Chryseobacterium piperi sp. nov., isolated from a freshwater creek

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As part of an undergraduate microbiology course, a yellow–orange pigmented, Gram-staining negative, rod-shaped, non-motile bacterial strain, designated CTM^T, was isolated from a creek in North-central Pennsylvania during the winter of 2006. The 16S rRNA gene sequence of the strain showed ~97 % similarity to that of *Chryseobacterium soldanellicola* PSD1-4^T and *Chryseobacterium soli* JS6-6^T, while the protein-coding *gyrB* gene sequence of strain CTM^T showed <87 % similarity to those of its two closest relatives. Using a polyphasic approach, strain CTM^T was characterized and compared to these and other closely related species of the genus *Chryseobacterium*. Strain CTM^T was similar to other strains of the genus *Chryseobacterium* in that it contained MK-6 as its major respiratory quinone, produced flexirubin-type pigments, oxidase and catalase, hydrolysed DNA, gelatin and aesculin and contained the fatty acids iso-C_{15:0}, iso-C_{17:1ω9c}, iso-C_{17:0} 3-OH and summed feature 3 (C_{16:1ω6c}, C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). Based on the results of this study, strain CTM^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium piperi* sp. nov. is proposed. The type strain is CTM^T (=ATCC BAA-1782^T =CCUG 57707^T =JCM 15960^T =DSM 22249^T =KCTC 23267^T).

The number of species with validly published names within the genus *Chryseobacterium* has increased dramatically in recent years. The genus was first described by Vandamme *et al.* (1994) with six species but when the description of the family *Flavobacteriaceae* was emended by Bernardet *et al.* (2002), there had been no novel validly published additions. Since that time, however, more than 40 novel species of the genus *Chryseobacterium* have been identified

from a variety of sources, including a water reservoir (Kim *et al.*, 2008), cow's milk (Hantsis-Zacharov *et al.*, 2008), diseased fish (Ilardi *et al.*, 2009), arthropod faeces (Kämpfer *et al.*, 2010) and many other environments (Bernardet *et al.*, 2006).

For decades, most undergraduate microbiology courses have included a series of experiments in which students must identify an unknown microbe. Over the past decade, the availability of universal primers for the amplification of 16S rRNA gene sequences and inexpensive DNA sequencing services have allowed students to identify truly novel organisms, as yet unknown to science, from the environment (Newman, 2000). Given that a significant percentage of culturable organisms have not yet been described in taxonomic literature, it is not surprising that novel organisms are encountered in these lab courses. Here, we describe a polyphasic study of one such organism.

Samples of sediment and water were obtained in January, 2006, from Loyalsock Creek (41.488° N 76.600° W), near Forksville, North-central Pennsylvania. After vigorous resuspension and the settling of large particulates, aqueous samples were serially diluted in tryptic soy broth (TSB; Remel) and spread on tryptic soy broth agar (TSBA) plates containing 16 g agar (Teknova) per l TSB. Well-separated colonies were selected and purified by streak plating for analysis by the students. Isolates were grown at 30 °C for 2 days unless otherwise noted, and permanent stocks were

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CTM^T is EU999735. GenBank/EMBL/DDBJ accession numbers for the *gyrB* gene sequences of strains CTM^T, *Chryseobacterium soldanellicola* DSM 17072^T, *Chryseobacterium soli* DSM 19298^T, *Chryseobacterium shigense* DSM 17076^T and *Chryseobacterium luteum* DSM 18605^T are HQ011910, HQ011912, HQ011913, HQ011911 and HQ011909, respectively.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

maintained at -80°C in TSB supplemented with 20% glycerol. For subsequent comparative studies, *Chryseobacterium soli* DSM 19298^T (Weon *et al.*, 2008), *Chryseobacterium soldanellicola* DSM 17072^T (Park *et al.*, 2006), *Chryseobacterium shigense* DSM 17076^T (Shimomura *et al.*, 2005) and *Chryseobacterium luteum* DSM 18605^T (Behrendt *et al.*, 2007) were purchased from the DSMZ and used as reference strains after confirming their identity by 16S rRNA sequencing as described below.

