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Chryseobacterium lineare sp. nov., isolated from a limpid stream

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Abstract

A Gram-stain-negative, aerobic, non-motile, rod-shaped, yellow-pigmented bacterial strain, XC0022^T, isolated from freshwater of a limpid stream in Zhejiang, China, was studied using a polyphasic approach. The phylogenetic analysis based on 16S rRNA gene sequences clearly showed an allocation to the genus *Chryseobacterium* with the highest sequence similarities of 98.0 % to *Chryseobacterium taeanense* PHA3-4^T, 97.2 % to *Chryseobacterium taihuense* THMBM1^T, 97.1 % to *Chryseobacterium rigui* CJ16^T and 97.1 % to *Chryseobacterium profundimaris* DY46^T. 16S rRNA gene sequence similarities to all other species of the genus *Chryseobacterium* were below 97.0 % (92.3–96.8 %). DNA–DNA hybridization results showed that strain XC0022^T was 55.3 %, 49.8 % and 31.1 % related to *C. taeanense* DSM 17071^T, *Chryseobacterium taichungense* DSM 17453^T and *Chryseobacterium gleum* JCM 2410^T, respectively. The quinone system was composed only of MK-6. Strain XC0022^T possessed iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, $C_{18:1}\omega9c$ and summed feature 3 (iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c$) as the major fatty acids. The polar lipids profile consisted of one phosphatidylethanolamine, one unidentified glycolipid, four unidentified aminolipids and two unidentified lipids. The G+C content of the genomic DNA was 29.7 mol%. On the basis of phenotypic, phylogenetic and chemotaxonomic data, strain XC0022^T (=KCTC 52364^T=MCCC 1K02723^T) represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium lineare* sp. nov. is proposed.

The genus *Chryseobacterium*, a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*, was established by Vandamme *et al.* [1]. At the time of writing, the genus *Chryseobacterium* contained 97 species isolated from various habitats, such as sediments [2–4], soil [5–8], water [9, 10], green tea [11], milk [12, 13], fish [14–16] and also clinical sources [17]. Members of the genus *Chryseobacterium* are Gram-stain-negative and aerobic. Phosphatidylethanolamine is the major polar lipid [18], and menaquinone 6 (MK-6) is the only respiratory quinone [19]. The predominant fatty acids include iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH [20].

The bacterial strain XC0022^T was isolated from a freshwater sample collected from a limpid stream (30° 24′ N 119° 33′ E) located in Zhejiang province, China. The stream is a source of drinking water, so we expected to isolate bacteria from it that may have an influence on human health. The standard dilution plating technique on PTYG agar was used. The PTYG agar medium contained (per litre distilled water): 5 g tryptone, 5 g soya peptone, 10 g yeast extract, 10 g glucose, 1 ml Tween 80, 0.05 g L-cysteine hydrochloride, 0.8 mg $CaCl_2$, 4 mg K_2HPO_4, 4 mg KH_2PO_4, 1.92 mg $MgSO_4 . 7H_2O$, 40 mg Na_2CO_3, 8 mg NaCl, 15 g agar, pH 6.8–7.0 adjusted with NaOH. Yellow-pigmented colonies formed after 3 days of incubation at 30 °C and were purified by repeated restreaking. Unless otherwise stated, strain XC0022^T was routinely cultured in PTYG broth or on PTYG agar at 30 °C for 24 h and preserved at −80 °C with 30.0 % (v/v) glycerol. According to the result of 16S rRNA gene sequence alignment, *Chryseobacterium taeanense* DSM 17071^T, *Chryseobacterium taichungense* DSM 17453^T and *Chryseobacterium gleum* JCM 2410^T (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen and Japan Collection of Microorganisms, respectively) were chosen as reference strains. They were routinely cultured and preserved in the same way as strain XC0022^T.

