. proteolytica can be misidentified as B. pseudomallei, it is important to emend the present descriptions with test results more customarily used in the identification of B. pseudomallei and other non-fermentative Gram-negative rods of medical importance, such as oxidative fermentation and growth on Ashdown’s medium. The strains in this study (Table S1) were subjected to the polyphasic procedure, as described above. The results were used to emend the present description of P. brenneri, P. gessardii and P. proteolytica. The type strains of P. brenneri (DSM 15294T), P. gessardii (DSM 17152T) and P. proteolytica (DSM 15321T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Because API 20 NE strips are replaced in most clinical laboratories by more automated methods, such as VITEK 2 and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, the strain set was also subjected to these two methods to investigate their ability to correctly identify P. brenneri, P. gessardii and P. proteolytica.

A cluster analysis of the 16S rRNA gene was carried out (Fig. 1). 16S rDNA analysis was performed using standard PCR equipment, with the following program: 94°C for 3 min, followed by 35 cycles consisting of 95°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec, with a combination of primers, which have been described previously (Brosius et al., 1978; Lane, 1991; Weisburg et al., 1991; Yoon et al., 1998). Sanger sequencing was performed using standard PCR equipment and a 3130xI Genetic Analyzer (Applied Biosystems), according to manufacturer’s instructions.

Sequences were submitted to GenBank, accession numbers are listed in Table S1. For cluster analysis, the neighbour-joining method using the Jukes and Cantor correction model (Jukes & Cantor, 1969) with 200x bootstrap resampling was performed with BioNumerics (Applied Maths NV). The 16S rDNA sequence of the type strains of P. proteolytica, B. pseudomallei and Franciscella tularensis were downloaded from NCBI (Fig. 1).

For fatty acid analysis, the selected strains were cultured on trypticase soy broth agar (TSBA) at 28°C for 24 h. Cells were harvested and fatty acids were extracted and identified according to instructions from the Sherlock Microbial Identification System, Version 6.1 (MIDI Labs). The fatty acid composition of P. brenneri, P. gessardii and P. proteolytica, is summarized in Table S2.

The phenotypic tests were performed as previously described (Ewing, 1986; Barrow, 1993) and incubated for 7 days. Growth requirements were tested on Columbia sheep agar (CSA), Levinthal serum agar (LSA) and GC agar with X and V factor. CSA were incubated aerobically at temperatures of 30°C, 37°C and 42°C, and anaerobically at 30°C. Ashdown’s medium with 4 mg l−1 gentamicin was prepared by bioTRADING Benelux B.V., as previously described (Ashdown, 1979), and a quality control was performed with positive growth of B. pseudomallei type strain NCTC 12939T (purchased from the National Collection of Type Cultures).

Ashdown’s medium was examined for growth after 72 h of incubation at 30°C. Salt tolerance was tested in Brain Heart Infusion (BHI) with different percentages (w/v) of NaCl. Oxidative-fermentative media, according to Hugh & Leifson (1953), were used to determine the production of acid from carbohydrates. Carbon source utilization tests and enzymatic tests were performed using API 20NE and API ZYM (BioMerieux), according to manufacturer’s instructions. The results of these tests are given in the emended descriptions and in Table 1.

With API 20 NE, twenty-five out of twenty-nine strains had a profile of 1156575, which corresponds to a low discrimination with a probability of 92.5% B. pseudomallei. Two strains scored 11567/575; this corresponds to a low discrimination with a probability of 88.1% B. pseudomallei. One strain scored 0146575, corresponding to a low discrimination with a probability of 2.0% B. pseudomallei. The only strain in this study that did not result in a probability of B. pseudomallei was the type strain of P. gessardii (DSM 17152T). The strains in this study scored B. pseudomallei in API 20 NE because P. brenneri, P. gessardii and P. proteolytica are not present in the database, and the results of their phenotypical tests are very similar to the results of B. pseudomallei.

The only distinguishing test present in API 20 NE was growth on phenylacetate as the sole carbon source; more than 90% of B. pseudomallei strains grew on phenylacetate (Wuthiekanun et al., 1996), whilst all P. brenneri, P. gessardii and P. proteolytica strains were negative (the present study).

All strains were identified using the VITEK 2 instrument (BioMerieux). Bacteria were grown on CSA at 30°C for 24 h, harvested, suspended and tested using GN ID cards according to manufacturer’s instructions. Only four strains scored a ‘very good identification’ using the VITEK 2 system; all four of them were identified as P. fluorescens. Two strains were correctly marked as unidentified organisms, because P. brenneri, P. gessardii and P. proteolytica are not present in the VITEK 2 database. Twenty-two strains scored a ‘low discrimination’ for diverse bacterial species. These
species comprise *P. fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas pseudoalcaligenes*, *Comamonas testosteroni*, *Oligella ureolytica*, *Aeromonas salmonicida*, *Achromobacter denitrificans*, various species from the group *Moraxella*, and *F. tularensis*.

