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Corynebacterium mendelii sp. nov., a novel bacterium isolated from Adélie penguin oral cavity

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Abstract

The taxonomic status of strain P5891^T, isolated from an Adélie penguin beak swab, was investigated. Based on the 16S rRNA gene sequence, the strain was identified as a potentially novel *Corynebacterium* species, with the highest sequence similarities to *Corynebacterium rouxii* FRC0190^T (96.7%) and *Corynebacterium epidermidicanis* DSM 45586^T (96.6%). The average nucleotide identity values between strain P5891^T and *C. rouxii* FRC0190^T and *C. epidermidicanis* DSM 45586^T were 68.2 and 69.2%, respectively. The digital DNA–DNA hybridization values between strain P5891^T and *C. rouxii* FRC0190^T and *C. epidermidicanis* DSM 45586^T were 23.7 and 21.4%, respectively. Phylogenetic trees based on the 16S rRNA sequence placed strain P5891^T in a separate branch with *Corynebacterium canis* 1170^T and *Corynebacterium freiburgense* 1045^T, while a phylogenomic tree based on the *Corynebacterium* species core genome placed the strain next to *Corynebacterium choanae* 200CH^T. Extensive phenotyping and genomic analyses clearly confirmed that strain P5891^T represents a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium mendelii* sp. nov. is proposed, with the type strain P5891^T (=CCM 8862^T=LMG 31627^T).

INTRODUCTION

The genus *Corynebacterium*, proposed by Lahmann and Neumann in 1896, is a large group of Gram-positive, non-spore-forming, rod-shaped bacteria. It belongs to the family *Corynebacteriaceae* within the phylum *Actinomycetota*. According to the List of Prokaryotic names with Standing in Nomenclature [1], the genus currently comprises 160 validly named species (https://lpsn.dsmz.de/genus/corynebacterium, accessed 21 November 2023). Corynebacteria can be found in a wide range of ecological niches, such as water, soil, foodstuffs, humans and animals. More than 50 species are of clinical significance, most notably *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, producers of diphtheria toxin and causative agents of diphtheria and other diseases in humans and animals [2]. Some *Corynebacterium* species can act as opportunistic pathogens, and some live in the human body as commensals [3–5]. Multiple *Corynebacterium* species have been isolated from animals, including various mammals, reptiles and birds. They are usually considered to be part of a normal microbiome, although some are suspected to be causative agents of infections [3].

Specifically in penguins, various *Corynebacterium* species appear to be an abundant part of the normal oral, cloacal and skin microbiome [6–11]. Multiple novel species have been isolated from penguins: *Corynebacterium sphenisci* [6] and *Corynebacterium spheniscorum* [7], both originating from apparently healthy Magellanic penguins, *Corynebacterium antarcticum*, *Corynebacterium marambiense*, *Corynebacterium meridianum* and *Corynebacterium pygosceleis* [11], originating from apparently healthy Adélie penguins, and *Corynebacterium megadyptis*, isolated from yellow-eyed penguin chicks with diphtheritic stomatitis [12]. Several studies have suggested a link between corynebacteria and various penguin diseases, such as diphtheritic stomatitis [8, 12, 13],

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Keywords: Adélie penguin; Antarctica; *Corynebacterium mendelii sp. nov.*; James Ross Island; taxonomy.

Abbreviations: ANI, average nucleotide identity; CDS, coding sequence; COG, cluster of orthologous groups; dDDH, digital DNA–DNA hybridization; TSA, tryptone soya agar; TSB, tryptone soya broth.

The GenBank/EMBL/DDBJ accession number for the strain P5891^T 16S rRNA gene sequence is OP099850; the whole-genome sequence accession number is JAFLEQ000000000.

Three supplementary figures and four supplementary tables are available with the online version of this article.

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bumblefoot infections [9], and ocular lesions [14]. However, data on their actual pathogenicity are limited and further research is required to clarify their role in the pathogenesis of these diseases.