A quick bacteria genomic DNA extraction kit (DongSheng Biotech) was used to obtain genomic DNA as a template. The 16S rRNA gene was amplified by PCR using bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCT-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were cloned into vector pMD 19-T (TaKaRa) for sequencing as described by Xu *et al.* [21]. The complete sequence was compared with sequences of closely related reference organisms obtained from the EzTaxon-e database [22]. Multiple sequence alignment was performed with CLUSTAL W 1.8 [23]. Phylogenetic trees were reconstructed using the neighbour-joining [24], maximum-likelihood [25] and maximum-parsimony [26] methods with the MEGA 5 program

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Two supplementary figures and one supplementary table are available with the online Supplementary Material.

package [27]. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model [28] for the neighbour-joining method. On the basis of 16S rRNA gene sequence alignment, strain XC0022^T was most closely related to *C. taeanense* PHA3-4^T with 98.0 % sequence similarity, and had 97.2 % sequence similarity to *Chryseobacterium taihuense* THMBM1^T, 97.1 % to *Chryseobacterium rigui* CJ16^T and 97.1 % to *Chryseobacterium profundimaris* DY46^T. 16S rRNA gene sequence similarities to all other species of the genus *Chryseobacterium* were below 97.0 % (92.3–96.8 %). Phylogenetic analysis, based on nearly full-length 16S rRNA gene sequences (1482 bp), showed that strain XC0022^T belonged to the genus *Chryseobacterium* and clustered with *C. taeanense* and *C. taichungense* in the neighbour-joining, maximum-likelihood and maximum-parsimony trees (Fig. 1).

Optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1203; JEOL) were used to examine motility (hanging drop method) and morphology of cells incubated in PTYG for 24 h. Cells of strain XC0022^T were sticky. It was hard to separate them from each other under transmission electron microscopy, and they always arranged themselves in a line (Fig. S1, available in the online Supplementary Material). The Gram reaction was performed as described by Reddy [29]. For pigment analysis, cells were disrupted by sonication, and the pigment was extracted with acetone/methanol (7 : 2, v/v). A spectrophotometer (TU-1810; PERSEE) was used to perform the pigment absorption spectrum analysis (from 310 nm to 800 nm). The absorption maxima were at 320 nm and 390 nm and were attributed to flexirubin-type pigments.

Growth was evaluated at 30 °C on several standard bacteriological media: PTYG, tryptic soy broth (BD), nutrient agar (contained peptone 10 g, NaCl 5 g, beef extract 3 g, agar 15 g per litre distilled water) and MacConkey agar (BD). The optimal conditions for growth were tested in PTYG broth. The temperature for growth was tested by incubation at 4, 15, 20, 22, 25, 28, 30, 32, 35, 40, 42, 43 and 45 °C. The pH range was tested in PTYG broth by adding four kinds of buffering agents (MES for pH 5.5–6.5, PIPES for pH 6.5–7.5, Tricine for pH 7.5–8.5, Capso for pH 9.0–10.0) at a concentration of 30 mM. The salt tolerance was determined in Na^+ and Cl^- ion free PTYG broth with various NaCl concentrations (0, 0.2, 0.5, 1, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 %). Anaerobic growth was determined with a microaerobic system (Anaero-Pack-MicroAero, 2.5 l; MGC) using PTYG, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate as electron acceptors, and 5 g L-arginine l^{-1} and 1 g cysteine l^{-1} as reductant were added [30].

The utilization of single carbon sources was examined in a culture medium based on the PTYG medium which contained only inorganic salts, L-cysteine hydrochloride (0.05 g l^{-1}) and the corresponding filter-sterilized sugars (1.0 %), alcohols (1.0 %) or organic acids (1.0 %). *p*-Aminodimethylaniline oxalate (1.0 %; SSS REAGENT) was used to detect oxidase

