Chryseobacterium pennae sp. nov., isolated from poultry feather waste

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Abstract

A Gram-stain-negative, rod-shaped, non-motile, non-sporulating, aerobic, yellow-pigmented bacterium was isolated from chicken feather waste collected from an abattoir in Bloemfontein, South Africa. A polyphasic taxonomy study was used to describe and name the bacterial isolate, strain 1_F178ᵀ. The 16S rRNA gene sequence analysis and sequence comparison data indicated that strain 1_F178ᵀ was a member of the genus Chryseobacterium and was closely related to Chryseobacterium jejune (99.1%) and Chryseobacterium nakagawai (98.7%). Overall genome similarity metrics (average nucleotide identity, digital DNA–DNA hybridization and average amino acid identity) revealed greatest similarity to the C. jejune and C. nakagawai type strains but were below the threshold for species delineation. Genome sequencing revealed a genome size of 6.18 Mbp and a G+C content of 35.6 mol%. The major respiratory quinone and most abundant polar lipid of strain 1_F178ᵀ were menaquinone-6 and phosphatidylethanolamine, respectively. Strain 1_F178ᵀ had a typical fatty acid composition for Chryseobacterium species. On the basis of physiological, genotypic, phylogenetic and chemotaxonomic data, strain 1_F178ᵀ constitutes a novel species of Chryseobacterium, for which the name Chryseobacterium pennae sp. nov. is proposed. The type strain is 1_F178ᵀ (=LMG 30779ᵀ=KCTC 62759ᵀ).

The genus Chryseobacterium initially belonged to the family Flavobacteriaceae and was first described by Vandamme et al. [1]. The genus Chryseobacterium has undergone several changes since its description and currently consists of 132 species [2]. Chryseobacterium has recently been reclassified into the family Weeksellaceae [3]. Another study suggested that nine Chryseobacterium species (Chryseobacterium arachidiradicis, Chryseobacterium bovis, Chryseobacterium caeni, Chryseobacterium hispanicum, Chryseobacterium hominis, Chryseobacterium hungaricum, Chryseobacterium molle, Chryseobacterium pallidum, Chryseobacterium zeae) should be transferred to the genus Epilithonimonas, 11 species (Chryseobacterium anthropi, Chryseobacterium antarcticum, Chryseobacterium carnis, Chryseobacterium chaponense, Chryseobacterium haiense, Chryseobacterium jeonii, Chryseobacterium montanum, Chryseobacterium palustre, Chryseobacterium solincola, Chryseobacterium treverense, Chryseobacterium yonginense) to the genus Kaistella and three (Chryseobacterium frigidisoli, Chryseobacterium humi, Chryseobacterium marina) to the genus Halpernia [4].

Chryseobacterial cells are Gram-stain-negative rods, aerobic, non-sporulating and non-motile. Colonies are circular, yellow-pigmented due to the presence of flexirubin, oxidase-positive and catalase-positive. Their predominant branched chain fatty acids are iso-C₁₅⁰, iso-C₁₇₀ 3-OH and iso-C₁₇₋₁₀ω₉c, with menaquinone-6 (MK-6) as the major respiratory quinone and phosphatidylethanolamine (PE) as the major polar lipid [5].

Chryseobacterium species have been isolated from a variety of food sources including fish, meat, poultry and dairy products [6–11]. Some species cause spoilage in milk due to lipolytic and...
proteolytic activities [12]. Chryseobacterium/Flavobacterium were also reported to cause spoilage in butter [13, 14] and creamed rice [15]. In contrast, Chryseobacterium species can also play a protective role, as evidenced by the presence of four Chryseobacterium balustinum strains on potatoes where they play an antagonistic role against plant-pathogenic fungi and a plant-parasitic nematode [16].

In this study, a putative novel species of the genus Chryseobacterium was isolated from chicken feather waste. A polyphasic taxonomy approach was followed to characterize strain 1_F178T.