ISOLATION AND ECOLOGY

Strain P5891^T was isolated from an oral swab of an apparently healthy adult Adélie penguin encountered near Mendel Polar Station (63.8007397° S 57.8834306° W) on James Ross Island, Antarctica, as part of a project investigating cultivable bacterial populations inhabiting the Maritime Antarctic environment and its flora and fauna. During the expedition, the collected swabs were stored in transport tubes containing Amies medium with charcoal (COPAN Italia) until processed in the field laboratory at Mendel station. The swabs were used to inoculate mannitol salt agar (HiMedia) plates. The plates were cultivated aerobically at 30°C for up to 5 days. Colonies with different morphologies were continuously picked and purified using the streak plate technique. The obtained pure cultures were transported to the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) and kept at −70°C as described previously [15] until they were processed.

16S rRNA GENE PHYLOGENY

To ascertain the phylogenetic position of P5891^T, a nearly complete sequence of the 16S rRNA gene was obtained using the primers pA 5′-AGAGTTTGATCCTGGCTCAG-3′ and pH 5′-AAGGAGGTGATCCAGCCGCA-3′ [16] as described previously [17]. After column-purification of the PCR product with the High Pure PCR Product Purification Kit (Roche), Sanger sequencing was performed with the same set of primers as for amplification by Eurofins Genomics (Ebersberg bei München, Germany). The obtained reads were assembled with the CAP3 tool (https://doua.prabi.fr/software/cap3/) [18]. The 1396 bp-long sequence was deposited in GenBank/EMBL/DDBJ under accession number OP099850. When compared to the EzBioCloud database (https://www.ezbiocloud.net/identify) [19], the sequence exhibited the highest similarity to *Corynebacterium rouxii* FRC0190^T (96.7%) and *Corynebacterium epidermidicanis* DSM 45586^T (96.6%). These values are well below the 98.7% similarity threshold suggested for delineating bacterial species [20, 21], and imply that strain P5891^T represents a new species.

The phylogenetic trees were reconstructed using the software MEGA X [22]. Evolutionary history was inferred using the maximum-likelihood (Fig. 1) [23], minimum-evolution (Fig. S1, available in the online version of this article) [24] and neighbour-joining methods (Fig. S2) [25] using the Kimura two-parameter model [26] and a bootstrap test [27] based on 1000 replications for all three methods. In all the trees, strain P5891^T formed a separate lineage together with *Corynebacterium canis* 1170^T and *Corynebacterium freiburgense* 1045^T.

GENOME FEATURES

A whole-genome sequence of strain P5891^T was obtained within the framework of the Global Catalogue of Microorganisms 10K type strain sequencing project [28], using the BGISEQ sequencing platform. Assembly was performed using the software SOAPdenovo (version JUL-2013) [29]. The sequence consisted of 18 scaffolds; its total length was 3090721 bp with a G+C content of 63.3 mol%. The sequence has been deposited at GenBank/EMBL/DDBJ under accession number JAFLEQ000000000.

The sequence was compared to the genome sequences of the type strains of the most closely related species, as designated by the 16S rRNA gene sequence similarity, phylogeny and core genome analysis. Average nucleotide identity (ANI) was calculated using the OrthoANI algorithm implemented in the software OAT [30] and digital DNA–DNA hybridization (dDDH) values were determined using Genome-to-Genome Distance Calculator version 3.0 (https://ggdc.dsmz.de/) [31], taking recommended formula 2 into account. The obtained values shown in Table 1 are well below the thresholds of 95–96 % (ANI) and 70% (dDDH) for differentiating bacterial species [20], confirming the taxonomic novelty of strain P5891^T.

Out of the 2326 coding sequences (CDSs) found in the genome of strain P5891^T, 1817 CDSs were assigned to known orthologous groups and corresponding clusters of orthologous group (COG) categories by eggNOG-mapper version 2.1.7 and eggNOG database version 5.0.2 [32] (Table S1). The most abundant category was 'S, function unknown', accounting for 14.3% of CDSs, which is not surprising for a novel species. Moreover, 509 genes (21.9%) were not assigned to any COG, which could be due to the fact that even the evolutionarily closest species are quite distant, so the orthologues of these genes may still be unknown. The other most abundant categories were those connected to metabolism, *e.g.* categories E, G, P and the expression of genetic information, *i.e.* categories J and K, each accounting for more than 5%. The genome also contained 40 genes in the category 'V, defence mechanisms', indicating that the bacterium possesses some genetic machinery to compete with other microorganisms. Strain P5891^T also possesses other genomic elements that can be understood as a kind of bacterial immunity. In particular, three clustered regularly interspaced short palindromic repeat (CRISPR) arrays were identified with CRISPRDetect version 2.4 [33] (Table S2). Moreover, no prophages were found in the genome of strain P5891^T by PHASTER (https://phaster.ca/) [34], suggesting that CRISPR-Cas systems may be active.

