Elizabethkingia anophelis sp. nov., isolated from the midgut of the mosquito *Anopheles gambiae*

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The taxonomic position, growth characteristics and antibiotic resistance properties of a slightly yellow-pigmented bacterial strain, designated R26^T, isolated from the midgut of the mosquito *Anopheles gambiae*, were studied. The isolate produced rod-shaped cells, which stained Gram-negative. The bacterium had two growth optima at 30–31 °C and 37 °C. Strain R26^T demonstrated natural antibiotic resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline. 16S rRNA gene sequence analysis revealed that the isolate showed 98.6 % sequence similarity to that of *Elizabethkingia meningoseptica* ATCC 13253^T and 98.2 % similarity to that of *Elizabethkingia miricola* GTC 862^T. The major fatty acids of strain R26^T were iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1ω7c/t}). Strain R26^T contained only menaquinone MK-6 and showed a complex polar lipid profile consisting of diphosphatidylglycerol, phosphatidylinositol, an unknown phospholipid and unknown polar lipids and glycolipids. DNA–DNA hybridization experiments with *E. meningoseptica* CCUG 214^T (=ATCC 13253^T) and *E. miricola* KCTC 12492^T (=GTC 862^T) gave relatedness values of 34.5 % (reciprocal 41.5 %) and 35.0 % (reciprocal 25.7 %), respectively. DNA–DNA hybridization results and some differentiating biochemical properties indicate that strain R26^T represents a novel species, for which the name *Elizabethkingia anophelis* sp. nov. is proposed. The type strain is R26^T (=CCUG 60038^T =CCM 7804^T).

The genus *Elizabethkingia* was proposed by Kim *et al.* (2005) for the two species *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* mainly on the basis of 16S rRNA gene sequence similarity studies. Strains of these species represent a separate lineage from the type strains of the *Chryseobacterium*–*Bergeyella*–*Riemerella* branch within the family *Flavobacteriaceae* (90.7–93.9 % similarities), which is also supported by phenotypic differences. Since 2005, no further species of this genus have been proposed. Representatives of the genus *Elizabethkingia* can be found in a wide variety of samples, including clinical sources (Bernardet *et al.*, 2006).

During the characterization of organisms from the midgut of *Anopheles gambiae* G3 originating from McCarthy Island,

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive extragenic palindromic-PCR.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain R26^T is EF426434.

The Gambia, a bacterial isolate was commonly recovered on Luria–Bertani (LB) agar containing ampicillin (100 µg ml^{−1}) at 37 °C (Lindh *et al.*, 2008) and was subsequently deposited by Dr William Collins at the Malaria Research Reference Resource Centre managed by the ATCC (MR4/ATCC). This bacterium was identified in 40 adults and two pupae of *A. gambiae*. Initial 16S rRNA gene sequencing and BLAST comparison showed that the isolate was most closely related to *Elizabethkingia meningoseptica* strains (GenBank accession nos EF426425–EF426434; Lindh *et al.*, 2008). Several other laboratories have since reported the isolation of bacteria from the gut of mosquitoes that share 16S rRNA gene sequence similarities of 99–100 % with *Elizabethkingia meningoseptica* (Dong *et al.*, 2009; Kajla *et al.*, 2010). The aim of the present study was to look further at this mosquito inhabitant. One of our isolates, originally designated strain R26^T (Lindh *et al.*, 2008), was selected for further study.

Isolate R26^T was maintained and subcultivated on nutrient agar (NA; Oxoid) 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, quinone and polar lipid patterns, further phenotypic characteristics, and DNA–DNA relatedness to those species most closely related on the basis of 16S rRNA gene sequence similarities. The type strains of *E. meningoseptica* CCUG 214^T and *E. miricola* KCTC 12492^T were studied in parallel.

Growth was investigated at 4, 11, 15, 20, 25, 30, 37, 45 and 50 °C in tryptone soy broth (TSB; Oxoid). Growth optima were determined between 27 and 41 °C in LB medium while shaking at 180 r.p.m. Oxidase activity was tested using the Oxidase reagent (bioMérieux) according to the instructions provided.