**ISOLATION AND ECOTOLOGY**

Strain 1_F178T was isolated from chicken feather waste. The samples were obtained from a chicken abattoir in Bloemfontein, Free State, South Africa (approx. 29.108405° S 26.183772° E). Ten grams of feather waste were added to 90 ml buffered peptone water (BPW; Merck 63725) and mixed vigorously. Serial dilutions were made in BPW and pour-plated in duplicate using nutrient agar (Oxoid CM3). The plates were incubated aerobically for 48 h at 25 °C. Yellow-pigmented colonies were enumerated and streaked on nutrient agar to obtain a pure culture of strain 1_F178T. The isolates were maintained as freeze-dried cultures on filter paper discs and stored in sealed Petri dishes at –20 °C. For short-term maintenance, they were cultured on nutrient agar slants, stored at 4 °C and re-streaked every 4–6 weeks.

**16S rRNA GENE PHYLOGENY**

The DNA of strain 1_F178T was extracted with the NucleoSpin Microbial DNA kit (Macherey-Nagel), according to the manufacturer’s instructions. The extracted genomic DNA quantity and quality was assessed using the Nanodrop ND-1000 spectrophotometer (version 3.3.0, Thermo Scientific).

The DNA was subjected to PCR amplification of the 16S rRNA gene according to the manufacturer’s instructions (Applied Biosystems). The forward primer used was 27F (5′-AGATTTTGATCCTGCTCAG-3′, Integrated DNA Technologies) and the reverse primer was 1492R (5′-GGTTACCTTGTTACGACTT-3′, Integrated DNA Technologies).

Sequence data was analysed and aligned using Geneious Pro R9 software (www.geneious.com) [17] and compared with sequences in the EzBioCloud (www.ezbiocloud.net) database [18] and in GenBank (www.ncbi.nlm.nih.gov) to identify closely related validly published species. The 16S rRNA gene sequence analysis clearly showed that strain 1_F178T was associated with members of the genus Chryseobacterium. Chryseobacterium jejuense (99.10%) and Chryseobacterium nakagawai (98.75%) showed the highest 16S rRNA sequence similarity values, which were above the threshold value (98.7%) for species delineation, hence further genomic analysis was conducted to determine whether strain 1_F178T was a novel species [19, 20].

Phylogenetic and molecular evolutionary analyses were conducted with MEGA software version 7 [21] using the neighbour-joining method [22] with Kimura’s two-parameter distance model to determine the relationship of the unidentifed isolate to the most similar validly named Chryseobacterium type strains (www.bacterio.net/chryseobacterium.html) (Fig. 1). Confidence values were estimated from bootstrap analysis of 1000 replicates [23]. Phylogenetic trees were also reconstructed with the maximum-likelihood method (Fig. S1, available in the online version of this article) [24]. The topological structure of both phylogenetic trees clearly indicated that strain 1_F178T clustered with C. jejuense and C. nakagawai, although with poor bootstrap support. These strains, together with Chryseobacterium gleum NCTC 11432T (type species of the genus) were chosen as the reference organisms for phenotypic and genomic comparisons. C. jejuense DSM 19299T was obtained from the Deutsche Sammlung fur Microorganism, C. nakagawai CCUG 60563T was obtained from the Culture Collection of the University of Göttingen and C. gleum NCTC 11432T was obtained from the National Collection of Type Cultures.

**GENOME FEATURES**

The genome of strain 1_F178T was sequenced and assembled as described by Oosthuizen et al. [11]. Assembly of reads resulted in 88 contigs with a total sequence length of 6.18 Mbp and a G+C content of 36.6 mol%. Assessment of the assembly quality with CheckM [25] as implemented in KBase [26] revealed a completeness score of 100% and ‘contamination’ score of 1.5%. The whole-genome shotgun project was deposited in DDBJ/ENA/GenBank under the accession QVNT0000000. The version described in this paper is QVNT01000000. The assembled 1_F178T genome was identified by the Genome Taxonomy Database (GTDB) [27, 28] as a representative of a new species. The bacterial phylogenomic tree reconstructed from alignment of 120 concatenated protein sequences from more than 45000 species level genomes was downloaded from the GTDB website (https://gtdb.ecogenomic.org/) and opened with NCBI Genome WorkBench version 3.5 [29]. The strain 1_F178T assembly accession (GCF 003385515)
was used to search the tree to identify the appropriate branch (Fig. S2). As with the 16S rRNA gene tree, C. nakagawai was confirmed as the closest relative, with C. jejuense present on the adjacent clade.