An incorrect identification as *F. tularensis* also has consequences for medical practice and public health, because it requires containment at Biosafety Level 3, and it is categorized by the CDC as a Bioterroristic Agent Category A and a Tier 1 agent (CDC, 2001). MALDI-TOF analysis of all strains was performed. Bacteria were grown on CSA agar at 30°C for 24 h. The colonies were spotted in duplicate on a polished steel target plate according to the manufacturer’s instructions (Bruker Daltonics).

The spots were overlaid with a saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile: trifluoroacetic acid (50:2.5, % vol/vol) matrix (HCCA matrix) and allowed to dry. The analysis was performed

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**Fig. 1.** Cluster analysis of the sequence of 16SrRNA genes. Neighbour-joining method, Jukes & Cantor correction, 200x bootstrapped. Asterisks indicate the strain number, or the accession number in the case of a type strain.
in a linear positive mode on a Microflex LT instrument (Bruker Daltonics) with a laser power of 30–40% in a mass-range of 2000–20 000 Da. The spectra obtained were analyzed with the MALDI Biotyper database (V3.2.1.1, 4110 msp).

A log score of ≥2.0 is considered to be a good identification at the species level, a log score of 1.7–2.0 is a good identification at the genus level, and a log score of ≤1.7 is considered to be unreliable for identification purposes. All strains were identified correctly with a log score of >2.0. Some of the strains had an inconsistent result. Inconsistent means that the first and the second best score had a log score of between 2.000 and 2.300 with various species. However, the difference in log score was considerable (>0.198). These second resulting species were all members of the P. fluorescens lineage.

Because the API 20 NE and VITEK 2 scored B. pseudomallei or F. tularensis, the strains were also tested for the presence of specific targets of these species. For the Burkholderia PCR, primers and probes were developed to target a Burkholderia-specific region of the 16SrRNA gene, the flfC-gene and Type Three Secretion (TTS) gene (Table S3). These targets allow for the identification and differentiation of B. pseudomallei, Burkholderia mallei, and Burkholderia thailandensis. Standard real-time PCR protocols based on the fluorescent resonance energy transfer (FRET) technique were performed using a Lightcycler 480 (Roche molecular diagnostics), according to the following program: 95°C for 10 min, followed by 45 cycles consisting of 95°C for 10 s, 57°C for 20 s and 72°C for 15 s. For the F. tularensis PCR, primers and probes were developed to target the FT-heli gene and the FTSE gene (Table S3) in order to identify and differentiate F. tularensis subspecies and F. philomiragia. Real-time PCR was performed as described above, except for with an annealing temperature of 59°C. Both molecular procedures were tested and validated with the appropriate strains. All strains in this study had negative results for B. mallei/pseudomallei and F. tularensis in the specific real-time PCRs, and were ruled out as possible identifications.

Table 1. Discriminative characteristics of P. gessardii, P. brenneri and P. proteolytica, including the typestrains DSM 15294T, DSM 17152T and DSM 15321T

<table>
<thead>
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<th>Characteristic</th>
<th>1 (n=12)</th>
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<tr>
<td>Growth at 37°C (7 days)</td>
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<td>Acid from:</td>
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<td>D-Maltose</td>
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<td>D-Xylose</td>
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<td>L-Rhamnose</td>
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<tr>
<td>D-Galactose</td>
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<td>100</td>
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<td>Malonate utilization</td>
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<td>0</td>
</tr>
<tr>
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<td>86</td>
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<td>0.1 % (w/v) nitrite reduction</td>
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<td>64</td>
<td>100</td>
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<td>0.01 % (w/v) nitrite reduction</td>
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<td>93</td>
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</tr>
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<td>Gas from nitrate</td>
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<td>50</td>
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<td>Lipase</td>
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<td>Growth in 5% (w/v) NaCl</td>
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<td>Naphthol-AS-BI-phosphohydrolase</td>
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</table>

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Emended description of Pseudomonas brenneri Baida et al., 2001

Pseudomonas brenneri (bren’ner.i. N.L. masc. gen. n. brenneri of Brenner, in honor of D. J. Brenner, an American microbiologist, for his contributions to the taxonomy of the families Enterobacteriaceae and Legionellaceae).