Physiological characterization and additional biochemical tests were performed as described previously (Kämpfer *et al.*, 1991, 2010). In addition, the following biochemical tests were performed: the production of hydrogen sulfide using lead acetate paper and triple-sugar-iron methods; the indole reaction with Ehrlich's and Kovacs' reagents; the 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); hydrolysis of casein, gelatin (plate method), starch and tyrosine; citrate utilization; and catalase activity (Smibert & Krieg, 1994). Anaerobic growth was tested in TSB-containing tubes sealed with sterile paraffin oil. All phenotypic tests were performed with strain R26^T and the type strains of *E. meningoseptica* and *E. miricola*; all three strains were maintained and grown under the same conditions.

Table 1. Characteristics differentiating strain R26^T from species of the genus *Elizabethkingia*

Strains: 1, R26^T; 2, *E. meningoseptica* CCUG 214^T; 3, *E. miricola* KCTC 12492^T. +, Positive; v, variable; –, negative. All data are from this study, except data in parentheses, which are from Kim *et al.* (2005). Acid production from arabinose, raffinose, salicin, sucrose and xylose is negative for all strains tested. Acid production from glucose, lactose, maltose, mannitol and trehalose is positive for all strains tested. Only characteristics that differ between the three strains are shown.

Characteristics	1	2*	3
Citrate utilization	–	– (v)	+
Acid production from:			
Cellobiose	+	– (–)	– (–)
Melibiose	–	+	–
Growth on MacConkey agar	–	+	+
Urea hydrolysis	–	– (–)	+

*Bernardet *et al.* (2006) reported that, for *E. meningoseptica*, acid production from cellobiose was found to vary between references and growth on MacConkey agar was strain-dependent. These findings were based on the study of a large number of *E. meningoseptica* strains.

Since *Elizabethkingia meningoseptica* is known to be resistant to several antibiotics and a putative pathogen that can cause meningitis in neonates (Bernardet *et al.*, 2006), the isolate was investigated for antibiotic resistance. MICs were tested in LB medium. The isolate was also tested for haemolytic activity on blood agar plates.

Cellular fatty acid analysis was performed for strain R26^T and the type strains of *E. meningoseptica* and *E. miricola* after extraction of whole cell hydrolysates according to Kämpfer & Kroppenstedt (1996). Menaquinones were extracted and analysed as described by Collins *et al.* (1977) and Groth *et al.* (1996). Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC as described by Collins & Jones (1980). For these analyses, strains were grown on tryptic soy agar (TSA; Difco) at 28 °C for 48 h.

The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003). DNA extraction was carried out using the GenElute Plant Genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions. The 16S rRNA gene was PCR-amplified using the primer pair 27F and 1492R (Lane, 1991) and the following cycle conditions: 95 °C for 3 min, 28 cycles of 94 °C for 1 min, 57.3 °C for 45 s, 72 °C for 2 min, and a final step of 72 °C for 15 min. The PCR product was purified with the QIAquick PCR purification kit

Table 2. Cellular fatty acids (%) of strain R26^T and species of the genus *Elizabethkingia*

Strains: 1, R26^T; 2, *E. meningoseptica* CCUG 214^T; 3, *E. miricola* KCTC 12492^T. All data are from this study, except data in parentheses, which are from Kim *et al.* (2005). Fatty acids (%) are listed using standard abbreviations (no. of carbon atoms: no. of double bonds). tr, Trace (<1.0 %); ECL, equivalent chain-length (i.e. the identity of the fatty acid is unknown). Fatty acids amounting to <1 % total fatty acids in all three strains are not shown.