DNA was isolated for analysis using a Qiagen Blood and Tissue kit. DNA G+C content was determined by the method of Mesbah *et al.* (1989) using a 4.6×150 mm Zorbax Eclipse Plus C18 column (3.5 μm particle size) (Agilent) on an Agilent 1200 liquid chromatograph. The DNA G+C content of strain CTM^T was 38.6 mol% and those of reference strains *C. soli* DSM 19298^T and *C. soldanellicola* DSM 17072^T were 39.5 and 38.9 mol%, respectively. While these values are in line with those previously reported for *C. soli* DSM 19298^T (39.9 mol%; Weon *et al.*, 2008) and many other strains of the genus *Chryseobacterium* (Weon *et al.*, 2008; Kim *et al.*, 2008; Park *et al.*, 2008; Herzog *et al.*, 2008), the value of 38.9 mol% obtained for *C. soldanellicola* DSM 17072^T was significantly higher in this study than the value of 28.8 mol% reported by Park *et al.* (2006) for this strain. This higher value is, however, consistent with the DNA G+C content of the *gyrB* gene fragment (38.1 mol%) as described below, as well as the that of *groEL/cpn60* and *rpoB* gene fragments (unpublished results). No other strains of the genus *Chryseobacterium* are reported to have DNA G+C contents below 30 mol%.

Primers 27f and 1492r (Lane, 1991) were used to amplify the 16S rRNA gene of strain CTM^T using high fidelity Ex-Taq DNA polymerase (Takara) and the following PCR conditions: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1 min; followed by a final extension step of 72°C for 5 min. PCR product (1477 bp) purification and sequencing were performed by Agencourt/Beckman-Coulter Genomics using primers 27f, 357f, 785f, 1119f, 1492r and 810r (Lane, 1991). Reads were assembled using the CAP3 sequence assembly program (<http://pbil.univ-lyon1.fr/cap3.php>; Huang & Madan, 1999).

Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were performed using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences of other species of the genus *Chryseobacterium* were downloaded from EzTaxon and aligned to the seed alignment by the Ribosomal Database Project (RDP) (Cole *et al.*, 2007, 2009); *Elizabethkingia meningoseptica* was used as an outgroup. Weighted neighbour-joining (Bruno *et al.*, 2000) trees inferred from the alignment (Fig. 1; Supplementary Fig. S1, available in IJSEM Online) using the RDP Tree-Builder program with 1000 resamplings to measure bootstrap support (Felsenstein, 1985), were compared to trees produced in the MEGA4 program (Tamura *et al.*, 2007)

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain CTM^T and closely related species of the genus *Chryseobacterium*. *Elizabethkingia meningoseptica* ATCC 13253^T (AJ704540) was used as an outgroup (not shown). Bootstrap values >70% (expressed as percentages of 1000 replications) are given at branch nodes. Dots indicate branch nodes that were also present in either the maximum-likelihood or maximum-parsimony trees. Bar, 0.005 substitutions per nucleotide position.

using the maximum-likelihood and maximum-parsimony methods (Nei & Kumar, 2000). A similar topology, grouping strain CTM^T with *C. soldanellicola* PSD1-4^T and *C. soli* JS6-6^T was obtained in all three instances, as indicated by filled circles on the neighbour-joining trees. The 16S rRNA gene sequence of strain CTM^T was similar to that of *C. soldanellicola* PSD1-4^T (97.3%), *C. soli* JS6-6^T, (96.8%) and those of other species of the genus *Chryseobacterium* (92.6–96.7%). These values were well below the 98.7–99.0% similarity threshold corresponding to a DNA–DNA hybridization value of 70% (Stackebrandt & Ebers, 2006). However, as an additional means to demonstrate genomic uniqueness and to improve the resolution with which we could distinguish the species genetically, we analysed the sequence of the *gyrB* gene encoding subunit B of DNA gyrase.