The genome was uploaded to the rast server [30] for annotation and analysed using the seed Viewer [31]. To identify closely related strains based on genes other than the 16S rRNA, the rpoB gene was retrieved from the genome and blast was used to identify similar sequences in the RefSeq database, which were downloaded, aligned by ClustalW and used to reconstruct a neighbour-joining tree with mega 7. The search identified a very similar sequence in a genome (RKHU01) labelled as 'Chryseobacterium nakagawai' BIGb0215 derived from a strain isolated from the nematode Caenorhabditis elegans. These genome sequences also clustered together in the genome blast distance phylogeny tree reconstructed by the Type Strain Genome Server [32].

Overall genomic relatedness indices (OGRIs) [30] of strain 1_F178 were calculated relative to the genome of closely related Chryseobacterium strains including the type species (Table S1). Average nucleotide identity (ANI) values were calculated using the Kostas Lab ANI calculator (http://enveomics.ce.gatech.edu/ani/). Digital DNA–DNA hybridization (dDDH) values were calculated using the Genome-t0-Genome Distance calculator [32]. Average amino acid identity (AAI) values were determined with a custom calculator (http://lycofs01.lycoming.edu/~newman/AAI.html).
Table 1. Differential characteristics of strain 1_F178T and the type strains of closely related species of the genus Chryseobacterium

| Characteristic                  | 1  | 2  | 3  | 4  |
|--------------------------------|----|----|----|----|
| **Growth at:**                 |    |    |    |    |
| 4 °C                           | +  | −  | −  | −  |
| 20 °C                          | +  | −  | −  | −  |
| 35 °C                          | w  | +  | +  | +  |
| 37 °C                          | −  | w  | +  | +  |
| **Acid production from:**      |    |    |    |    |
| Glucose                        | −  | −  | +  | −  |
| Mannitol                       | w  | −  | −  | −  |
| Glycerol                       | w  | −  | −  | −  |
| Maltose                        | w  | −  | w  | −  |
| Trehalose                      | −  | +  | +  | +  |
| Simmon's citrate               | +  | −  | +  | +  |
| **Reduction of:**              |    |    |    |    |
| Nitrate                        | −  | +  | +  | −  |
| Nitrite                        | −  | −  | −  | +  |
| **Hydrolysis of:**             |    |    |    |    |
| Tween 20                       | −  | +  | −  | −  |
| Tyrosine                       | +  | +  | −  | +  |
| **Production of:**             |    |    |    |    |
| Amylase                        | −  | +  | −  | −  |
| **Growth on:**                 |    |    |    |    |
| Mannitol salt agar             | −  | w  | +  | +  |
| Cetrimide                      | −  | +  | −  | +  |

Organisms that are members of the same species have dDDH values of 70 % or greater [33], ANI values greater than 95 % [34] and AAI values above 95 % [35]. Comparison of the genome sequences of strain 1_F178T and ‘Chryseobacterium nakagawai’ BIGb0215 (RKHU01) with each other revealed values (Table S1) above the species thresholds, indicating that they are members of the same species. Comparison to the genomes of validly published species revealed values below the species thresholds, indicating that these two strains represent a single novel species. Most notably, despite the assignment of strain BIGb0215 to C. nakagawai in GenBank, the ANI value of this strain relative to the type strain was only 87.1%, clearly indicating that these are different species.