Fatty acid	1	2*	3†
iso-C _{13:0}	1.8	2.8 (1.3 ± 0.3)	2.1 (2.0 ± 0.5)
ECL 13.566	6.8	6.3 (1.9 ± 0.3)	4.9 (1.5 ± 0.2)
iso-C _{15:0}	45.8	40.8 (43.9 ± 2.0)	44.7 (46.4 ± 2.2)
iso-C _{15:0} 3-OH	2.9	3.1 (2.8 ± 0.3)	3.6 (3.0 ± 0.6)
anteiso-C _{15:0}	1.8	3.9 (1.1 ± 0.8)	1.5 (1.0 ± 0.6)
C _{16:0}	tr	1.8 (tr)	1.1 (1.2 ± 0.1)
C _{16:0} 3-OH	2.1	3.4 (2.6 ± 0.4)	2.4 (3.0 ± 0.6)
iso-C _{16:0} 3-OH	tr	1.3 (tr)	tr (tr)
ECL 16.580	1.2	1.1 (1.6 ± 0.1)	1.4 (1.3 ± 0.6)
iso-C _{17:0} 3-OH	12.8	12.4 (14.6 ± 1.0)	14.6 (15.3 ± 0.2)
iso-C _{17:1} ω9c	4.2	4.0 (7.8 ± 1.3)	4.5 (6.6 ± 0.2)
Summed feature 4‡	15.6	14.2 (19.6 ± 1.0)	15.7 (17.0 ± 1.3)

*Data in parentheses are means ± SD derived from results for five strains.

†Data in parentheses are means ± SD derived from results for two strains.

‡Summed feature 4 comprises iso-C_{15:0} 2-OH and/or C_{16:1} ω7c/t.

(Qiagen) according to the manufacturer's instructions and was sequenced with standard sequencing primers for the 16S rRNA gene (sequence length 1475 bp). Phylogenetic analysis was performed using the ARB software package (December 2007 version; Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 95 database (July 2008 release; Pruesse *et al.*, 2007). Trees were reconstructed using the maximum-likelihood method with fastDNAmI (Olsen *et al.*, 1994) without filters. The almost complete 16S rRNA gene sequences (1312 bp) of the three strains were compared by distance calculations (pairwise distances) using the ARB software package (December 2007 version; Ludwig *et al.*, 2004).

DNA–DNA hybridization experiments were performed between strain R26^T and the type strains of the two species of the genus *Elizabethkingia* according to the method of Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during 3 h of incubation at 15 °C.

Four repetitive extragenic palindromic (rep)-PCRs [ERIC-, REP-, BOX- and (GTG)₅-PCR (Versalovic *et al.*, 1994)] and randomly amplified polymorphic DNA (RAPD)-PCR (Welsh & McClelland 1990; Williams *et al.*, 1990) were tested to compare strain R26^T and the type strains of both species of the genus *Elizabethkingia*. ERIC- and rep-PCR could not be used because amplification products were not observed for all strains. All PCRs were performed in a total volume of 10 µl including 40 ng genomic DNA, 1 × *Taq* buffer with (NH₄)₂SO₄ (Fermentas), 1.5 mM MgCl₂, 1.25 mM dNTPs, 2 µM of each primer, 0.4 µg bovine serum albumin µl⁻¹ and 0.8 U DreamTaq DNA polymerase (Fermentas). rep-PCRs were performed with primers ERIC1R and ERIC2, REP-1R-I and REP-2-I BOXA1R, and (GTG)₅ (Versalovic *et al.*, 1994). PCR conditions for BOX- and (GTG)₅-PCR were as follows: 3 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 53 °C, and 8 min (BOX-PCR) or 3 min [(GTG)₅-PCR] at 70 °C, and finished with 8 min at 70 °C. RAPD-PCR analysis was performed with primer A and PCR conditions as described by Ziemke *et al.* (1997). PCR products were separated on 1.5% agarose gels in 1 × Tris-borate-EDTA buffer for 2.5 h at 2.7 V cm⁻¹, stained with ethidium bromide and documented using a Fluor-S MultiImager (Bio-Rad).

