Psychrobacter pasteurii and Psychrobacter piechaudii sp. nov., two novel species within the genus Psychrobacter

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Abstract
Six Gram-negative, non-motile, non-spore-forming, non-pigmented, oxidase- and catalase-positive bacterial strains were deposited in 1972, in the Collection of the Institut Pasteur (CIP), Paris, France. The strains, previously identified as members of the genus Moraxella on the basis of their phenotypic and biochemical characteristics, were placed within the genus Psychrobacter based on the results from comparative 16S rRNA gene sequence studies. Their closest phylogenetic relatives were Psychrobacter sanguinis CIP 110993T, Psychrobacter phenylpyruvicus CIP 82.27T and Psychrobacter lutiphocae CIP 110018T. The DNA G+C contents were between 42.1 and 42.7 mol%. The predominant fatty acids were C18:1ω9c, C16:0, C12:0 3-OH, and C18:0. Average nucleotide identity between the six strains and their closest phylogenetic relatives, as well as their phenotypic characteristics, supported the assignment of these strains to two novel species within the genus Psychrobacter. The proposed names for these strains are Psychrobacter pasteurii sp. nov., for which the type strain is A1019T (=CIP 110853T =CECT 9184T), and Psychrobacter piechaudii sp. nov., for which the type strain is 1232T (=CIP110854T =CECT 9185T).

The genus Psychrobacter is part of the family Moraxellaceae which includes two other genera: Moraxella and Acinetobacter. It was described by Juni and Heym [1] to accommodate obligate or facultative psychrophilic bacteria that lacked motility and possessed strictly oxidative metabolism. At the time of writing, 36 species have been classified as members of this genus. Most of the species of the genus Psychrobacter have been frequently found to be associated with the Antarctic and marine environments [2] as well as with poikilothermic animals, but some of them have also been isolated from humans [3] and have been found to be responsible for human infections [4] including ocular infection [5], bacteremia, endocarditis [6] and meningitis [7–9].

We selected six strains (A1019T, 1232T, A390, 5542, GROSJEAN and 9413) deposited in 1972 at the Collection of the Institut Pasteur (CIP) of human origin, which had been previously identified as representing members of the genus Moraxella on the basis of their phenotypic and biochemical characteristics. In the present study, these strains were re-identified, their taxonomic position is described and the description of two novel species is proposed.

The strains were preserved freeze-dried and pre-cultivated on brain–heart infusion (BHI) broth (bioMérieux) and subsequently sub-cultivated on BHI agar supplemented with 5% horse blood, aerobically at 37°C for 24 h. All the type strains of species of the genus Psychrobacter used for comparative studies were grown on the same media. Growth at different NaCl concentrations [0, 5, 10, 15 and 20% (w/v)] and temperatures (0, 4, 20, 30 and 37°C) were examined by using BHI as the basal medium. Enzymatic reactions and acid productions were studied by means of the API 20NE and API ZYM Systems (bioMérieux). Carbon utilization assays were performed using the BIOLOG PM1 and PM2 Phenotype MicroArray Panels (BIOLOG).

DNA of the novel species strains and of type strains of species of the genus Psychrobacter was obtained using the Wiz

 Ard Genomic DNA Purification Kit (Promega). The extracted DNA was used for PCR amplification of the rrs gene coding for the 16S rRNA. Primers and PCR conditions

