Chryseobacterium culicis sp. nov., isolated from the midgut of the mosquito *Culex quinquefasciatus*

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A yellow-pigmented bacterial strain, R4-1A^T, isolated from the midgut of the mosquito *Culex quinquefasciatus* (a vector of lymphatic filariasis), was studied using a polyphasic approach. Cells of the isolate were rod-shaped and stained Gram-negative. A comparison of the 16S rRNA gene sequence of this organism with sequences of type strains of the most closely related species clearly showed an allocation to the genus *Chryseobacterium*, with the highest sequence similarities (all 97.9%) to *Chryseobacterium jejuense* JS17-8^T, *C. indologenes* ATCC 29897^T, *C. arthrosphaerae* CC-VM-7^T and *C. aquifrigidense* CW9^T. 16S rRNA gene sequence similarities to type strains of other *Chryseobacterium* species were below 97.5%. The fatty acid profile of strain R4-1A^T included the major fatty acids iso-15:0, summed feature 4 (comprising iso-15:0 2-OH and/or 16:1 ω 7c), iso-17:1 ω 9c and iso-17:0 3-OH. DNA–DNA hybridizations with *C. jejuense* KACC 12501^T, *C. indologenes* CCUG 14556^T, *C. arthrosphaerae* CC-VM-7^T and *C. aquifrigidense* KCTC 12894^T resulted in relatedness values of 38.3% (reciprocal 30.5%), 29.4% (32.1%), 23.2% (37.2%) and 29.5% (47.1%), respectively. These results and the differentiating biochemical and chemotaxonomic properties show that strain R4-1A^T represents a novel species, for which the name *Chryseobacterium culicis* sp. nov. is proposed. The type strain is R4-1A^T (=LMG 25442^T =CCM 7716^T).

The genus *Chryseobacterium* was described by Vandamme *et al.* (1994) and at that time contained only five species, *Chryseobacterium gleum* (the type species), *C. balustinum*, *C. indologenes*, *C. indoltheticum* and *C. scophthalmum*. The number of species descriptions has since increased tremendously, and more than 30 species have been described in the last 15 years (e.g. Hugo *et al.*, 2003; Li *et al.*, 2003; de Beer *et al.*, 2005; Shen *et al.*, 2005; Shimomura *et al.*, 2005; Young *et al.*, 2005; Park *et al.*, 2006; Tai *et al.*, 2006; Herzog *et al.*, 2008; Behrendt *et al.*, 2008; Weon *et al.*, 2008; Szoboszlay *et al.*, 2008; Hantsis-Zacharov *et al.*, 2008; Kim *et al.*, 2005, 2008; Kämpfer *et al.*, 2009, 2010). Representatives of the genus *Chryseobacterium* can be found in a wide variety of samples, including environmental but also clinical sources (Bernardet *et al.*, 2006).

A yellow-pigmented strain from the midgut of the mosquito *Culex quinquefasciatus* Say was isolated on

tryptic soy agar (TSA; Oxoid). This strain, R4-1A^T, was maintained and subcultivated on nutrient agar (NA) at 30 °C for 48 h and subsequently analysed for its 16S rRNA gene sequence, fatty acid methyl ester composition of the whole cell hydrolysate, further phenotypic characteristics and DNA–DNA relatedness to the type strains of those species most closely related on the basis of 16S rRNA gene sequence similarity.

Cultural and morphological characteristics were observed on NA. The Gram reaction was tested by the modified method of Gerhardt *et al.* (1994) and motility was assessed under the light microscope on cells grown for 3 days in nutrient broth (NB; Oxoid) at 30 °C. Growth was investigated at 4, 11, 30, 37 and 45 °C in NB. Salt tolerance was also investigated at 0.5–8.0% (w/v) NaCl in tryptic soy broth.

