Chryseobacterium palustre sp. nov. and *Chryseobacterium humi* sp. nov., isolated from industrially contaminated sediments

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Two Gram-staining-negative bacterial strains, designated 3A10^T and ECP37^T, were isolated from sediment samples collected from an industrially contaminated site in northern Portugal. These two organisms were rod-shaped, non-motile, aerobic, catalase- and oxidase-positive and formed yellow colonies. The predominant fatty acids were iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:1ω9c} and iso-C_{17:0} 3-OH. The G + C content of the DNA of strains 3A10^T and ECP37^T was 43 and 34 mol%, respectively. The major isoprenoid quinone of the two strains was MK-6. 16S rRNA gene sequence analysis revealed that strains 3A10^T and ECP37^T were members of the family *Flavobacteriaceae* and were related phylogenetically to the genus *Chryseobacterium*. Strain 3A10^T showed 16S rRNA gene sequence similarity values of 97.2 and 96.6 % to the type strains of *Chryseobacterium antarcticum* and *Chryseobacterium jeonii*, respectively; strain ECP37^T showed 97.3 % similarity to the type strain of *Chryseobacterium marinum*. DNA–DNA hybridization experiments revealed levels of genomic relatedness of <70 % between strains 3A10^T and ECP37^T and between these two strains and the type strains of *C. marinum*, *C. antarcticum* and *C. jeonii*, justifying their classification as representing two novel species of the genus *Chryseobacterium*. The names proposed for these organisms are *Chryseobacterium palustre* sp. nov. (type strain 3A10^T =LMG 24685^T =NBRC 104928^T) and *Chryseobacterium humi* sp. nov. (type strain ECP37^T =LMG 24684^T =NBRC 104927^T).

The genus *Chryseobacterium* was proposed by Vandamme *et al.* (1994). It belongs to the family *Flavobacteriaceae* and, at the time of writing, comprises 37 recognized species (Kämpfer *et al.*, 2009). *Chryseobacterium* species may be found in soil and water environments and in clinical and dairy sources (Bernardet *et al.*, 2006). Three flavobacteria previously classified as *Sejongia antarctica*, *Sejongia jeonii* (Yi *et al.*, 2005) and *Sejongia marina* (Lee *et al.*, 2007) have subsequently been transferred to the genus *Chryseobacterium* (Kämpfer *et al.*, 2009).

Recently, the bacterial diversity of sediments collected from an industrially polluted site at Estarreja in northern Portugal was investigated. Sediments at the site have high

levels of contamination, especially by heavy metals (Costa & Jesus-Ryding, 2001; Oliveira *et al.*, 2001; Carvalho *et al.*, 2002). Two different sampling occasions yielded two unidentified organisms that were related phylogenetically to the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). In the present study, a detailed classification of the two strains is provided on the basis of a polyphasic study, including analysis of morphological and physiological characteristics, cellular fatty acid profiling, DNA–DNA hybridization experiments and phylogenetic analysis of 16S rRNA gene sequences. On the basis of the results obtained, strains 3A10^T and ECP37^T are considered to represent two novel species of the genus *Chryseobacterium*.

Sediment samples were serially diluted in saline solution (0.85 %, w/v, NaCl) and were inoculated on trypticase soy agar (TSA; Oxoid), adjusted to pH 7.0, at 30 °C. Strains

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 3A10^T and ECP37^T are EU360967 and EU360966, respectively.

3A10^T and ECP37^T, selected on the basis of colony morphology and colour, were purified by subculturing and were preserved at -80 °C in modified Luria-Bertani broth (Tiago *et al.*, 2004), supplemented with 15 % (v/v) glycerol. Unless stated otherwise, all morphological and tolerance tests were performed by using TSA.

Cell morphology and gliding motility were examined by phase-contrast microscopy. Flagellar motility was tested as described by Alexander & Strete (2001). Gram staining and catalase and cytochrome oxidase tests were performed as described by Murray *et al.* (1994) and Smibert & Krieg (1994). Cell size was determined by using a Leica DM4000B light microscope equipped with Leica Applied Suite software. Presence of flexirubin pigments was investigated by using the bathochromatic test with a 20 % (w/v) KOH solution as described by Bernardet *et al.* (2002).