GyrB amino acid sequences were derived from genomic sequences of a variety of species of the phylum *Bacteroidetes* present in GenBank, including *Chryseobacterium gleum* (accession no. NZ_ACKQ02000000), and were aligned to identify conserved sequences for the design of the degenerate primers GyrB-350aaF (5'-CARTTYG-ARGGNCARCANAA-3') and GyrB-600aaR (5'-TTCATY-TCNCCNARNCCYTTRTA-3'). A ~750 bp fragment was amplified by PCR from a variety of species of the phylum *Bacteroidetes* using the same cycling conditions as those used to amplify 16S rRNA gene sequences, except that the annealing temperature was increased to 55°C for the first five cycles to improve specificity.

The *gyrB* sequences generated in this study with strain CTM^T and the four reference strains were aligned in the GenBank database with sequences of other *Flavobacteriaceae* *gyrB* genes using the CLUSTAL W program in MEGA 4. The alignment was manually edited and used to reconstruct neighbour-joining (Supplementary Fig. S2), maximum-likelihood and maximum-parsimony trees (complete deletion option). All of the *Chryseobacterium* *gyrB* sequences

clustered together and the pairwise similarities between *gyrB* sequences of strain CTM^T and other members of the genus *Chryseobacterium* (85.8–87.8%) were less than the pairwise similarity value between *C. gleum* and *C. indologenes* (88.6%) or between *C. luteum* and *C. shigense* (89.5%). While comparing *gyrB* sequences and DNA–DNA hybridizations in other members of the family *Flavobacteriaceae*, specifically members of the genera *Tenacibaculum* and *Cytophaga*, Suzuki *et al.* (2001) noted that a 70% DNA–DNA hybridization level correlated with *gyrB* gene sequence similarity values as low as 88.8% but, in most cases, similarity values were significantly higher.

Strain CTM^T was initially characterized by visual observation of colony colour, texture and morphology, Gram stain reaction by the Hucker method (Gerhardt *et al.*, 1994), endospore staining using the Schaeffer–Fulton method (Gerhardt *et al.*, 1994) and motility in hanging drop wet mounts using bright-field microscopy. The metabolic characteristics of strain CTM^T, *Chryseobacterium soli* DSM 19298^T and *C. soldanellicola* DSM 17072^T were determined following the methods described by Gerhardt *et al.*, (1994) unless otherwise noted. Examination of carbohydrate metabolism included testing for citrate utilization with Simmons citrate agar, acetoin production via the Vogues–Proskauer test and fermentative acid and gas production in a phenol-red broth base supplemented with 0.75% glucose, lactose, sucrose, mannitol, arabinose or galactose. Nitrogen metabolism studies included nitrate reduction tests using tryptic nitrate broth, indole and hydrogen sulfide production assays using SIM medium and the phenylalanine deaminase assay on phenylalanine agar. The requirement for oxygen was tested by growth on TSBA in a Gas-Pak jar made anaerobic by inclusion of an AnaeroPack pouch (Mitsubishi Gas Chemical). The presence of cytochrome oxidase was determined by smearing a freshly grown culture onto a filter paper disk and adding a few drops of oxidase reagent (Difco-BBL). The presence of catalase was detected by observing oxygen bubbles produced after submerging bacteria on a cotton swab into 3% hydrogen peroxide solution. Production of exoenzymes was determined after 2 days of growth on the following media: caseinase on skimmed milk agar, lecithinase on spirit blue agar supplemented with lipase reagent (Difco), amylase on starch agar that was flooded with Gram's iodine after growth, DNase on agar supplemented with 0.2% DNA that was flooded with 1 M HCl after growth, Tween 80 hydrolase on TSBA supplemented with 1% Tween 80, gelatinase in nutrient broth supplemented with 12% gelatin, and haemolysis on BUG (Biolog) and TSA (Remel) media supplemented with 5% sheep's blood. Growth on Endo, EMB, MacConkey, phenylethanol and mannitol salt agars (Difco) as well as on lab prepared R2A (van der Linde *et al.*, 1999) and cetrimide (Difco Manual, 1984) agars was also tested.