The protein-coding gene content of strain 1_F178T was compared to those of the related strains. Coding sequences identified as bidirectional best hits by the seed Viewer sequence-based comparison tool [30] were used to generate a Venn diagram (Fig. S3) using a calculator available at http://lycofs01lycomingedu/~newman/CurrentResearch.html. Strain 1_F178T had 743 unique genes, of which 219 encoded proteins with assigned functions. These included Tra genes associated with a two separate conjugative transposons, several phage genes (e.g. WP_115971329, WP_115971998) and many genes annotated as ‘mobile element proteins’ (e.g. WP_115971419). Given that strain 1_F178T was isolated from feather waste, proteases would be expected to play an important role in this niche. Unique genes present were predicted to encode enzymes such as cysteine proteases (e.g. WP_115971399), serine proteases (e.g. WP_115973573, WP_115973076) and zinc metalloproteases (WP_115973574, WP_115973075). Interestingly, the only other Chryseobacterium genome currently in RefSeq encoding orthologs of WP_115973076 and WP_115973075, is derived from Chryseobacterium arthrosphaerae CC-VM-7T. One unique gene found in strain 1_F178T that is not seen in any other Weeksellaceae genomes in RefSeq, encodes an aquaporin (WP_115970958). Orthologs of this gene are seen in other Bacteroidota, and the gene is close to genes encoding an integrase (WP_115970810) and a tetracycline resistance element mobilization regulatory protein (WP_115970813), suggesting that it was acquired by horizontal gene transfer.

There were 496 genes shared by strains 1_F178T and BIGb0215 but absent in the other three strains and there were 3059 genes shared by all five strains.

**PHYSIOLOGY AND CHEMOTAXONOMY**

For phenotypic comparison, reference strains C. jejuense and C. nakagawai, together with the type species, C. gleum were used. Gram-staining and the production of oxidase and catalase were determined [36]. The cultures produced a fruity odour, similar to that produced by closely related species. Motility was determined by phase-contrast examination of wet mounts from nutrient broth (NB; CM67; Oxoid). Gliding motility was determined according to the protocol of Jooste [37]. The presence of flexirubin-type pigments was assessed by flooding the colonies with 20% (w/v) KOH solution [23].
NCTC 11432 T. All results are from this study. Values correspond to Chryseobacterium nakagawai CCUG 60563 T; 4, (Difco 0691326). A multi-inoculation device was used to perform the inoculations.

Table 2. Cellular fatty acid profiles (%) of strain 1_F178⁷ and reference strains

| Fatty acid | 1          | 2          | 3          | 4          |
|------------|------------|------------|------------|------------|
| Saturated: |            |            |            |            |
| C₁₆:0      | 2.2        | 3.7        | 3.6        | 1.3        |
| Unsaturated: |            |            |            |            |
| iso-C₁₅:0 9c | 24.6       | 15.6       | 30.3       | 23.6       |
| Branched:   |            |            |            |            |
| iso-C₁₅:0 3-OH | 37.9       | 34.1       | 38.9       | 35.7       |
| iso-C₁₆:0 3-OH | 2.2        | 3.0        | 2.9        | 2.8        |
| iso-C₁₇:0 3-OH | TR         | 1.2        | 1.6        | ND         |
| iso-C₁₇:3 3-OH | 15.1       | 12.3       | 19.9       | 15.1       |
| Summed feature 3 | 9.31       | 10.7       | 10.4       | ND         |
| Unknown ECL 16.587 | 1.2        | TR         | 1.9        | 1.7        |
| Unknown ECL 13.591 | 4.5        | 1.39       | 4.5        | 2.8        |

Furthermore, physiological and biochemical features and enzymatic activities were assessed using API ZYM and API 20NE test strips (bioMérieux) and the Biolog Omnilog GEN III identification system in accordance with the manufacturer’s instructions.

Strain 1_F178⁷ could be distinguished from C. jejune and C. nakagawai by its ability to grow at 4 and 20 °C (weakly) on nutrient agar, produce acid from glycerol and mannitol, inability to grow at 37 °C and not producing acid from trehalose, and ability to grow on mannitol salt agar but not on cetrime agar (Table 1).