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Keywords: Psychrobacter; Novel species; Average Nucleotide Identity; Whole genome sequencing.
Abbreviation: ANI, average nucleotide identity.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains CIP 110853T and CIP 110854T are KY292376 and KY292375, respectively. The annotated genomic sequences of strains CIP 110853T and CIP 110854T were deposited in the European Nucleotide Archive and are available under accession numbers FUGD01000001–FUGD01000015 and FUGE010001–FUGE01000200, respectively.
Three supplementary tables and one supplementary figure are available with the online Supplementary Material.
Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA sequences, reconstructed using IQ-Tree [13] with evolutionary model GTR+Γ+Γ indicating the relationship of strains of Psychrobacter pasteurii and Psychrobacter piechaudii (in bold type) with the type strains of species of the genera Psychrobacter and Moraxella. The Psychrobacter ingroup was rooted by using 13 16S rRNA sequences of members of the genus Moraxella as the outgroup. Bootstrap values (based on 1000 replicates) are shown as percentages near each internal tree branch. Bar, 0.04 substitutions per nucleotide position.
were the same as those described by Clermont et al. [10].
PCR products were sent to Eurofins Genomics (Ebersberg, Germany) to be sequenced. The PCR products were sequenced using A, H and two other sequencing primers (Escherichia coli numbering system): B, 5’-CTCCTACGGAGGGACGAGT-3’, positions 339 to 358, and G, 5’-GCATGGTTAATTCGA-3’, positions 947 to 964, using the dideoxy chain-termination method [11]. Sequences were assembled using BioNumerics version 6.6 (Applied-Maths). These 16S rRNA sequences were used together with other those of members of the genera Psychrobacter and Moraxella retrieved from GenBank to perform a multiple sequence alignment with R-Coffee [12]. Characters containing more than 50% gaps were discarded and a phylogenetic analysis was performed by IQ-Tree [13] with evolutionary model GTR+I+

The resulting phylogenetic tree indicated that strains A1019 and A390 are closely related to Psychrobacter lutiphocae CIP 110018T, and strains 1232, 5542, GROSJEAN and 9413 are closely related to Psychrobacter sanguinis CIP 110993T and Psychrobacter phenylpyruvicus CIP 82.27T (Fig. 1). A

### Table 1. Cellular fatty acid compositions of strains of members of the genus Psychrobacter and their closest phylogenetic neighbours

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<th>6</th>
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<td>0.4</td>
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<td><strong>Summed Features</strong></td>
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<td>0.7</td>
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</table>

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C<sub>16</sub>:1ω7c and/or C<sub>16</sub>:1ω6c. Summed feature 5 consisted of C<sub>18</sub>:ω6,9c and/or anteiso-C<sub>18</sub>:0. Summed feature 8 consisted of C<sub>18</sub>:1ω7c and/or C<sub>18</sub>:1ω6c.
The whole-genome sequencing of the six selected novel strains of the genus *Psychrobacter*, as well as of their related type strains, was carried out at the Mutualized Microbiology Platform (P2M) of the Pasteur International Bioresources network (PIBnet) of the Institut Pasteur, Paris, France, using the Nextera XT DNA sample preparation kit (Illumina) for 2 × 150 bp paired-end sequencing as per the manufacturer’s instructions. All sequenced paired-ends reads were clipped and trimmed with AlienTrimmer [16], corrected with Musket [17], merged (if needed) with FLASH [18], and subjected to a digital normalization procedure with khmer [19]. For each sample, remaining processed reads were assembled and scaffolded with SPAdes [20] (see Table S1, available in the online Supplementary Material for the *de novo* assembly details).

To determine with confidence the species-level identification, we performed a pairwise ANI calculation using the JSpecies Web server [21]. ANI calculated with BLAST (ANIb) values are presented in Fig. S1. Strains A1019$^T$ and A390 displayed a high genomic similarity (ANIb=98.7%), as expected for strains representing the same species. The ANIb values between these two strains and their closest relative *P. lutiphocae* CIP 110018$^T$ were 77.2 and 77 %, respectively, well below the species cut-off value. In the same manner, the genome sequences of strains 1232$^T$, 5542, GROSJEAN and 9413 displayed high pairwise similarities (ANIb >98%). The ANIb values of these four strains when compared against *P. sanguinis* CIP 110093$^T$ and *P. phenylpyruvicus* CIP 82.27$^T$, was approximately 82 % and approximately 88 %, respectively. These values are highly supported by the ANI calculated with MUMer (ANIm) and TETRA estimates (Tables S2 and S3, respectively).