Phenotypic tests listed below were performed on strains 3A10^T and ECP37^T and on their closest phylogenetic neighbours, namely *Chryseobacterium marinum* NBRC 103143^T, *Chryseobacterium antarcticum* AT1013^T and *Chryseobacterium jeonii* AT1047^T. The pH range for growth was determined in buffered trypticase soy broth (TSB) adjusted to pH 3–10 (at 1 pH unit intervals). Turbidity of the cultures grown in an orbital shaker at 25 °C was measured at 610 nm. All buffer solutions used to adjust the pH of TSB were prepared from 1 M stock solutions according to Gomori (1990). Citrate buffer was used for pH 3–6, phosphate buffer for pH 7, Tris buffer for pH 8, and a carbonate-bicarbonate buffer for pH 9 and 10. Growth temperature ranges were determined on TSA incubated at 4, 10, 15, 20, 25, 30, 37 and 50 °C.

Growth in the presence of 0–20 % (w/v) NaCl (at 1 % intervals up to 10 %, then at 12, 15 and 20 %) was examined in TSB adjusted to pH 7 and incubated at 25 °C. The ability to grow under anaerobic conditions was evaluated by incubating TSA plates in an anaerobic jar (with 9–13 % CO₂) in an oxygen-deprived atmosphere by using AnaeroGen (Oxoid) at 25 °C for 7 days.

Acid production from carbohydrates was examined in API 50 CH test strips by using API 50 CHB/E medium (bioMérieux) according to the manufacturer's instructions. Single carbon source assimilation was also determined in API 50 CH test strips (bioMérieux) by using cells suspended in 0.1 M phosphate buffer (pH 7) supplemented with 0.7 % yeast nitrogen base (Difco) and 0.05 % NH₄Cl (Tiago *et al.*, 2005, 2006). Bacterial cells were suspended in sterilized water to reach a turbidity corresponding to McFarland No. 6 standard. Cell suspensions (3 ml) were then added to 60 ml medium and inoculated in the API 50 CH test strip wells, according to the manufacturer's recommendations (bioMérieux). Nitrate reduction, indole production and the presence of β -galactosidase, L-arginine dihydrolase and urease activities were determined by using API 20 NE strips and API AUX medium (bioMérieux). All the above results were recorded

after 24, 48, 72 and 120 h incubation at 25 °C. Hydrolysis of arbutin, gelatin, casein, Tweens 20 and 80 and starch was tested on TSA as described by Hudson *et al.* (1986) and Smibert & Krieg (1994). Antibiotic susceptibility was examined on TSA at 25 °C for 72 h with Oxoid discs (see species descriptions for details) following the interpretation criteria proposed by the Comité de l'Antibiogramme de la Société Française de Microbiologie (1998). The phenotypic characteristics of strains 3A10^T and ECP37^T are given in Table 1 and in the species descriptions.

Genomic DNA for determination of the G + C content was obtained as described by Cashion *et al.* (1977). G + C ratios were estimated by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) by HPLC as described by Mesbah *et al.* (1989). Fatty acid methyl esters were obtained from fresh wet biomass, grown on TSA at 28 °C for 24 h, by saponification, methylation and extraction as described by Kuykendall *et al.* (1988), and the fatty acids were separated, identified and quantified according to the protocol of the Microbial Identification System, Sherlock version 4.6 (MIS-MIDI). The closest phylogenetic neighbours of strains 3A10^T and ECP37^T were not included in the fatty acid analysis because they could not be grown under the same conditions as the new isolates. Analysis of respiratory quinones was also carried out at the DSMZ.

The major respiratory lipoquinone of strains 3A10^T and ECP37^T was menaquinone 6 (MK-6), which is in accordance with all recognized members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2006). The fatty acid profile of strains 3A10^T and ECP37^T, like those of recognized species of the genus *Chryseobacterium*, was dominated by branched components, namely iso-C_{15:0}, iso-C_{17:1}ω9c, anteiso-C_{15:0} and iso-C_{17:0} 3-OH. The detailed fatty acid profiles of strains 3A10^T and ECP37^T are compared with those reported in the literature for related *Chryseobacterium* species (Yi *et al.*, 2005; Lee *et al.*, 2007) in Table 2. The G + C content of the DNA of strains 3A10^T and ECP37^T was 43 and 34 mol%, respectively. The DNA G + C content of strain ECP37^T is in line with those reported for recognized *Chryseobacterium* species (Yi *et al.*, 2005; Bernardet *et al.*, 2006; Lee *et al.*, 2007), but that of strain 3A10^T is significantly higher (Bernardet *et al.*, 2006), being similar to that of *Kaistella koreensis* (Kim *et al.*, 2004).

Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey *et al.* (1996). Cloning of the amplicons into pGEM T-Easy vector (Promega) and cycle sequencing were performed at MacroGen Inc. (Seoul, Republic of Korea) by using universal bacterial 16S rRNA primers (f27, f357, f519, f1114, r519, r800, r1056, r1492) (Lane, 1991). For phylogenetic analyses, the sequences were aligned by using the BioEdit program (version 7.0.5.3) (Hall, 1999) and

Table 1. Differential characteristics between strains 3A10^T and ECP37^T and related species of the genus *Chryseobacterium*

Strains: 1, 3A10^T; 2, ECP37^T; 3, *C. marinum* NBRC 103143^T; 4, *C. antarcticum* AT1013^T; 5, *C. jeonii* AT1047^T. All data are from the present study. +, Positive; –, negative; w, weakly positive. Data in parentheses were reported in the original descriptions of the species.

Characteristic	1	2	3	4	5
Colony pigmentation on TSA	Yellow	Yellow	Yellow	Pale yellow	Yellow
Cell length (µm)	1.5–2.1	1.6–2.5	0.6–1.4	1.3–3.1	1.0–2.9
Cell width (µm)	0.6	0.5–0.6	0.5–0.7	0.4–0.6	0.4–0.5
Catalase	+	w	+	+	+
Oxidase	+	+	–	+	+
Growth temperature (°C)					
Range	10–37	4–37	5–25	5–25	5–25
Optimum	30	25–30	15	20	20
pH for growth					
Range	6–9	6–9	7–9	6–10	6–10
Optimum	7	7–8	7	7–8	7–8
Growth with NaCl (% w/v)					
Range	0–6	0–7	0–3	0–3	0–2
Optimum	2–3	2	0–1	1	0
Indole production	–	–	–	+	– (+)
Hydrolysis of:					
Casein	+	+	–	– (+)	w
Starch	+	+	+	w	+
Gelatin	+	+	+	– (+)	– (+)
Arbutin	+	+	–	–	–
Tween 20	–	–	+	–	+
Tween 80	–	–	– (+)	– (+)	+
Acid production from:					
Galactose	w	–	+	– (+)	–
Lactose	–	w	–	–	–
Gluconate	–	–	+	–	–
L-Arabinol	–	–	+	–	–
Cellobiose	–	+	–	–	–
D-Mannose	w	–	–	–	– (+)
Starch	w	+	+	+	+
Glycogen	–	+	–	+	+
Amygdalin	–	+	–	–	–
Arbutin	–	w	–	–	–
Salicin	–	w	–	–	–
5-Ketogluconate	–	–	+	–	–
Xylitol	–	w	–	–	–
Gentiobiose	–	+	–	–	–
DNA G + C content (mol%)	43	34	35	34	36

were analysed via the DNAML, SEQBOOT, DNAPARS, DNADIST (Kimura's two-parameter correction), NEIGHBOR, FITCH and CONSENSE programs of the PHYLIP package (Felsenstein, 1995). 16S rRNA gene sequences of other members of the family *Flavobacteriaceae* were obtained from the NCBI database (Benson *et al.*, 2007). A manually corrected and degapped alignment of 31 sequences of 1249 nt was used. The robustness of the phylogenetic tree was confirmed by using bootstrap analysis based on 100 resamplings of the sequences (1000 for the neighbour-joining analysis). Non-homologous and ambiguous nucleotide positions were excluded from the calculations. In the neighbour-joining phylogenetic tree (Fig. 1), strains 3A10^T and ECP37^T

formed a monophyletic group with *C. antarcticum* AT1013^T, *C. jeonii* AT1047^T and *C. marinum* NBRC 103143^T supported by high bootstrap values (82–99%). The other phylogenetic trees showed essentially the same topology (not shown). These results show that the two novel strains belong to the genus *Chryseobacterium*. Strain 3A10^T showed 16S rRNA gene sequence similarities of 97.2 and 96.6% to the type strains of *C. antarcticum* and *C. jeonii*, respectively, while strain ECP37^T showed 97.3% similarity to the type strain of *C. marinum*.

For DNA–DNA hybridization experiments, the genomic DNA of strains 3A10^T and ECP37^T was hybridized and

Table 2. Fatty acid contents of strains 3A10^T and ECP37^T and the type strains of related *Chryseobacterium* species

Strains: 1, 3A10^T; 2, ECP37^T; 3, *C. marinum* NBRC 103143^T (data from Lee *et al.*, 2007); 4, *C. antarcticum* AT1013^T (Yi *et al.*, 2005); 5, *C. jeonii* AT1047^T (Yi *et al.*, 2005). Not all strains were cultivated under the same conditions. Values are percentages of the total fatty acids. Cellular fatty acids that amount to <1 % of the total fatty acid content in all strains are not shown. –, Not detected; tr, trace (<0.5 %).