The isolate formed visible, slightly yellowish colonies with a diameter of ~2 mm on NA within 48 h at 30 °C. The Gram reaction was negative as tested by the modified Hucker method according to Gerhardt *et al.* (1994). No motility was observed under the light microscope on cells grown for 3 days in nutrient broth (Oxoid) at 30 °C. The growth optimum in LB medium was observed from 31 to 37 °C with a doubling time of 42 min. No growth was observed below 10 °C or above 37 °C. The flexirubin test (KOH method; Reichenbach, 1992) was negative. Oxidase activity was positive. Good growth was observed on NA, R2A agar and TSA, but no growth was visible on MacConkey agar (Oxoid).

Isolate R26^T did not utilize the carbon sources tested, which is similar to the type strains of both species of the

genus *Elizabethkingia*, but was positive for hydrolysis of some chromogenic substrates. Differential characteristics of the three strains are summarized in Table 1.

The fatty acid profile was similar to those found in other species of the genus *Elizabethkingia*, with the most abundant fatty acids being iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7*c/t*). The complete fatty acid pattern of strain R26^T is shown in Table 2 along with those of the type strains of the two species of the genus *Elizabethkingia*.

Strain R26^T showed a complex polar lipid profile consisting of diphosphatidylglycerol, phosphatidylinositol, one

Fig. 1. Total polar lipid profiles, obtained after staining with molybdenum blue, of strain R26^T (a), *E. meningoseptica* CCUG 214^T (b) and *E. miricola* KCTC 12492^T (c). DPG, Diphosphatidylglycerol, PI, phosphatidylinositol, PL1, unidentified phospholipid; L1–L8, unidentified polar lipids; GL1, GL2, unidentified glycolipids.

Fig. 2. Phylogenetic analysis of species most closely related to strain R26^T based on 16S rRNA gene sequences available from EMBL (accession nos are given in parentheses). Tree building was performed with the maximum-likelihood algorithm without filters for 53 sequences. For clarity, only a subset of sequences (those with 93.3 % or higher sequence similarity to strain R26^T) are shown for the genus *Chryseobacterium*. *Flexithrix dorotheae* ATCC 23190 was used as an outgroup. Bar, 0.10 substitutions per nucleotide position.

unidentified phospholipid, four unidentified polar lipids and two glycolipids. *E. meningoseptica* CCUG 214^T contained four additional polar lipids with higher relative front values than diphosphatidylglycerol (L5–L8), only traces of which were detectable in strain R26^T and *E. miricola* KCTC 12492^T (Fig. 1). The only menaquinone of strain R26^T was

MK-6 (99 %); this menaquinone was also detected in *E. meningoseptica* CCUG 214^T and *E. miricola* KCTC 12492^T.

A maximum-likelihood tree for the 16S rRNA gene is shown in Fig. 2. Strain R26^T formed a separate cluster with the type strains of species of the genus *Elizabethkingia*. Further calculations confirmed that the closest relatives of the isolate were *E. meningoseptica* ATCC 13253^T and *E. miricola* GTC 862^T, which showed sequence similarities of 98.6 and 98.2 %, respectively, to the isolate. DNA–DNA hybridizations of strain R26^T with *E. meningoseptica* CCUG 214^T and *E. miricola* KCTC 12492^T gave similarities of 34.5 % (reciprocal 41.5 %) and 35.0 % (reciprocal 25.7 %), respectively. Genomic fingerprints generated by BOX-, (GTG)₅- and RAPD-PCR showed clear differences between strain R26^T, *E. meningoseptica* CCUG 214^T and *E. miricola* KCTC 12492^T (Fig. 3).

Although the chemotaxonomic data demonstrate a high similarity between strain R26^T and the type strains of both species of the genus *Elizabethkingia*, hybridization experiments, genomic fingerprint analysis and physiological data allow a clear separation of strain R26^T from the other species. On the basis of the reported results, a novel species, *Elizabethkingia anophelis* sp. nov., is proposed to accommodate strain R26^T.