The DNA G+C content of A1019$^T$ and A390 genome sequences was 42.7% and it was 42.1–42.2% for the genomes of strains 1232$^T$, 5542, GROSJEAN, and 9413. These values were similar to those found in the related species of the genus *Psychrobacter*, such as *P. sanguinis* CIP

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**Table 2. Differential characteristics of the two novel species and their closest phylogenetic neighbours**


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percentage similarity of 98 % for the 16S rRNA sequences was found between strains A1019$^T$ and A390 and *P. lutiphocae* CIP 110018$^T$, and a percentage similarity of 99 % was found between strains 1232$^T$, 5542, GROSJEAN and 9413 and *P. sanguinis* CIP 110093$^T$ and *P. phenylpyruvicus* CIP 82.27$^T$.

Even though 16S rRNA has become a standard for prokaryotic taxonomy, the low evolutionary rate of its rrs gene does not provide enough resolution at shallow taxonomic scales [14]. Therefore, a whole-genome sequencing approach using an average nucleotide identity (ANI) boundary of 95–96 % has been recommended for circumscribing prokaryotic species, and can be reinforced by high oligonucleotide frequencies [e.g. tetranucleotide (TETRA)] [15].
The analysis of cellular fatty acids was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), using the Sherlock MIDI system following the protocol of the Microbial Identification System [22, 23]. The cellular fatty acid profile was consistent with that of the members of the genus <i>Psychrobacter</i> (Table 1). The oleic acid C<sub>18:1ω9c</sub> was predominant in all the strains. Its percentage was between 28.3 and 38.6 %, except for strain GROSJEAN in which it was present in greater abundance (71.1 %). The predominance of monounsaturated fatty acids, such as oleic acid, has been postulated to maintain cell membrane permeability at low temperatures, a characteristic of the members of the genus <i>Psychrobacter</i> [24]. The other predominant fatty acids found in the six strains were C<sub>16:0</sub>, C<sub>12:0 3-OH</sub>, C<sub>18:0</sub> and summed feature 3 (composed of C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>). Comparison of fatty acid profiles of the six strains and their nearest phylogenetic neighbours (P. <i>sanguinis</i> CIP 110993<sup>T</sup>, <i>P. phenylpyruvic</i>us CIP 82.27<sup>T</sup> and <i>P. lutiphocae</i> CIP 110018<sup>T</sup>) revealed variability in the abundance of fatty acids (Table 1).

The six studied strains were Gram-negative, non-motile, non-pigmented, catalase and oxidase-positive and non-spore-forming rods, features that are characteristic of members of the genus <i>Psychrobacter</i>. They were able to grow at 0–37 °C and tolerated 0–15 % (w/v) NaCl. A1019<sup>T</sup> and A390 could be distinguished from <i>P. sanguinis</i> CIP 110993<sup>T</sup> by their ability to tolerate 5 and 10 % (w/v) NaCl, their capacity to use dextrin as a carbon source and the presence of lipase. Strains 1232<sup>T</sup>, 5542, GROSJEAN, and 9413 could be distinguished from <i>P. phenylpyruvic</i>us CIP 82.27<sup>T</sup> by their ability to tolerate 10 % (w/v) NaCl except for the GROSJEAN strain, their absence of alkaline phosphatase and their ability to use D-glucosamine and 5-keto-D-gluconic acid as carbon source. Similarly, the six strains studied could be distinguished from <i>P. lutiphocae</i> CIP 110018<sup>T</sup> thanks to their ability to tolerate 10 % (w/v) NaCl and their inability to produce acid from malate. The characteristics that distinguish the six strains from the reference type strains of closely related species are detailed in Table 2.