Analysis of fatty acids was done according to the method of Sasser [44]. For fatty acid methyl ester (FAME) analysis, strains were cultivated on trypticase soy broth agar at 25 °C. To ensure a standardized physiological age of the cells, a section of choice from a quadrant streak on the agar plate was taken. The cells were saponified, methylated and extracted according to the standard protocol of the Sherlock Microbial Identification System (MIS, version 6.1; mdi). The FAMEs were analysed on an Agilent 6850 gas chromatograph using the method RT5B6 of the MIS. The major fatty acids of strain 1_F178⁷ were iso-C₁₅:0, iso-C₁₇:0 3-OH and iso-C₁₇:0 3-OH, which were also predominant in the related species. The differences in cellular fatty acid content between strain 1_F178⁷ and related species of the genus Chryseobacterium are shown in Table 2.

Polar lipids were extracted from wet cells and analysed according to the methods of Nguyen et al. [45]. Extraction was done with chloroform–methanol–saline (6:10:3, v/v/v) and the lipids were separated using aluminium 20×20 cm silica gel 60 F254 plates (Merck Millipore), by two-dimensional chromatography using chloroform–methanol–water (65:25:4, v/v/v) for the first chromatography dimension, and chloroform–glacial acetic acid–methanol–water (40:7.5:6.2, v/v/v/v) in the second dimension. The plates were sprayed with the appropriate reagents to detect different lipids. Total lipid profiles were detected with phosphomolybdic acid, aminolipids and phospholipid groups were detected with ninhydrin reagent and glycolipids with α-naphthol–sulphuric acid–methanol reagent. The major polar lipid of strain 1_F178⁷ was PE, along with several unidentified lipids (L1–5), glycolipids (G1–2) and other unidentified amino lipids (AL1–3) (Fig. S5).

Cell morphology was determined using scanning (JSM-7800F Extreme-resolution Analytical Field Emission SEM) and transmission (Philips CM100 Transmission Electron Microscope, FEI) microscopy (Fig. S4). These were performed by the Centre of Microscopy at the University of the Free State [11]. Phenotypic and biochemical tests were carried out as reported by Oosthuizen et al. [11]. Strains were cultivated in NB at 25 °C for 48 h. The cells were centrifuged at 3000 g for 10 min (Eppendorf). The supernatant was discarded and the cell pellets were washed twice with phosphate buffer (0.1 M, pH 7). The cell pellets were re-suspended in 10 ml fresh phosphate buffer and standardized in comparison with a McFarland number 2 density (6×10⁸ c.f.u. ml⁻¹) standard (Difco 0691326). A multi-inoculation device was used to perform the inoculations.

The following range of phenotypic tests were done according to Cowan [38] and MacFaddin [36] unless otherwise stated: oxidative or fermentative metabolism of glucose; methyl red and Voges–Proskauer reactions; gluconate oxidation; potassium cyanide tolerance; malonate utilization; growth in 0–5% (w/v) sodium chloride; growth at 4, 20, 32, 35, 37 and 42 °C; growth on cetrimide agar (Merck 5284), MacConkey agar (Oxoid CM0007) and Simon’s citrate agar (Oxoid CM155); growth in 0–6% (w/v) sodium chloride; reduction of 0.4% selenite [39]; nitrate and nitrite reduction; production of acid from 10% (w/v) glucose and lactose; alkaline reaction on Christensen’s citrate [40]; production of ammonia from arginine; lysine decarboxylase, ornithine decarboxylase, deoxyribonuclease (Oxoid CM321+ 0.01% toluidine blue), β-galactosidase (ONPG), hydrogen sulphide (TSI method), indole (Kovac’s reagent), 3-ketolactose, phenylalanine deaminase, urease on Christensen’s urea agar [39, 41]; hydrolysis of aesculin [42], casein, gelatin (plate method), starch [43], Tween 20, Tween 80 [38] and tyrosine [38]; and acid production in D-mannitol, L-arabinose, trehalose, ethanol and D-xylene. The sugars were incorporated at a final concentration of 1% (w/v).
Respiratory quinones were extracted from wet cells and analysed by the TLC method described by Komagata and Suzuki [46]. The only quione was MK-6, which is the major menaquinone in the *Chryseobacterium* species [5].