Strain R4-1A^T showed Gram-negative staining behaviour and formed visible (diameter about 2 mm) yellowish colonies within 48 h at 30 °C. No growth was observed below 5 °C or above 37 °C. Strain R4-1A^T grew in the presence of up to 3.0% NaCl. Colonies were translucent

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R4-1A^T is FN554975.

and shiny with entire edges but, after prolonged incubation, the colonies were not visible as single entities, probably because of the profuse production of extracellular substances. A bright-yellow pigment of the flexirubin type (KOH method according to Reichenbach, 1989) was produced on NA. Oxidase activity was tested using the bioMérieux oxidase reagent according to the instructions of the manufacturer; strain R4-1A^T showed a positive oxidase reaction. Cells were non-motile, non-spore-forming rods (1 µm wide, 2 µm long). Strain R4-1A^T grew well on NA, brain heart infusion agar and TSA and not on MacConkey agar (Oxoid).

Physiological characterization and additional biochemical tests were performed to assess the carbon source utilization pattern and the hydrolysis of chromogenic substrates was investigated as described by Kämpfer *et al.* (1991). The following additional biochemical tests were performed: production of hydrogen sulphide, using lead acetate paper and triple-sugar-iron methods, indole reaction with Ehrlich's and Kovács' reagent, activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, DNase (Oxoid CM321; supplemented with 0.01 % toluidine blue), β -galactosidase (ONPG) and urease on Christensen's urea agar and hydrolysis of casein, gelatin (plate method), starch and tyrosine (Smibert & Krieg, 1994). Strain R4-1A^T utilized few carbon sources, similar to all *Chryseobacterium* species, but was able to hydrolyse many chromogenic substrates. Urease, indole production and hydrolysis of casein, gelatin, starch and tyrosine were positive, while hydrogen sulphide production and activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β -galactosidase (ONPG) were negative. The results of biochemical and physiological tests are given in Table 1 and in the species description.

Table 1. Comparison of characteristics of strain R4-1A^T with those of the type strains of the most closely related *Chryseobacterium* species

Strains: 1, *Chryseobacterium culicis* sp. nov. R4-1A^T; 2, *C. jejuense* KACC 12501^T; 3, *C. indologenes* CCUG 14556^T; 4, *C. aquifrigidense* KCTC 12894^T; 5, *C. arthrosphaerae* CC-VM-7^T (data from Kämpfer *et al.*, 2010). Data were obtained in this study unless indicated. +, Positive; (+), weakly positive; –, negative. All strains were able to produce acid from D-glucose and maltose, hydrolysed aesculin and produced indole from tryptophan. None of the strains produced acid from D-fructose, sucrose, L-arabinose, lactose or D-xylose.

Characteristic	1	2	3	4	5
Growth on MacConkey agar	–	+	+	+	–
Acid production from:					
D-Mannitol	+	–	–	–	(+)
Trehalose	+	+	+	+	–
Nitrate reduction	–	–	+	–	+
Urease activity	+	+	–	+	+

Cellular fatty acid analysis of whole-cell hydrolysates was performed according to Kämpfer & Kroppenstedt (1996) and revealed that the following fatty acids were most abundant: iso-15:0, summed feature 4 (iso-15:0 2-OH and/or 16:1 ω 7c), iso-17:1 ω 9c and iso-17:0 3-OH. The complete fatty acid pattern of strain R4-1A^T is shown in Table 2 in comparison with those of the type strains of the most closely related *Chryseobacterium* species.

The 16S rRNA gene of strain R4-1A^T was sequenced as described by Kämpfer *et al.* (2003). Subsequent analysis was performed using the ARB software package (Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 95 database (version July 2008; Pruesse *et al.*, 2007) as well as the software package MEGA version 4.0 (Tamura *et al.*, 2007) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). The sequenced length of the 16S rRNA gene was 1387 bp. Nucleotide sequence

Table 2. Long-chain fatty-acid compositions of strain R4-1A^T and type strains of related *Chryseobacterium* species

Strains: 1, *C. culicis* sp. nov. R4-1A^T; 2, *C. jejuense* KACC 12501^T; 3, *C. indologenes* CCUG 14556^T; 4, *C. aquifrigidense* KCTC 12894^T; 5, *C. arthrosphaerae* CC-VM-7^T. Values are percentages of total fatty acids; components amounting to less than 1 % of the total fatty acids in all strains are not included. tr, Trace (less than 1.0 %); ND, not detected. Data in column 5 were taken from Kämpfer *et al.* (2010) and data in parentheses in column 3 (obtained from cells grown on TSA at 20 °C for 24h) are means $\pm$ SD taken from a survey of 45 strains (Hugo *et al.*, 2003); other data were obtained in this study.