Fatty acid	1	2	3	4	5
iso-C _{12:0}	–	–	–	0.5	1.0
iso-C _{13:0}	3.1	5.8	0.6	2.5	2.9
anteiso-C _{13:0}	tr	tr	tr	tr	tr
iso-C _{14:0}	0.9	0.7	0.8	1.5	5.0
C _{15:0}	–	–	–	2.6	1.5
C _{15:0} 2-OH	0.8	1.0	2.9	1.9	1.9
iso-C _{15:0}	40.0	45.0	17.0	13.6	12.2
iso-C _{15:0} 3-OH	2.0	6.4	3.2	1.0	1.3
anteiso-C _{15:0}	17.0	8.0	33.8	15.2	24.2
anteiso-C _{15:1}	–	–	–	6.6	–
iso-C _{16:0}	tr	tr	0.6	2.8	5.7
iso-C _{16:0} 3-OH	1.4	1.0	2.9	5.1	9.0
iso-C _{16:1} H	tr	–	2.3	3.6	9.1
C _{16:1} ω5c	tr	tr	–	–	–
C _{17:0} 2-OH	0.8	0.5	5.9	3.3	2.3
iso-C _{17:0} 3-OH	6.0	10.8	5.0	5.6	4.4
iso-C _{17:1} ω9c	18.7	8.7	8.8	21.3	8.9
anteiso-C _{17:1} ω9c	tr	–	2.3	2.5	1.9
C _{18:1} ω5c	1.5	1.1	1.5	1.5	0.8
Unknown 11.543*	tr	tr	–	–	–
Unknown 13.565*	0.7	1.4	–	0.7	0.4
Unknown 16.582*	0.7	1.0	–	0.5	tr
Summed feature 3†	3.8	6.8	–	–	–
Summed feature 4†	0.5	0.6	–	2.7	2.6

*Unknown fatty acid; numbers indicate equivalent chain-length.

†Summed features are groups of two or three fatty acids that cannot be reliably separated by GLC with the MIDI system. Summed feature 3 comprised iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; summed feature 4 comprised iso-C_{17:1} I and/or anteiso-C_{17:1} B.

each was hybridized with the DNA of the type strains of their closest phylogenetic neighbours, namely *C. antarcticum* AT1013^T, *C. jeonii* AT1047^T and *C. marinum* NBRC 103143^T. DNA–DNA hybridization was performed at the DSMZ, as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983), by using a model Cary 100 Bio UV/visual spectrometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). DNA was isolated by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977).

DNA–DNA hybridization experiments revealed levels of genomic relatedness of 15.2, 29.5 and 21.8 % between

strain 3A10^T and *C. marinum* NBRC 103143^T, *C. antarcticum* AT1013^T and *C. jeonii* AT1047^T, respectively. Levels of DNA–DNA relatedness between strain ECP37^T and *C. marinum* NBRC 103143^T, *C. antarcticum* AT1013^T and *C. jeonii* AT1047^T were 10.1, 11.0 and 7.4 %, respectively. DNA–DNA relatedness between strains 3A10^T and ECP37^T was 19.8 %.

The data presented herein demonstrate that strains 3A10^T and ECP37^T represent two novel species of the genus *Chryseobacterium*, for which the names *Chryseobacterium palustre* sp. nov. and *Chryseobacterium humi* sp. nov., respectively, are proposed. The new isolates can be differentiated from closely related species based on a combination of phenotypic features (Tables 1 and 2).

Description of *Chryseobacterium palustre* sp. nov.

Chryseobacterium palustre (pa.lus'tre. L. neut. adj. *palustre* pertaining to a marsh).