All strains grow on MacConkey agar, Salmonella Shigella (SS agar and Cetrimide and in BHI) with 3% (w/v) NaCl. Variable growth in BHI with 5% (w/v) NaCl. No growth in BHI with 7, 9 and 10% (w/v) NaCl. All strains grow at 4°C and 30°C, with weak growth at 37°C and no growth at 41°C. Does not grow anaerobically on TSA. Beta-haemolysis negative. Acid is produced from D-glucose, L-rhamnose, D-mannitol, D-mannose, D-fructose, D-galactose, but not from lactose, D-maltose and D-sucrose. Acid formation from D-xylose is variable. Positive test results are obtained from all strains for Simmon’s citrate, denitrification and caseinase activity. Negative test results are obtained from all strains for ONPG, Na-malonate utilization, H₂S-production from TSI, indole-production, esculin hydrolysis, methyl red, phenylalanine deamination, starch hydrolysis and gelatin liquefaction. Variable test results are obtained for gas from nitrate and nitrite and lipase activity. All strains utilize N-acetyl-glucosamine and adipate as the sole carbon source, but not phenyl acetate. The following enzymes are produced in API ZYM: esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not produced. Variable test results are obtained for the production of alkaline phosphatase, esterase (C4) and valine arylamidase. The fatty acid compositions comprise substantial amounts of 16:0 and a summed feature of 16:0 and a summed feature of 16:1ω7c/16:1ω7c, and less substantial amounts 10:3 OH, 12:0, 12:0 2OH, 12:0 3OH, 16:0, 17:0 cyclo and a summed feature of 18:1ω7c/18:1ω7c.

Emended description of Pseudomonas gessardii Verhille et al., 1999

Pseudomonas gessardii (ges.sar’di.i. N.L. gen. masc. n. gessardi after C. Gessard, who isolated ‘Bacterium aeruginosum’ for the first time and studied its pigment).

All strains grow on MacConkey agar, SS agar and Cetrimide and in BHI with 3% (w/v) NaCl. Variable growth in BHI with 5% (w/v) NaCl. No growth in BHI with 7, 9 and 10% (w/v) NaCl. All strains grow at 4°C and 30°C; no or weak growth at 37°C and no growth at 41°C. Does not grow anaerobically on CSA. Beta-haemolysis negative. Acid is produced from D-glucose, D-mannitol, D-mannose, D-fructose, but not from lactose and D-sucrose. Acid formation from D-maltose, D-xylose, L-rhamnose and D-galactose is variable. Positive test results are obtained from all strains for Simmon’s citrate. Negative test results are obtained from all strains for ONPG, Na-malonate utilization, H₂S-production from TSI, urease activity, methyl red, lipase activity, starch hydrolysis and gelatin liquefaction. Variable test results are obtained for caseinase activity. All strains use adipate as the sole carbon source. The following enzymes are produced in API ZYM: leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Alkaline phosphatase, lipase (C14), cystine arylamidase trypsin and α-chymotrypsin are not produced. Variable results are obtained for the production of valine arylamidase. The fatty acid compositions comprise substantial amounts of 16:0 and a summed feature of 16:1ω7c/16:1ω7c, and less substantial amounts 10:3 OH, 12:0, 12:0 2OH, 12:0 3OH, 16:0, 17:0 cyclo and a summed feature of 18:1ω7c/18:1ω7c.

Emended description of Pseudomonas proteolytica Reddy et al., 2004

Pseudomonas proteolytica (pro.te.o.ly’ti.ca. N.L. fem. adj. proteolytica proteolytic).

All strains grow on MacConkey agar, SS agar and Cetrimide and in BHI with 3% (w/v) NaCl. Variable growth in BHI with 5% (w/v) NaCl. No growth in BHI with 7, 9 and 10% (w/v) NaCl. All strains grow at 4°C and 30°C with weak growth at 37°C and no growth at 41°C. Does not grow anaerobically on CSA. Beta-haemolysis negative. Acid is produced from D-glucose, D-xylose, L-rhamnose, D-mannitol, D-mannose, D-fructose, D-galactose, but not from lactose, D-maltose and D-sucrose. Positive test results are obtained from all strains for Simmon’s citrate, denitrification, gas from nitrate and nitrite, and caseinase activity. Negative test results are obtained from all strains for ONPG, Na-malonate utilization, H₂S-production from TSI, indole-production, esculin hydrolysis, methyl red, phenylalanine deamination, starch hydrolysis and gelatin liquefaction. Variable test results are obtained for lipase activity. All strains utilize N-acetyl-glucosamine and adipate as the sole carbon source, but not phenyl acetate. The following enzymes are produced in API ZYM: esterase (C4), esterase Lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not produced. Variable results are obtained for the production of alkaline phosphatase. The fatty acid compositions comprise substantial amounts of 16:0 and a summed feature of 16:1ω7c/16:1ω7c, and less substantial amounts 10:3 OH, 12:0, 12:0 2OH, 12:0 3OH, 17:0 cyclo and a summed feature of 18:1ω7c/18:1ω7c.

References


Emended descriptions of Pseudomonas brenneri, gessardii and proteolytica