Fatty acid	1	2	3	4	5
iso-13:0	tr	1.0	ND (tr)	ND	tr
Unknown 13.566*	8.7	8.5	13.1 (21 $\pm$ 0.7)	4.2	20.5
iso-15:0	33.9	31.2	28.5 (34.3 $\pm$ 4.9)	36.2	32.3
anteiso-15:0	tr	tr	ND	ND	ND
iso-15:0 3-OH	2.5	2.5	2.1 (2.6 $\pm$ 0.2)	3.0	1.0
iso-15:1 F	tr	tr	3.4 (ND)	ND	4.6
16:0	1.8	1.7	1.2 (tr)	1.8	1.6
15:0 3-OH	1.0	ND	ND	ND	ND
16:0 3-OH	1.3	1.3	tr (1.0 $\pm$ 0.2)	2.3	ND
Unknown 16.580*	1.3	1.2	1.3 (1.7 $\pm$ 0.2)	1.2	1.7
17:0 2-OH	ND	ND	ND (ND)	ND	2.0
iso-17:0	1.3	1.5	1.1 (tr)	1.0	2.0
iso-17:0 3-OH	15.5	15.4	14.4 (19.2 $\pm$ 1.8)	18.2	3.5
iso-17:1 ω 9c	20.7	21.9	23.4 (24.2 $\pm$ 3.1)	17.3	18.9
Summed feature 4†	10.0	11.0	9.8 (11.1 $\pm$ 1.3)	13.8	5.5
Summed feature 5†	ND	ND	tr (tr)	ND	1.0

*Unknown fatty acids; numbers indicate the equivalent chain-length.

†Fatty acids that could not be separated by GC using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 4 contains iso-15:0 2-OH and/or 16:1 ω 7c. Summed feature 5 contains iso-17:1 I and/or anteiso-17:1 B.

distances were calculated (distance options according to the Kimura-2 model) using the software package MEGA version 4.0 (Tamura *et al.*, 2007).

The tree shown in Fig. 1 is based on a maximum-likelihood reconstruction (only alignment columns in which the frequency of the most abundant nucleotide was $\geq 50\%$ were included in the calculation). The topologies of the neighbour-joining and maximum-parsimony trees were congruent (not shown). Strain R4-1A^T clearly grouped most closely with *Chryseobacterium jejuense* JS17-8^T.

According to the sequence similarity calculations, the type strains most closely related to strain R4-1A^T were *C. jejuense* JS17-8^T, *Chryseobacterium indologenes* ATCC

29897^T, *Chryseobacterium aquifrigidense* CW9^T and *Chryseobacterium arthrosphaerae* CC-VM-7^T, all showing 97.9% sequence similarity, while sequence similarities were below 97.5% for the type strains of other *Chryseobacterium* species.

DNA–DNA hybridization experiments were performed between strain R4-1A^T and the type strains of the four most closely related *Chryseobacterium* species according to the method of Ziemke *et al.* (1998) (except that, for nick translation, 2 µg DNA was labelled for 3 h at 15 °C). Strain R4-1A^T showed relatively low DNA–DNA hybridization to *C. jejuense* KACC 12501^T (38.3%; reciprocal 30.5%), *C. indologenes* CCUG 14556^T (29.4/32.1%), *C. aquifrigidense*

Fig. 1. Phylogenetic analysis of strains most closely related to strain R4-1A^T based on 16S rRNA gene sequences available from the EMBL database (accession numbers in parentheses). The tree was constructed using the ARB software package (version December 2007; Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 95 database (version July 2008; Pruesse *et al.*, 2007). The sequence of *Flexibacter aggregans* ATCC 23190 was used as an outgroup. Tree building was performed using the maximum-likelihood method with fastDNAmI (Olsen *et al.*, 1994). Bar, 0.10 substitutions per nucleotide position.