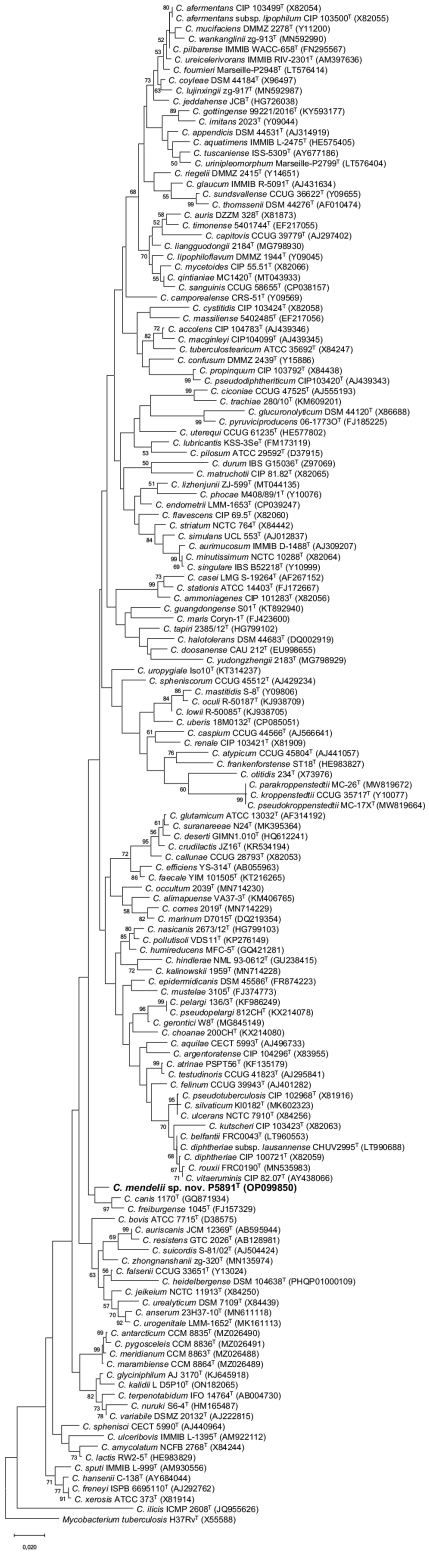

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA sequences comparison showing the position of strain P5891^T within the genus *Corynebacterium*. The percentage of bootstrap replicate trees (1000 replicates) in which the associated taxa clustered together is indicated by the values shown next to the branches. All positions with less than 95 % site coverage were eliminated (partial deletion option). The final dataset contained a total of 1213 positions. *Mycobacterium tuberculosis* H37Rv^T was used as an outgroup. Bar, 0.02 nucleotide substitutions per site.

Table 1. Percentages of ANI and dDDH between strain P5891^T and five closest *Corynebacterium* species

Species	ANI	dDDH
C. canis DSM 45402^T (NZ_CP047080.1)	70.2	21.6
C. choanae 200CH^T (NZ_CP033896.1)	71.0	22.4
C. epidermidicanis DSM 45586^T (NZ_CP011541.1)	69.2	21.4
C. freiburgense DSM 45254^T (NZ_CP047355.1)	67.6	21.8
C. rouxii FRC0190^T (NZ_LR738855.1)	68.2	23.7

Strain P5891^T possessed no known antibiotic resistance genes when compared to the comprehensive antibiotic resistance database using resistance gene identifiers [35]. This means that the observed resistance to penicillin (see below) is probably mediated by one or more genes not included in the database. The virulence potential of the strain is questionable, as the genome lacks genes coding surface-anchored proteins *spaB*, *spaC*, *spaD*, *spaE* and *spaF*, which form pili responsible for adhesion to host cells and belong to widespread virulence factors in corynebacteria [36]. Strain P5891^T is unlikely to form pili or flagella, as only seven genes were assigned to the category 'N, cell motility', consistent with the observed non-motile phenotype (see below). On the other hand, two putative genes, *rpfI* (JZY06_02330) and *cwlH* (JZY06_00280), coding for Rpf-interacting protein and cell wall-associated hydrolase, respectively, were found in the genome with more than 40% amino acid sequence similarity to known genes with BLAST searches [37]. These genes were previously found in *Corynebacterium* strains responsible for diphtheritic stomatitis in yellow-eyed penguins [13]. No other significant hits (sequence similarity >40%) for known virulence factors were found.