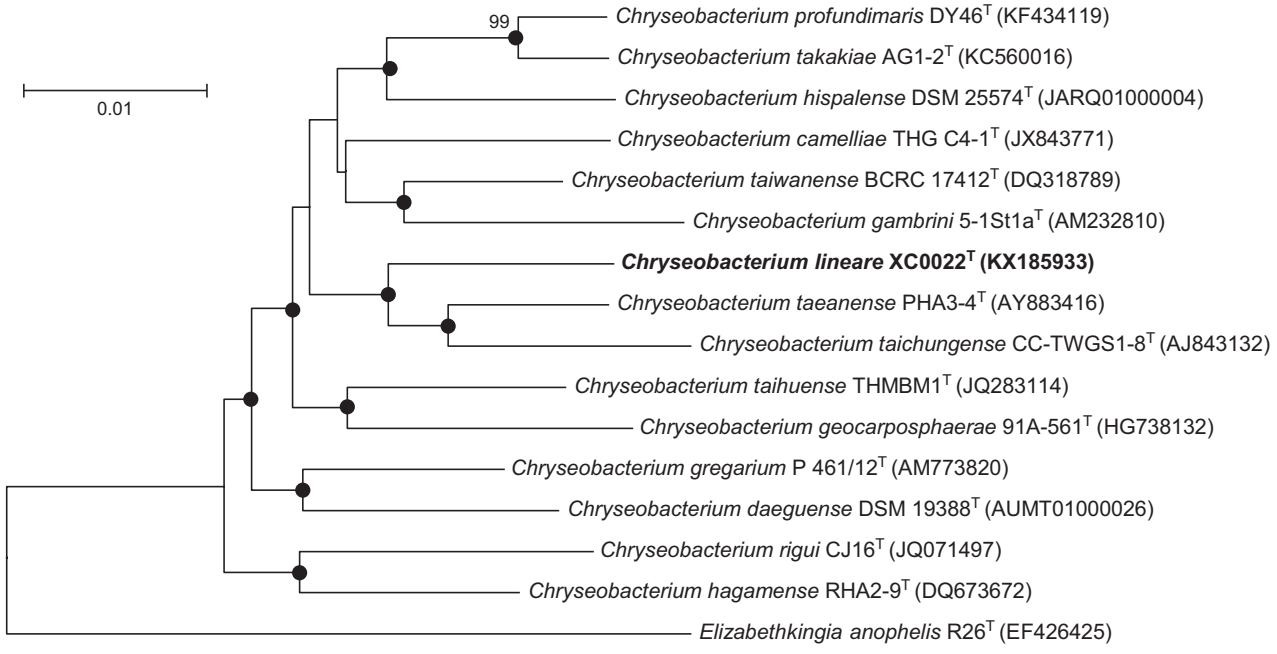

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain XC0022^T among species of the genus *Chryseobacterium*. Bootstrap values (expressed as percentages of 1000 replications) >70 % are shown at branching points. *Elizabethkingia anophelis* R26^T (GenBank accession no. EF426425) was used as an outgroup. Filled circles indicate nodes also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

activity. Catalase activity was determined by observing bubble production in 3.0 % (v/v) H_2O_2 (Enox) solution. The hydrolysis of Tweens 20, 40, 60 and 80, H_2S production, and methyl red and Voges–Proskauer reactions were tested based on the methods of Han *et al.* [31]. All experiments were performed in triplicate. For the acid production test, Leifson modified MOF medium [32] was used to suspend the cells for the inoculation of API 50CH (bioMérieux) strips. Other biochemical characteristics and enzyme activities were tested using API 20NE and API ZYM strips (bioMérieux) following the manufacturer's instructions. Phenotypic characteristics of strain XC0022^T are given in Tables 1 and S1, and in the species description.

Cells used for polar lipid, fatty acids and isoprenoid quinones analysis were grown in PTYG medium at 30 °C for 24 h to reach the exponential stage of growth. Isoprenoid quinones were extracted from freeze-dried cells with chloroform/methanol (2 : 1, v/v), purified by TLC and analysed by reversed-phase HPLC [33]. The only respiratory quinone detected in strain XC0022^T was MK-6, which is also in accordance with the genus *Chryseobacterium* [1].

For fatty acids methyl esters (FAMEs) analysis, cells were freeze-dried and then extracted according to the standard protocol of the Sherlock Microbial Identification System (MIDI, Microbial ID) with the standard MIS Library Generation Software version 4.5. The fatty acids were analysed by GC (6890N; Agilent) and identified by using the TSBA40 database. The major cellular fatty acids (>10.0 %) of strain XC0022^T were iso-$C_{15:0}$ (26.5 %), iso-$C_{17:0}$ 3-OH (15.3 %), $C_{18:1}\omega 9c$ (14.2 %) and iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega 7c$ (summed feature 3; 10.6 %). The results of the fatty acids analysis were also consistent with previous research [1]. A comparison of

Table 1. Differential characteristics between strain XC0022^T and related members of the genus *Chryseobacterium*

Strains: 1, XC0022^T; 2, *C. taeanense* DSM 17071^T; 3, *C. taichungense* DSM 17453^T; 4, *C. gleum* JCM 2410^T. +, Positive reaction; −, negative reaction; w, weakly positive reaction; ND, no data. Data were obtained from this study unless stated otherwise. All strains were positive for catalase, oxidase, H_2S production and hydrolysis of Tweens 20, 40, 60, 80.