After initial screening suggested that strain CTM^T might be a novel species of the genus *Chryseobacterium*, more extensive general tests and *Flavobacteriaceae*-specific tests were conducted on strain CTM^T as well as on the reference

strains *C. soli* DSM 19298^T (Weon *et al.*, 2008), and *C. soldanellicola* DSM 17072^T (Park *et al.*, 2006). All assays were repeated at least twice and metabolic profiles of the three strains were generated using GenIII plates (Biolog) incubated at 30 °C, API 20 NE strips incubated at 30 °C and API ZYM test strips (bioMérieux) incubated at 37 °C according to the manufacturers' instructions (see Table 1 and Supplementary Tables S1 and S2). Growth temperature requirements were tested on TSA plates at 5, 20, 30, 37 and 45 °C. Tolerance of NaCl was determined in NaCl-free TSB supplemented with 0, 1, 2, 3, 4, 5 or 6% (w/v) NaCl. Growth at pH 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0 was determined in TSB adjusted with either HCl or NaOH and retested with pH paper after autoclaving. For NaCl and pH tolerance studies, cells were incubated in the shaker and growth was monitored spectrophotometrically at OD₆₀₀ for 48 h. The presence of flexirubin-type pigments was determined by the red colour shift response in the presence of 20% KOH (Bernardet *et al.*, 2002) and respiratory quinones were

Table 1. Phenotypic characteristics of strain CTM^T and closely related species of the genus *Chryseobacterium*

Strains: 1, CTM^T; 2, *C. soli* DSM 19298^T; 3, *C. soldanellicola* DSM 17072^T. +, Positive; w, weakly positive; –, negative. All data from this study.

Characteristic	1	2	3
Growth on cetrimide agar	+	+	–
Growth on Endo agar	+	w	–
DNase activity	+	+	–
β-Haemolysis	+	–	–
API 20 NE strip			
Urease	+	+	–
PNPG hydrolysis	+	–	–
Utilization of (GenIII plate):			
Dextrin	+	+	–
Sucrose	+	+	–
α-D-Glucose	+	+	–
D-Mannitol	+	–	–
Glycerol	+	+	–
D-Glucose-6-phosphate	+	–	w
D-Fructose-6-phosphate	+	–	w
D-Aspartic acid	+	–	–
L-Pyrogutamic acid	+	–	–
L-Serine	+	+	–
D-Glucuronic acid	+	–	w
Citric acid	+	–	–
Tween 40	+	+	–
Acetoacetic acid	+	w	–
Formic acid	+	–	–
Resistance to (GenIII plate):			
Rifamycin SV	+	–	+
Lincomycin	w	–	+
Reduction of (GenIII plate):			
Tetrazolium violet	+	–	–

analysed by TLC using the method of Komagata & Suzuki (1987). Fatty acid profiles were determined by GLC (MIDI Sherlock version 6.1, method RTSBA6) using cells grown on TSBA for 24 h at 30 °C and extracted using the standard method of Sasser (1990). The fatty acid composition of strain CTM^T (Table 2) was typical of species of the genus *Chryseobacterium* (Bernardet *et al.*, 2006) in that the most abundant fatty acids were iso-C_{15:0}, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3 (comprising C_{16:1}ω6c/C_{16:1}ω7c/iso-C_{15:0} 2-OH). Other biochemical characteristics of strain CTM^T are listed in the species description, while phenotypes that can be used to distinguish strain CTM^T from related species are listed in Table 1. The combination of phenotypic and genotypic characteristics that differentiate strain CTM^T from previously described species suggests that strain CTM^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium piperi* sp. nov. is proposed.

Description of *Chryseobacterium piperi* sp. nov.

Chryseobacterium piperi (pi'per.i. N.L. gen. masc. *piperi* of Piper, in honour of John F. Piper Jr, recently retired Academic Dean of Lycoming College).