As chemotaxonomic traits of the genus *Corynebacterium* are well characterized, the presence of the most prominent markers was investigated by in silico inference performed by the identification of metabolic pathways based on searching for orthologues in the KEGG database [38] using BlastKOALA (https://kegg.jp/blastkoala/) [39], while annotation was completed manually using online BLAST searches [37]. The analysed genome contains gene machinery responsible for the synthesis of menaquinones *via* the isochorismate pathway, which is typical for corynebacteria, specifically the genes *menA* (JZY06_04590), *menB* (JZY06_04600), *menC* (JZY06_04605), *menD* (JZY06_04610), *menE* (JZY06_04595), *menF* (JZY06_08370) and *menG* (JZY06_04620). Moreover, the presence of the *menJ* gene (JZY06_04625) suggests that strain P5891^T is able to produce saturated menaquinones [40]. Additionally, the genes responsible for the synthesis of mycolic acid, a long-chain fatty acid produced by the majority of corynebacteria, were found: *fadD32* (JZY06_11625), encoding the long-chain fatty acid AMP ligase, *pks13* (JZY06_11620), encoding polyketide synthase 13, and *cmrA* (JZY06_05875), encoding mycolate reductase. The adjacent genes *fadD32* and *pks13* were located in the same operon predicted by the Operon-mapper (https://biocomputo.ibt.unam.mx/operon_mapper/) [41]. Despite the absence of the *accD4* gene, previously reported to be part of the operon [42], the genome contains multiple putative *accD* subunits (JZY06_11600, JZY06_07745, JZY06_07730, JZY06_03315 and JZY06_08615) with high sequence similarity to *accD* subunits that are essential for mycolic acid synthesis [42–44]. Furthermore, several genes coding for enzymes responsible for polar lipid synthesis were found, specifically the genes *cls* (JZY06_11040), encoding cardiolipin synthetase, *pgsA* (JZY06_01815), encoding phosphatidylglycerophosphate synthase, *ptfP1* (JZY06_01820), encoding phosphatidylinositol mannoside acyltransferase, and *pimA* (JZY06_01825), and *pimB* (JZY06_00285), both encoding phosphatidylinositol mannosyltransferase. The latter three genes code for key proteins in the synthesis of phosphatidylinositol mannoside and phosphatidylinositol dimannoside [45], polar lipids characteristic of many *Corynebacterium* species.

The analysed genome was compared to 139 reference genomes of the genus *Corynebacterium* available in the RefSeq database (9 September 2022, Table S3) [46]. The core genome was reconstructed with BPGA version 1.3 [47] with amino acid sequences clustered using USEARCH version 11.0.667 [48], with an identity cut-off of 50%. A phylogenomic tree based on the core genes was calculated with the BPGA tool [47] using the neighbour-joining method [25], and visualized with the iTOL version 6 tool (https://itol.embl.de/) [49]. The core genome comprised 103 genes shared by all the analysed species. The genome of strain P5891^T carried 764 unique genes not found in any other analysed genome. This is a high number, as the average number of unique genes per genome in the dataset was 301. It is important to mention that 58 of the reference genomes used for this comparison are draft genomes, which may negatively influence the accuracy of the analysis. Analysis of the core genes confirmed the similarity of strain P5891^T to *Corynebacterium* species present in birds. Phylogenomic analysis based on the concatenated sequences of 103 core genes (Fig. 2) showed that the closest relative of strain P5891^T is *Corynebacterium choanae*, which was isolated from the northern bald ibis [50].