Characteristic	1	2	3	4
NaCl range for growth (%) (optimal)	0–4 (0)	0–6 (ND)*	ND	ND
Temp. range for growth (°C) (optimal)	4–42 (30)	5–42 (25–30)*	11–36 (15–36)†	5–42 (ND)‡
pH range for growth (optimal)	5–10 (6–6.5)	5–9 (5)*	6–9 (7–8)†	ND
Growth on MacConkey agar	−	−	−	+
API ZYM				
Esterase (C4)	w	−	w	w
Esterase lipase (C8)	+	−	+	w
Trypsin	+	+	w	+
Chymotrypsin	+	+	w	+
β-Galactosidase	+	−	w	w
β-Fucosidase	−	−	−	w
Arginine dihydrolase	−	−	−	+
API 50CH				
D-Arabinose	+	+	−	−
D-Galactose	+	w	w	−
D-Fructose	+	w	w	−
L-Sorbose	w	−	−	−
N-Acetylglucosamine	+	+	+	−
Aesculin ferric citrate	+	+	+	−
Salicin	+	+	w	−
Cellobiose	+	+	w	−
Melibiose	+	−	w	−
Raffinose	−	−	+	−
Xylitol	+	−	+	−
API 20NE				
Hydrolysis of urea	−	−	−	+
Reduction of nitrate	−	−	−	+
DNA G+C content (mol%)	29.7	32.1*	ND	38‡

*Data from Park *et al.* [40].

†Data from Shen *et al.* [6].

‡Data from Holmes *et al.* [41].

Table 2. Cellular fatty acid contents (percentages) of strain XC0022^T and related species of the genus *Chryseobacterium*

Strains: 1, XC0022^T; 2, *C. taeanense* DSM 17071^T; 3, *C. taichungense* DSM 17453^T; 4, *C. gleum* JCM 2410^T. All data are from this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not included. TR, trace (<1 %). Major components (≥10 %) are highlighted in bold type.

Fatty acid	1	2	3	4
Straight-chain				
$C_{16:0}$	6.5	6.5	8.0	6.1
anteiso-$C_{15:0}$	TR	TR	1.1	TR
iso-$C_{15:0}$	**26.5**	**27.6**	**27.0**	**27.4**
iso-$C_{17:0}$	4.4	3.0	1.4	1.6
Unsaturated				
iso-$C_{17:1}\omega 9c$	6.6	4.2	5.7	5.8
$C_{18:1}\omega 9c$	**14.2**	**16.0**	**14.6**	**19.0**
$C_{18:1}\omega 7c$	1.1	2.1	1.6	1.0
Hydroxy				
iso-$C_{15:0}$ 3-OH	2.8	3.0	2.8	2.4
$C_{16:0}$ 3-OH	1.2	1.3	2.5	2.2
iso-$C_{17:0}$ 3-OH	**15.3**	**13.3**	**14.2**	**11.3**
Summed feature 3*	**10.6**	9.4	**10.7**	**11.5**
Unknown 13.565†	3.8	5.6	4.0	3.9

*As indicated by Montero-Calasanz *et al.* [18], summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain-lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 3 was listed as iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$.

†Unknown fatty acids, numbers indicate the ECL.

the fatty acid profiles showed small differences between strain XC0022^T and the reference strains, such as less iso-$C_{15:0}$, less $C_{18:1}\omega 9c$ and more iso-$C_{17:0}$ 3-OH (Table 2).

Polar lipids were extracted by the method of Sun *et al.* [34] and separated by two-dimensional TLC with silica gel 60 F_{254} aluminium-backed thin layer plates (10×10, 5554; Merk) which had been activated at the temperature of 55 °C for 30 min. According to Cui *et al.* [35], chloroform/methanol/water (65 : 24 : 4, by vol.) as the first dimension and chloroform/glacial acetic acid/methanol/water (80 : 15 : 12 : 4, by vol.) as the second dimension were used. Polar lipids were identified as described by Han *et al.* [31]. Phosphomolybdic acid (5.0 %, heating at 160 °C), ninhydrin (0.5 %, heating at 55 °C), aubepine and molybdenum blue (Sigma) were used to detect total lipids, aminolipids, glycolipids and phospholipids, respectively. The polar lipid profile of XC0022^T comprised one phosphatidylethanolamine, one unidentified glycolipid, four unidentified aminolipids and two unidentified lipids (Fig. S2). The result was in accordance with the genus description that sphingophospholipids were absent [1].