The genotypic and phenotypic characteristics of strains A1019<sup>T</sup>, 1232<sup>T</sup>, A390, 5542, GROSJEAN, and 9413 fully demonstrate that they represent two novel species of the genus <i>Psychrobacter</i> for which we propose the names <i>Psychrobacter pasteurii</i> sp. nov. and <i>Psychrobacter piechaudii</i> sp. nov. with A1019<sup>T</sup> and 1232<sup>T</sup> as type strains, respectively.

**DESCRIPTION OF PSYCHROBACTER PASTEURII SP. NOV.**

<i>Psychrobacter pasteurii</i> (pas.teu’ri.i. N.L. gen. n. pasteurii honoring the French microbiologist Louis Pasteur).

Cells are Gram-negative, non-motile, non-pigmented and non-spore-forming coccobacilli. Aerobic, catalase-positive and oxidase-positive. Cells are urease-positive and are able to reduce nitrate to nitrite. Colonies are translucent, bright and approximately 1–2 mm in diameter after 48 h at 30 °C on blood agar. Growth occurs with 0–15 % (w/v) NaCl and at 0–37 °C (optimum 30 °C). Cells are negative for acid production from D-glucose, arabinose, mannose, citrate and malate. They are positive for esterase and lipase, and negative for cystine arylamidase. Strains may be positive or negative for alkaline phosphatase. Cells use D-xyllose, D-ribose, L-lyxose, L-arabinose, D-arabinose, dextrin, D-glucosamine, Tween 20, Tween 40, Tween 80 and 5-keto-D-gluconic acid as sources of carbon. The predominant cellular fatty acids are C<sub>18:1ω9c</sub>, C<sub>12:0 3-OH</sub>, C<sub>16:0</sub>, C<sub>10:0</sub> and C<sub>18:0</sub>.

The type strain is A1019<sup>T</sup> (=CIP 110853<sup>T</sup>=CECT 9184<sup>T</sup>) and was deposited in the Collection of Institut Pasteur, France, in 1972. The genomic DNA G+C content of the type strain is 42.7 mol%.

**DESCRIPTION OF PSYCHROBACTER PIECHAUDII SP. NOV.**

<i>Psychrobacter piechaudii</i> (pie.chau’di.i N.L. gen. n. piechaudii from M. Piechaud, the curator of the Collection of the Institut Pasteur-CIP, France).

Cells are Gram-negative, non-motile, non-pigmented and non-spore-forming coccobacilli. Aerobic, catalase-positive and oxidase-positive. Cells are not able to reduce nitrate to nitrite. Urease activity varies between strains. Colonies are translucent, bright and approximately 1–2 mm in diameter after 48 h at 30 °C on blood agar. Growth occurs at 0–37 °C (optimum 30 °C) and with 0–15 % (w/v) NaCl, some strains are not able to grow with 10 % NaCl. Cells are negative for acid production from D-glucose, arabinose, mannose, citrate and malate. They are negative for alkaline phosphatase and for cystine arylamidase and positive for esterase. Lipase activity varies between strains. Cells use D-xyllose, D-ribose, L-lyxose, L-arabinose, 2-deoxy-D-ribose, D-glucosamine, Tween 20, Tween 40, Tween 80 and 5-keto-D-gluconic acid as sources of carbon. Most strains are not able to use dextrin as source of carbon, while some are able to use it. The most predominant cellular fatty acid is C<sub>18:1ω9c</sub>. The other predominant fatty acids are C<sub>16:1ω7c</sub>, C<sub>18:0</sub>, C<sub>12:0 3-OH</sub> and summed feature 5 (C<sub>18:2ω6c</sub> and/or anteiso-C<sub>18:0</sub>).

The type strain is 1232<sup>T</sup> (=CIP 110854<sup>T</sup>=CECT 9185<sup>T</sup>) and was deposited in the Collection of Institut Pasteur, France, in 1972. The genomic DNA G+C content of the type strain is 42.3 mol%.

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

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