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 CTMT, 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 PSD1-4T and Chryseobacterium soli JS6-6T, while the protein-coding gyrB gene sequence of strain CTMT showed ~87% similarity to those of its two closest relatives. Using a polyphasic approach, strain CTMT was characterized and compared to these and other closely related species of the genus Chryseobacterium. Strain CTMT 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-C15:0, iso-C17:1o6c, iso-C17:0 3-OH and summed feature 3 (C18:1o6c, C18:1o7c and/or iso-C15:0 2-OH). Based on the results of this study, strain CTMT represents a novel species of the genus Chryseobacterium, for which the name Chryseobacterium piperi sp. nov. is proposed. The type strain is CTMT (=ATCC BAA-1782T =CCUG 57707T =JCM 15960T =DSM 22249T =KCTC 23267T).

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 1 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...
maintained at -80 °C in TSB supplemented with 20 %
glycerol. For subsequent comparative studies, Chryseobac-
terium soli DSM 19298T (Weon et al., 2008), Chryseobac-
terium soldanellicola DSM 17072T (Park et al., 2006),
Chryseobacterium shigense DSM 17076T (Shimomura et al.,
2005) and Chryseobacterium luteum DSM 18605T
(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 CTMT (38.6 mol%) and those of reference
strains C. soli DSM 19298T and C. soldanellicola DSM 17072T
were 39.5 and 38.9 mol%, respectively. While these values are in line with those
previously reported for C. soli DSM 19298T (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 17072T 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 CTMT 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,
319f, 806f, 1006f, 1492r, were aligned to identify conserved sequences for the design
of the degenerate primers GyrB-350aaF (5′-CARTTYG-
9-CARTTTYA-3′) and GyrB-600aaR (5′-TTCATY-
9-TCRTATT-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
CTMT and the four reference strains were aligned in the
GenBank database with sequences of other
Chryseobacterium piperi sp. nov.

using the maximum-likelihood and maximum-parsimony
methods (Nei & Kumar, 2000). A similar topology,
grouping strain CTMT with C. soldanellicola PSD1-4T
and C. soli JS6-6T was obtained in all three instances, as
indicated by filled circles on the neighbour-joining trees. The 16S rRNA gene sequence of strain CTMT was similar
to that of C. soldanellicola PSD1-4T (97.3 %), C. soli JS6-6T,
(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 Chryseobac-
terium 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′-TTCTAY-
TCNCCNARNCCYTTYRTA-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
CTMT and the four reference strains were aligned in the
GenBank database with sequences of other Flavobacteriaciae
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 dele-
tion option). All of the Chryseobacterium gyrB sequences

| Chryseobacterium piperi CTMT (EU999735) |
|---------------------|---------------------|
| Chryseobacterium soli JS6-6T (EF591302) |
| Chryseobacterium soldanellicola PSD1-4T (AY983415) |
| Chryseobacterium piciola YO-6316a (EU669190) |
| Chryseobacterium aquaticum 10-46 (AM748690) |
| Chryseobacterium balustinum ATCC 33487T (M85771) |
| Chryseobacterium piscium LMG 23089 (AM040439) |
| Chryseobacterium scopophilum LMG 13028 (AJ271006) |
| Chryseobacterium indolithecum LMG 4025T (AY468448) |

Fig. 1. Neighbour-joining phylogenetic tree based on 16S
rRNA gene sequences showing the phylogenetic relationship
between strain CTMT and closely related species of the
genus Chryseobacterium. Elizabethkingia meningoseptica ATCC
13253T (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.
clustered together and the pairwise similarities between gyrB sequences of strain CTMT1 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 CTMT1 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 CTMT1, Chryseobacterium soli DSM 19298T and C. soldanellicola DSM 17072T 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 Voges–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 TSA 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 paper disk and adding 1 M 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>
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<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Growth on citramide agar</td>
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<td>Growth on Endo agar</td>
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<td>Dnase activity</td>
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<td>API 20 NE strip</td>
<td>Urease</td>
<td>+</td>
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<tr>
<td>PNPG hydrolysis</td>
<td>+</td>
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<td>Sucrose</td>
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<td></td>
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<td>Tetracycline</td>
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Table 1. Phenotypic characteristics of strain CTMT1 and closely related species of the genus Chryseobacterium

Strains: 1, CTMT1; 2, C. soli DSM 19298T; 3, C. soldanellicola DSM 17072T. +, Positive; w, weakly positive; −, negative. All data from this study.
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 CTMT (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_{16:1ω6c}/C_{16:1ω7c}/iso-C_{15:0} 2-OH). Other biochemical characteristics of strain CTMT were listed in the species description, while phenotypes that can be used to distinguish strain CTMT from related species are listed in Table 1. The combination of phenotypic and genotypic characteristics that differentiate strain CTMT from previously described species suggests that strain CTMT 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 x 3 μm. Grow on R2A, tryptic soy, Endo and cetrimide agars, but not on MacConkey, EMB, phynethanol 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, aesculnin, 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, glucoronidase, 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 leupepe, leucose-, 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, β-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, glycy1-L-proline, L-aspartic acid, L-glutamic acid, L-propyglutamid 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-methylglucose, D- and L-fucos, L-thamnosine, 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. Tetrozolium blue and tetrazolium violet are reduced. The major fatty acids are iso-C_{15:0} 3-OH, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3 (comprising C_{16:1ω6g}, C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). Menaquinone MK-6 is the predominant respiratory quinone.

The type strain, CTMT (USATC 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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