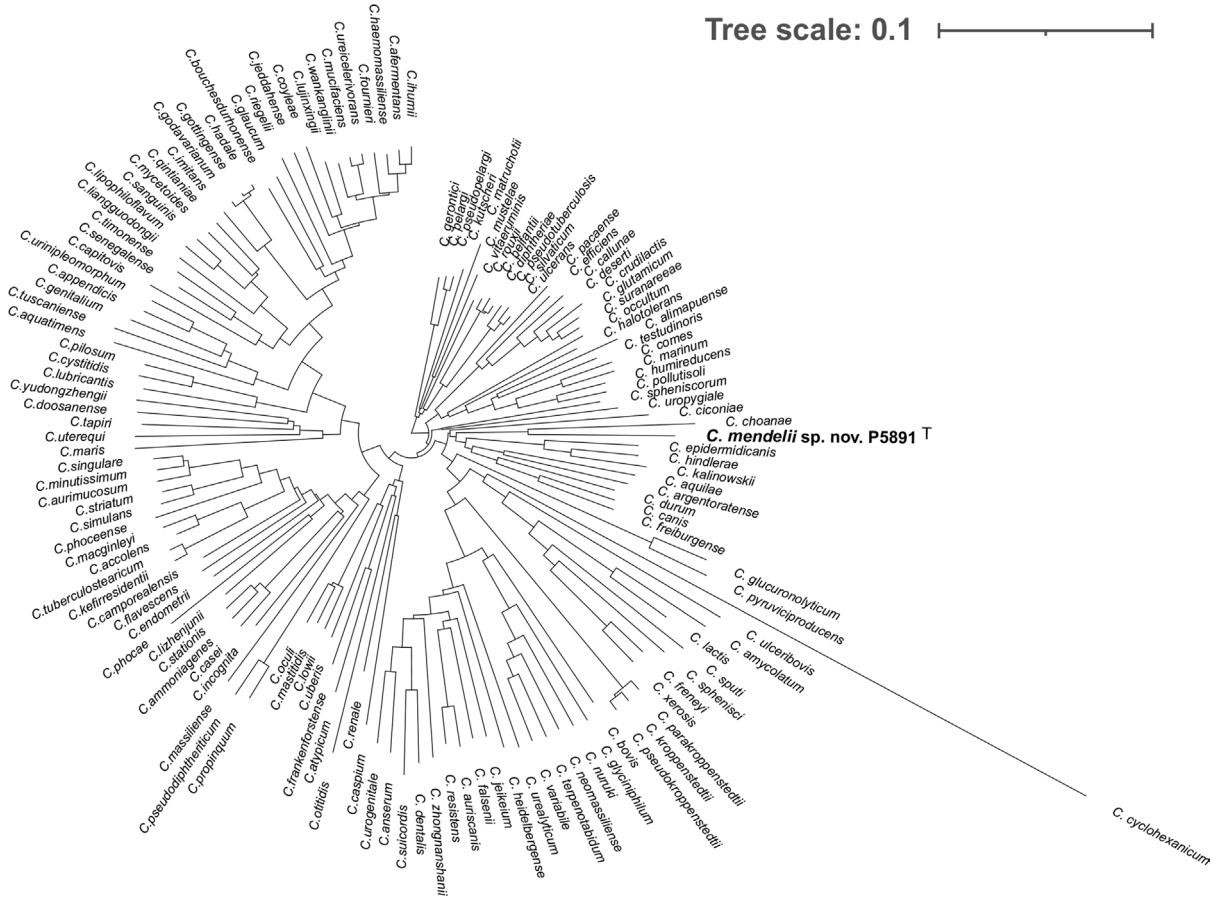

Fig. 2. Phylogenomic analysis based on concatenated sequences of 103 core genes of analysed representative genomes. The tree, reconstructed using the neighbour-joining method, was calculated using the Bacterial Pan Genome Analysis tool (BPGA), visualized with iTOL.

PHENOTYPE

The Gram stain was carried out using the Poly Stainer System (IUL Instruments), and cell morphology was observed using a BX53 light microscope (Olympus). The observed cells were Gram-stain-positive, club-shaped short rods. The KOH lysis test method [51] was used to confirm the Gram-staining results. Cellular morphology was further studied using transmission electron microscopy. Cells were transferred onto a copper grid (TedPella) coated with Formvar/carbon, negatively stained with 1% ammonium molybdate solution, and then viewed under a Philips Morgagni 268D transmission electron microscope (FEI) at ×8000 magnification and an accelerating voltage of 80 kV. The observed cells had an irregular shape and formed clusters (Fig. S3).