KCTC 12894^T (23.2/37.2 %) and *C. arthrosphaerae* CC-VM-7^T (29.5/47.1 %).

On the basis of the results of this polyphasic study, it is clear that strain R4-1A^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium culicis* sp. nov. is proposed.

Description of *Chryseobacterium culicis* sp. nov.

Chryseobacterium culicis [cu.li'cis. L. n. *culex* -icis a gnat, a midge, and also a genus of mosquito (*Culex*, family Culicidae); N.L. gen. n. *culicis* of *Culex*, referring to the isolation of the type strain from *Culex quinquefasciatus*].

Cells are Gram-staining-negative, non-motile, non-spore-forming rods, approx. 1 µm wide and 2 µm long. Aerobic, oxidase-positive and catalase-positive. Good growth occurs at 11–36 °C after 48 h on NA, brain heart infusion agar and TSA (all Oxoid). No growth on MacConkey agar (Oxoid) at 28 °C. The type strain is unable to grow at 4 or 45 °C. Grows in the presence of 0.5–3.0 % NaCl. Colonies on NA are smooth, yellowish, circular, translucent and shiny with entire edges. Colonies become mucoid and cannot be identified as single entities after prolonged incubation. The yellow pigment of the flexirubin type is non-diffusible and non-fluorescent. Produces acid weakly from D-glucose, trehalose and D-mannitol. No acid is produced from adonitol, L-arabinose, D-arabitol, dulcitol, erythritol, myo-inositol, lactose, maltose, melibiose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose or D-xylose. Urease activity, indole production and hydrolysis of casein, gelatin, starch, DNA and tyrosine are positive, while hydrogen sulphide production and activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β-galactosidase (ONPG) are negative. D-Glucose, maltose, D-mannose, D-fructose and trehalose are utilized as sole sources of carbon. The following compounds are not utilized as sole sources of carbon: acetate, propionate, N-acetylgalactosamine, N-acetylglucosamine, L-arabinose, L-arbutin, cellobiose, D-galactose, gluconate, glycerol, D-mannitol, maltitol, melibiose, L-rhamnose, D-ribose, sucrose, salicin, D-xylose, adonitol, myo-inositol, D-sorbitol, putrescine, *cis*- and *trans*-aconitate, 4-aminobutyrate, adipate, azelate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, 2-oxoglutarate, pyruvate, suberate, citrate, mesaconate, L-alanine, β-alanine, L-ornithine, L-phenylalanine, L-serine, L-aspartate, L-histidine, L-leucine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The chromogenic substrates *p*-nitrophenyl (pNP) α-D-glucopyranoside, pNP β-D-glucopyranoside (weak), pNP β-D-galactopyranoside, bis-pNP phosphate, bis-pNP phenylphosphonate, bis-pNP phosphorylcholine, 2-deoxythymidine-2'-pNP phosphate, L-alanine *p*-nitroanilide (pNA), γ-L-glutamate pNA and L-proline pNA are hydrolysed. pNP β-D-xylopyranoside and pNP β-D-glucuronide are not hydrolysed. The major cellular fatty acids (≥ 10 %) are iso-15:0, summed feature 4

(iso-15:0 2-OH and/or 16:1ω7c), iso-17:1ω9c and iso-17:0 3-OH.

The type strain is R4-1A^T (=LMG 25442^T =CCM 7716^T), isolated from the midgut of the mosquito *Culex quinquefasciatus* (a vector of lymphatic filariasis) collected from the filarial endemic region of Raipur (Chattishgarh province), India.

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