Based on the results obtained from the taxonomic and morphological analyses, strain 1_F178T shares characteristics (iso C15:0 as the major fatty acid, MK-6 as the major quione and PE as major polar lipid) with other described species in the genus *Chryseobacterium*. Moreover, all the OGRI values of strain 1_F178T were less than the cut-off values for species delineation, hence confirming the affiliation of strain 1_F178T as representing a novel species in the genus *Chryseobacterium*, for which the name *Chryseobacterium pennae* sp. nov. is proposed.

**DESCRIPTION OF *CHRYSEOBACTERIUM PENNAE* SP. NOV.**

*Chryseobacterium pennae* (pen’nae. L. gen. n. pennae of a feather).

Gram-stain-negative, rod-shaped (approximately 1–3 μm long devoid of flagella), non-gliding, non-motile and non-spore-forming cells with rounded ends. The colonies are yellowish and circular when cultivated at 25 °C for 48 h on nutrient agar, produce a fruity odour, are translucent, have a flat and smooth entire surface, and become mucoid on prolonged incubation. Cells are non-fluorescent, produce flexirubin-type pigment, are strictly aerobic and grow at 4 °C but not at 37 °C and 42 °C. Optimal growth is at 25–30 °C. Grows on MacConkey, tryptic soy agar, Simmons citrate, Christensen’s citrate, nutrient agar and β-hydroxybutyrate, but not on cetrimide and mannitol salt agars. Grows in 0–3% NaCl (optimal at 0% NaCl), but not in 4–6% NaCl; growth at pH 5–8 (optimal at pH 6) but not at pH less than 5; produces acid weakly from mannitol, glycerol and maltose, but not from glucose, fructose, arabinose, lactose, trehalose, sucrose or xylose. Strain 1_F178T produces caseinase, DNase, gelatinase, urease and tryptophanase but not anylase, lecithinase, β-galactosidase (ONPG) and phenylalanine deaminase. Negative for the production of 3-ketolactose and H₂S, and unable to utilize malonate.

In the Biolog GEN III system, strain 1_F178T is susceptible to troleandomycin, rifamycin SV, lincomycin, vancomycin (weakly), nalidixic acid (weakly) and aztreonam. The strain has a high reducing power to tetrazolium violet and tetrazolium blue. Strain 1_F178T gave a positive response in wells containing the following amino acids: n-serine, glycyl-l-proline, l-alanine, l-arginine, l-aspatic acid, l-glutamic acid, l-histidine, l-pyroglutamic acid and l-serine. On the API 20 NE test strip, positive for indole, urease and cytochrome oxidase production, aesculin and gelatin hydrolysis, assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose and potassium gluconate. On the API ZYM test strip, positive for alkaline phosphatase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-β-glucosaminidase. The predominant cellular fatty acids are iso-C₁₅:₀, iso-C₁₇:₁ω9c and iso-C₁₇:₃-3-OH. PE is the most abundant polar lipid with MK-6 as the only respiratory quinone.

The type strain is 1_F178T (=LMG 30779T =KCTC 62759T), isolated from chicken feather waste collected from an abattoir in Bloemfontein, Free State, South Africa. The genome comparisons in this study indicated that the *C. 'nakagawai'* strain BIGb0215 (RKHU01) is a member of the same species, *C. pennae*.

General features of the genome assembly are as follows: genome size, approximately 6187872 bp; number of contigs, 88; coding sequences, 5682; N50 value, 162953; coverage, 25.0×. The DNA G+C content is 35.6 mol%.
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