Growth on tryptone soya agar (TSA; Oxoid), nutrient agar (Oxoid), brain heart infusion agar (Oxoid) and Mueller–Hinton agar (Merck) at 30°C was assessed. The growth temperature range was determined in tryptone soya broth (TSB; Oxoid) incubated at 5, 10, 15, 20, 30, 37, 42 and 45°C, and tolerance to salinity was tested in TSB enriched with 5, 6.5, 10, 11 and 12% NaCl (w/v). Tolerance to pH range was tested in TSB adjusted to pH 4–11 in 1 pH unit increments using a buffer system (pH 4–8, 0.1 M KH_2PO_4/0.1 M NaOH; pH 9–10, 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11–12, 0.05 M Na_2HPO_4/0.1 M NaOH). Conventional tests were performed to determine phenotypic properties: motility [52, 53], the production of oxidase (OXItest, Erba Lachema), catalase (ID Colour Catalase, bioMérieux), acetoin (VPtest, Erba Lachema), pyrrolidonyl arylamidase (PYRAtest, Erba-Lachema), β-galactosidase (ONPG test) [54], urease [55] and lecithinase (egg yolk reaction) [56], the reduction of nitrate [57], and hydrolysis of DNA (CM321, Oxoid), aesculin [57], Tween 80, gelatin [58], tyrosine [59] and starch [57]. Further phenotypic testing was done using the API 50CH, API Coryne and API ZYM kits (bioMérieux) according to the manufacturer's instructions. The results of the phenotypic tests allowed for the differentiation from closely related species determined by 16S rRNA gene sequence similarity (Table 2). The complete characteristics of strain P5891^T are stated in the protologue. It is acknowledged that some features could be strain-specific, and the description may change as further strains of the species are isolated.

Table 2. Phenotypic characteristics allowing differentiation of strain P5891^T from its close relatives determined by 16S rRNA gene similarity

All the results were obtained in this study. +, Positive; w, weakly positive; –, negative.

Characteristic	Strain P5891^T	*Corynebacterium rouxii* CCM 9205^T	*Corynebacterium epidermidicanis* CCM 9052^T
Tween 80 hydrolysis	–	–	+
β-Galactosidase	+	–	–
DNA hydrolysis	–	+	+
Tyrosine hydrolysis	–	–	+
Growth at/with:			
10°C	w	–	w
42°C	–	–	+
6.5% NaCl	+	–	+
10% NaCl	+	–	–
pH 5	+	–	+
pH 9	+	–	+
pH 10	–	–	+
API Coryne:			
Pyrazinamidase	w	–	+
Alkaline phosphatase	–	–	+
β-Galactosidase	+	–	–
α-Glucosidase	+	+	–
API ZYM:			
Alkaline phosphatase	–	–	w
Esterase (C4)	–	–	w
Leucine arylamidase	–	w	+
β-Galactosidase	+	–	–
α-Glucosidase	w	+	–
API 50CH:			
Glycerol	–	+	–
Maltose	+	–	+
Trehalose	–	–	w
Glycogen	–	–	+
D-Tagatose	+	–	–
Potassium 5-ketogluconate	+	w	–

Susceptibility to antibiotics was tested using the disc diffusion method on Mueller–Hinton agar (Oxoid) according to the EUCAST standards [60]. The following antibiotics were tested (amount per disc): penicillin G (1 IU), ciprofloxacin (5 µg), gentamicin (10 µg), vancomycin (30 µg), clindamycin (2 µg), tetracycline (30 µg), linezolid (10 µg) and rifampicin (5 µg) (Oxoid). Strain P5891^T was susceptible to all the antibiotics tested except for penicillin G. This corresponded well with the recently described *Corynebacterium* species isolated from Adélie penguins described by Švec *et al.* [11], in which all the strains of *C. marambiense*, *C. meridianum* and *C. pygosceleis* and one strain of *C. antarcticum* also exhibited resistance to penicillin G, while being susceptible to most of the other antibiotics tested (except clindamycin).