Cells are strictly aerobic, non-spore-forming, non-motile Gram-reaction-negative rods, 0.5 × 3 µm. Grows on R2A, tryptic soy, Endo and cetrimide agars, but not on MacConkey, EMB, phenylethanol or mannitol-salt agars. Colonies on TSA are translucent and shiny with entire edges. Yellow–orange non-diffusible flexirubin-type pigments are produced. Grows well at 20–30 °C and weakly at 37 °C, but

not at 5 °C or 42 °C. The pH range for growth is pH 6–10 (optimum pH 6.5–8.0). Grows in the presence of 0–2 % (w/v) NaCl, but not in 3 % NaCl. Cells produce indole on SIM medium and exhibit oxidase, catalase, amylase, caseinase, DNase, gelatinase, Tween 80 hydrolase and β-haemolytic activities. Negative for phenylalanine deaminase and lipase activities, nitrate reduction, utilization of citrate, production of hydrogen sulfide and acetoin and production of acid from fermentation of glucose, lactose, sucrose, mannitol, arabinose and galactose. In the API 20 NE system; positive for indole production and hydrolysis of urea, aesculin, gelatin and PNPG but negative for nitrate reduction, production of acid from D-glucose, arginine dihydrolase activity and the utilization of L-arabinose, N-acetylglucosamine, gluconic acid, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. Weakly utilizes α-D-glucose, D-mannose, D-mannitol, and maltose. In the API ZYM system; positive for alkaline phosphatase, C4 esterase (weakly), C8 esterase lipase, leucine-, valine-, and cystine (weakly) arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-β-glucosaminidase activities; negative for lipase (C14), trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities. On the GenIII plate; positive for utilization of dextrin, maltose, trehalose, gentiobiose, sucrose, α-D-glucose, D-mannose, D-fructose, D-mannitol, glycerol, D-glucose-6-phosphate, D-fructose-6-phosphate, D-aspartic acid, gelatin, glycyl-L-proline, L-aspartic acid, L-glutamic acid, L-pyrogutamic acid, L-serine, pectin, D-galacturonic acid, D-glucuronic acid, citric acid, Tween-40, acetoacetic acid, acetic acid and formic acid; weakly positive for utilization of D-galactose, 3-methyl glucose, D- and L-fucose, L-rhamnose, L-alanine, mucic acid, quinic acid, D-lactic acid methyl ester and α-keto-glutaric acid; negative for the remaining utilization tests. Also on the GenIII plate; resistant to pH 6, 1 % NaCl, 1 % Na-lactate, rifamycin SV and aztreonam; weakly resistant to D-serine, lincomycin, guanidine HCl, and K-tellurite; sensitive to the remaining inhibitory conditions. Tetrazolium blue and tetrazolium violet are reduced. The major fatty acids are iso-C_{15:0}, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3 (comprising C_{16:1}ω6c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). Menaquinone MK-6 is the predominant respiratory quinone.

The type strain, CTM^T (=ATCC BAA-1782^T =CCUG 57707^T =JCM 15960^T =DSM 22249^T =KCTC 23267^T), was isolated from a freshwater creek in North-central Pennsylvania, USA. The DNA G+C content of the type strain is 38.6 mol%.

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Table 2. Cellular fatty acid profiles (%) of strain CTM^T and closely related species of the genus *Chryseobacterium*

Strains: 1, CTM^T; 2, *C. soli* DSM 19298^T; 3, *C. soldanellicola* DSM 17072^T. tr=Trace (<1 %); fatty acids amounting to <1 % of the total fatty acids in all three strains are not shown. All strains were grown on TSBA at 30 °C for 24 h. All data from this study.

Fatty acid	1	2	3
iso-C _{13:0}	1.1	1.0	1.8
iso-C _{15:0}	36.6	36.5	40.4
anteiso-C _{15:0}	tr	2.6	2.7
Summed feature 3*	12.6	11.9	11.8
C _{16:0}	1.1	1.6	1.4
iso-C _{15:0} 3-OH	4.2	2.8	3.6
iso-C _{17:1} ω9c	22.0	16.9	12.2
iso-C _{17:0}	tr	1.3	tr
C _{16:0} 3-OH	1.8	1.3	1.6
iso-C _{17:0} 3-OH	17.9	20.7	19.8
C _{17:0} 2-OH	tr	1.1	tr

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises C_{16:1}ω6c, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

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