The DNA base composition (G+C content) was determined by reversed-phase HPLC [36]. The genomic DNA G+C content of strain XC0022^T was 29.7 mol% (HPLC), which was within the range of 29–39 % for the members of the genus *Chryseobacterium* [37].

DNA–DNA hybridization experiments were performed between strain XC0022^T and the type strains of the most closely related species to confirm the taxonomic status of the novel strain. As described by Sun *et al.* [38], a thermal denaturation (at 100 °C) and renaturation (at 64.9 °C) method with the Beckman DU800 spectrophotometer was used. The DNA–DNA hybridization values between strain XC0022^T and related strains were lower than 70 % [39] (55.3 %, 49.8 % and 31.1 % with *C. taeanense* DSM 17071^T, *C. taichungense* DSM 17453^T and *C. gleum* JCM 2410^T, respectively).

Phenotypic and molecular studies, especially the examination of growth pH range, the activity of β-galactosidase, the utilization of raffinose, DNA G+C content and DNA–DNA hybridization, indicate that strain XC0022^T can be clearly distinguished from its closest relatives.

On the basis of the phenotypic, phylogenetic and chemotaxonomic characteristics described above, strain XC0022^T is considered to represent a novel species within the genus *Chryseobacterium* for which the name *Chryseobacterium lineare* sp. nov. is proposed.

DESCRIPTION OF *CHRYSEOBACTERIUM LINEARE* SP. NOV.

Chryseobacterium lineare sp. nov. (li.ne.a′re. L. neut. adj. *lineare* linear, arranged like a line under transmission electron microscopy).

Cells are Gram-staining-negative, aerobic, rod-shaped, 0.8–1.0 µm in diameter and 2.0–3.0 µm in length, non-flagellated, non-motile and non-spore-forming. Colonies

are circular, convex with a shiny, smooth surface and entire edges, orange–yellow and produce a non-diffusible flexirubin-type yellow pigment. After prolonged incubation, the colonies become mucoid and unidentifiable as single entities. Good growth can be observed after 24 h of incubation on PTYG, TSB and nutrient agar at 30 °C. No growth occurs on MacConkey agar (BD) at 30 °C. Cells grow at 4 °C, but not at 43 °C. The optimum temperature for growth is 30 °C. The pH range for growth is pH5.0–10, and optimum pH is pH 6.0–6.5. Growth occurs at NaCl concentrations of 0–4 % (w/v) (optimum 0 %). Cells are positive for catalase and oxidase activities, H_2S production and hydrolysis of Tweens 20, 40, 60 and 80. Negative in the methyl red and Voges–Proskauer tests. The following compounds are utilized as sole sources of carbon: D-fructose, lactose, D-xylose, maltose, sucrose, L-arabinose, D-mannose, cellobiose, raffinose, starch, L-rhamnose and ethanol. The following compounds are not utilized as sole carbon sources: trehalose, D-galactose, citrate, malate, pyruvate, formate, acetate, lactate, gluconate, sorbitol and glycerol. With API 50CH strips, acid is produced from D-arabinose, D-galactose, D-fructose, L-sorbose (weakly), *N*-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, melibiose, starch, xylitol and potassium gluconate. In the API 20NE system, hydrolysis of aesculin, hydrolysis of gelatin and β-galactosidase activity are positive. Nitrate reduction, indole production, hydrolysis of urea and arginine dihydrolase activity are negative. In API ZYM tests, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase are positive. Esterase (C4) and cystine arylamidase are weakly positive. Lipase (C14), α-galactosidase, β-glucuronidase, α-mannosidase and β-fucosidase are negative. The predominant respiratory quinone is MK-6. The major cellular fatty acids include iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, $C_{18:1}\omega9c$ and iso-$C_{15:0}$ 2-OH/$C_{16:1}$ $\omega7c$ (summed feature 3). The polar lipids include one phosphatidylethanolamine, one unidentified glycolipid, four unidentified aminolipids and two unidentified lipids.

The type strain, XC0022^T (=KCTC 52364^T=MCCC 1K02723^T), was isolated from a freshwater sample collected from a limpid stream, Zhejiang province, China. The genomic DNA G+C content of the type strain is 29.7 mol% (HPLC).

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

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