Analysis of fatty acid methyl esters was performed from biomasses of all three compared strains: P5891^T, *C. rouxii* FRC0190^T and *C. epidermidicanis* CCM 9052^T. All strains were grown under the same conditions, on TSA at 30±2°C for 48 h, to reach the late exponential stage of growth according to the four-quadrant streak method described by Sasser [61]. Separation and identification of the fatty acids were performed with an Agilent 7890B gas chromatograph according to the Standard Protocol of the Sherlock Identification System (MIDI Sherlock version 6.2, MIDI database RTSBA 6.21).

Strain P5891^T could be clearly distinguished from its closest relatives by a specific combination of the major fatty acids (>10%), *cis*-9-octadecenoic (oleic) $C_{18:1}$ *ω9c* (74.2%) and hexadecanoic (palmitic) $C_{16:0}$ (17.6%) acids (Table S4). *C. rouxii* FRC0190^T differed from strain P5891^T by having significantly higher amounts of $C_{16:0}$ (41.2%), while *C. epidermidicanis* DSM 45586^T differed by the presence of four major fatty acids. Tuberculostearic acid (10-methyl $C_{18:0}$) was not present. The fatty acid profile and major fatty acids detected in strain P5891^T were in agreement with those of other *Corynebacterium* species, and the high amount of 'oleic' acid allowed clear differentiation from its closest phylogenetic relatives [3].

DESCRIPTION OF *CORYNEBACTERIUM MENDELII* SP. NOV.

Corynebacterium mendelii (men.de'li.i. N.L. gen. n. *mendelii* of Mendel, named in honour of Johann Gregor Mendel, the founder of genetics, an eponym of Johann Gregor Mendel Antarctic station, where the type strain was isolated).

Cells are Gram-stain-positive, non-spore-forming, irregular short rods, 0.9–2×0.7 µm, occurring predominantly in clusters. Grows well on TSA, nutrient agar, brain heart infusion agar and Mueller–Hinton agar at 30°C. Colonies on TSA plates are creamy, circular with entire margins, slightly convex, smooth and glistening when cultivated at 30°C for 2 days. Grows on TSA at 10–37°C, but not at 5 and 42°C. Grows in TSB containing 10% NaCl, but not 11%. Grows in TSB with pH adjusted to 5–9; pH 10 and pH 4 inhibit growth. Catalase positive and oxidase negative. Produces *β*-galactosidase (ONPG test). Negative for the production of pyrrolidonyl arylamidase, acetoin, urease and lecithinase (egg-yolk reaction), reduction of nitrate, and hydrolysis of Tween 80, gelatin, starch, aesculin, DNA, tyrosine and casein. The major fatty acids are $C_{18:1}$ ω9*c* and $C_{16:0}$.

Enzymatic reactions tested with the API ZYM kit gave positive results for *β*-galactosidase and *α*-glucosidase, weakly positive results for esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase, and negative results for alkaline phosphatase, esterase (C4), lipase (C14), leucine arylamidase, 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.

The API Coryne kit gave positive results for the production of *β*-galactosidase and *α*-glucosidase, and fermentation of glucose and maltose, and a weakly positive result for the production of pyrazinamidase. Results for the reduction of nitrates, production of pyrrolidonyl arylamidase, alkaline phosphatase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, aesculin, urease and gelatinase, fermentation of ribose, xylose, mannitol, lactose, sucrose and glycogen were negative.

The API 50CH kit gave positive results for the utilization of D-galactose, D-glucose, D-mannose, maltose, D-tagatose and potassium 5-ketogluconate, a weakly positive result for the utilization of D-fructose, and negative results for the utilization of glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-xylose, methyl *β*-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl *α*-D-mannopyranoside, methyl *α*-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate.

The type strain, P5891^T (=CCM 8862^T= LMG 31627^T), was isolated from a beak swab of an Adélie penguin (*Pygoscelis adeliae*) on James Ross Island, Antarctica. The genomic G+C content of the type strain is 63.3 mol%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of the type strain is OP099850, and the whole-genome sequence accession number is JAFLEQ000000000.

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

Ethical statement

Ethical approval was not required for non-invasive sampling performed in this study according to Czech legislation on the protection of animals against cruelty. A permit for the collection of samples was issued on 15 November 2013 by the Ministry of Environment of the Czech Republic in compliance with the Act on Antarctica.

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