Cells are Gram-staining-negative, aerobic, chemoheterotrophic rods (1.5–2.1 µm long and 0.6 µm in diameter). No flagellar or gliding motility. Colonies grown on TSA for 3 days are 0.3–1.2 mm in diameter, circular with regular edges and yellow. Flexirubin-type pigments are absent. Oxidase- and catalase-positive. Growth occurs at 10–37 °C (optimum, about 30 °C), at pH 6.0–9.0 (optimum, pH 7) and in the presence of 0–6.0 % NaCl (optimum, 2–3 %). Nitrite and nitrate are not reduced. Casein, gelatin, arbutin, aesculin and starch are hydrolysed. Positive for the Voges–Proskauer reaction (API 20NE). Urease and β-galactosidase activities are absent. Assimilates D-glucose, L-arabinose, D-mannose, N-acetylglucosamine, maltose, adipic acid, malic acid, trisodium citrate, L-xylose, D-fructose, L-sorbose, L-rhamnose, inositol, D-mannitol, D-sorbitol, lactose, sucrose, glycogen, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Acid is produced from galactose, D-glucose, D-mannose, maltose and starch. Assimilation or acid production is negative for the other carbon sources in the API 50CH and API 20NE strips. The type strain is resistant to discs containing penicillin G (10 µg), polymyxin B (300 µg), gentamicin (10 µg), sulfamethoxazole (25 µg), colistin sulfate (50 µg) and meticillin (5 µg), but susceptible to erythromycin (15 µg), ceftazidime (30 µg), cephalothin (30 µg), tetracycline (30 µg), amoxicillin (25 µg), ciprofloxacin (5 µg), ticarcillin (75 µg), sulfamethoxazole/trimethoprim (1.25/23.75 µg), vancomycin (30 µg), meropenem (10 µg), streptomycin (10 µg), lincomycin (2 µg), rifampicin (30 µg), cefoxitin (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg) and ampicillin (10 µg). The major respiratory lipoquinone is MK-6. The major cellular fatty acids (≥6 % of the total) are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{17:1}ω9c and iso-C_{17:0} 3-OH. Other cellular fatty acids are listed in Table 2. The G+C content of the genomic DNA of the type strain is 43 mol%.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains 3A10^T and ECP37^T and representative members of the family *Flavobacteriaceae*. Bootstrap values >80% are shown at nodes. The sequence of *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

The type strain, 3A10^T (=LMG 24685^T =NBRC 104928^T), was isolated from a rhizosphere sediment sample collected near a stream at a polluted site located in the industrial complex of Estarreja, northern Portugal.

Description of *Chryseobacterium humi* sp. nov.

Chryseobacterium humi (hu'mi. L. gen. n. *humi* of earth, soil).

Cells are Gram-staining-negative, aerobic, chemoheterotrophic rods (1.6–2.5 µm long and 0.5–0.6 µm in diameter). No flagellar or gliding motility. Colonies grown on TSA for 3 days are 0.2–1.4 mm in diameter, circular with regular edges and yellow. Flexirubin-type pigments are absent. Oxidase- and catalase-positive. Growth occurs at 4–37 °C (optimum, 25–30 °C), at pH 6.0–9.0 (optimum, pH 7.0–8.0) and in the presence of 0–7.0% NaCl (optimum, 2%). Nitrite and nitrate are not reduced. Casein, gelatin, arbutin, aesculin and starch are hydrolysed. Positive for the Voges–Proskauer reaction (API 20NE). Urease and β-galactosidase activities are absent. Assimilates D-glucose, L-arabinose, D-mannose, maltose, potassium gluconate, amygdalin, cellobiose, maltose, glycogen and β-gentiobiose. Acid is produced from lactose, D-glucose, cellobiose, starch, glycogen, amygdalin, arbutin, salicin, xylitol and gentiobiose. Assimilation or acid production is negative for the other carbon sources in the API 50CH and API 20NE strips. The type strain is resistant to discs containing sulfamethoxazole (25 µg), vancomycin (30 µg), meticillin (5 µg), gentamicin (10 µg), polymyxin B (300 µg) and penicillin G (10 µg), but susceptible to erythromycin (15 µg), ceftazidime (30 µg), cephalothin (30 µg), tetracycline (30 µg), amoxicillin (25 µg), cipro-

floxacin (5 µg), colistin sulfate (50 µg), ticarcillin (75 µg), sulfamethoxazole/trimethoprim (1.25/23.75 µg), meropenem (10 µg), streptomycin (10 µg), lincomycin (2 µg), rifampicin (30 µg), cefoxitin (30 µg), cephalothin (30 µg), amoxicillin/clavulanic acid (20/10 µg), chloramphenicol (30 µg) and ampicillin (10 µg). The major respiratory lipoquinone is MK-6. The major cellular fatty acids (≥8% of the total) are iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{17:1} ω⁹c and anteiso-C_{15:0}. Other cellular fatty acids are listed in Table 2. The G+C content of the genomic DNA of the type strain is 34 mol%.

The type strain, ECP37^T (=LMG 24684^T =NBRC 104927^T), was isolated from a soil sample collected at a polluted site located in the industrial complex of Estarreja, northern Portugal.

Acknowledgements

C. P. and M. F. C. acknowledge research grants from Fundação para a Ciência e a Tecnologia (FCT), Portugal (SFRH/BD/25493/2005 and SFRH/BPD/44670/2008, respectively), and FCT projects POCI/AMB/60131/2004 and POCI/V.5/A0105/2005. We are indebted to Milton da Costa (University of Coimbra, Coimbra, Portugal) and to Fernanda Nobre for help with fatty acid analysis. We also thank the Unit of Research and Development of Nephrology (Faculty of Medicine, University of Porto, Porto, Portugal) for help with cell measurements.

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