Description of *Elizabethkingia anophelis* sp. nov.

Elizabethkingia anophelis (a.no.phe'lis. N.L. gen. n. *anophelis* of/from a mosquito of the genus *Anopheles*, as the type strain was isolated from the midgut of *Anopheles gambiae*).

Cells are aerobic Gram-reaction-negative, non-motile, non-spore-forming rods, approximately 1 µm in width

Fig. 3. Genomic fingerprint patterns generated by (GTG)₅-, BOX- and RAPD-PCR; ethidium bromide-stained fingerprint patterns separated on agarose gels are shown. Lanes: 1, strain R26^T; 2, *E. meningoseptica* CCUG 214^T; 3, *E. miricola* KCTC 12492^T; 4, no template control; M, GeneRuler 100 bp Plus DNA ladder (Fermentas).

and 2 µm in length. Oxidase- and catalase-positive. Good growth occurs after 48 h on NA, R2A agar and TSA (all Oxoid) at 11–36 °C. No growth occurs on MacConkey agar (Oxoid) at 28 °C. Unable to grow at temperatures below 10 °C or above 37 °C. Two growth optima are detected on LB medium: 30–31 °C with a doubling time of 50 min; and 37 °C with a doubling time of 42 min. Colonies on NA are smooth, yellowish, circular, translucent and shiny with entire edges. The non-diffusible and non-fluorescent yellow pigment is not of the flexirubin-type (KOH test-negative). Resistant to a number of antibiotics; MICs in LB medium are >400 µg ml⁻¹ for ampicillin, >250 µg ml⁻¹ for kanamycin, >250 µg ml⁻¹ for streptomycin, >30 µg ml⁻¹ for chloramphenicol and >10 µg ml⁻¹ for tetracycline. Tests for haemolytic activity on blood agar plates show no lytic zones around the colonies. Acid is produced from D-glucose, lactose (weak), D-mannitol, maltose, L-rhamnose, sucrose, trehalose and cellobiose (weak). No acid is produced from adonitol, L-arabinose, D-arabitol, dulcitol, erythritol, *i*-inositol, melibiose, methyl α -D-glucoside, raffinose, salicin, D-sorbitol or D-xylose. Aesculin hydrolysis, indole production from tryptophan and β -galactosidase activity (ONPG) are positive. Urease activity, hydrolysis of casein, gelatin, starch, DNA and tyrosine, hydrogen sulfide production, activity of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, and utilization of malonate are negative. The following compounds are not utilized as sole sources of carbon: *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, L-arbutin, cellobiose, D-fructose, D-glucose, maltose, D-galactose, gluconate, glycerol, D-mannose, D-mannitol, maltitol, α -melibiose, L-rhamnose, D-ribose, sucrose, salicin, trehalose, D-xylose, adonitol, inositol, D-sorbitol, putrescine, acetate, propionate, *cis*-aconitate, *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)- β -D-glucopyranoside, pNP- β -D-galactopyranoside, pNP- α -D-glucopyranoside, bis-pNP-phosphate, bis-pNP-phenyl-phosphonate, bis-pNP-phosphoryl-choline, 2-deoxythymidine-2'-pNP-phosphate, L-alanine-*p*-nitroanilide (pNA), γ -L-glutamate-pNA and L-proline-pNA are hydrolysed but not pNP- β -D-xylopyranoside or pNP- β -D-glucuronide. Major cellular fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH and summed feature 4 (iso-C_{15:0} 2-OH and/or C_{16:1} ω 7*cd*t). The only menaquinone is MK-6. The major polar lipids are diphosphatidylglycerol, phosphatidylinositol, a characteristic unknown phospholipid, and unknown polar lipids and glycolipids.

The type strain is R26^T (=CCUG 60038^T=CCM 7804^T), isolated from the midgut of *Anopheles gambiae* G3, originating from McCarthy Island, The Gambia, and deposited by Dr William Collins at Malaria Research Reference Resource Centre.

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