Ignatzschineria indica sp. nov. and *Ignatzschineria ureiclastica* sp. nov., isolated from adult flesh flies (Diptera: Sarcophagidae)

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Two Gram-negative-staining, aerobic, non-motile, rod-shaped bacteria, designated strains FFA1^T and FFA3^T, and belonging to the class *Gammaproteobacteria* were isolated from the gastrointestinal tract of adult flesh flies (Diptera: Sarcophagidae). Phylogenetic analysis of 16S rRNA gene sequence data placed these two strains within the genus *Ignatzschineria* with similarities of 98.6 % (FFA1^T) and 99.35 % (FFA3^T) to *Ignatzschineria larvae* L1/68^T. The level of gene sequence similarity between strains FFA1^T and FFA3^T was 99 %, 97.15 % and 78.1 % based on the 16S rRNA, 23S rRNA and *gyrB* gene sequences, respectively. Strains FFA1^T and FFA3^T shared 24 % DNA–DNA relatedness. DNA–DNA hybridization revealed a very low level of relatedness between the novel strains (22 % for strain FFA1^T and 44 % for strain FFA3^T) and *I. larvae* L1/68^T genomic DNA. The respiratory quinone was Q-8 in both novel strains. The DNA G + C contents were 41.1 mol% and 40.1 mol% for strains FFA1^T and FFA3^T, respectively. The cell membrane of both strains consisted of phosphatidylglycerol, phosphatidylethanolamine, phospholipids and aminophospholipid. The major fatty acids for both strains were C_{16:0}, summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c), CyC_{19:0}ω8c and C_{14:0}. The results of DNA–DNA hybridization between the two new strains and *I. larvae* L1/68^T, in combination with phylogenetic, chemotaxonomic, biochemical and electron microscopic data, demonstrated that strains FFA1^T and FFA3^T represented two novel species of the genus *Ignatzschineria* for which the names *Ignatzschineria indica* sp. nov. (type strain FFA1^T=DSM 22309^T=KCTC 22643^T=NCIM 5325^T) and *Ignatzschineria ureiclastica* sp. nov. (type strain FFA3^T=DSM 22310^T=KCTC 22644^T=NCIM 5326^T) are proposed.

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Abbreviations: CMCCase, carboxymethylcellulase; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, 23S rRNA and *gyrB* gene sequences of strains FFA1^T and FFA3^T are EU008088, EU008089, HM162934, HM162935, FJ966119 and FJ966120, respectively. The GenBank/EMBL/DDBJ accession numbers for 23S rRNA and *gyrB* gene sequences of strains *Ignatzschineria larvae* L1/68^T and *Wohlfahrtiimonas chitiniclastica* S5^T are HM162936, HM162937, FJ966121 and FJ966122, respectively.

Supplementary figures and tables are available with the online version of this paper.

The genus *Ignatzschineria* (formerly known by the illegitimate name *Schineria*) was first described by Tóth *et al.* (2001, 2007). This genus belongs to the class *Gammaproteobacteria* and, at present, comprises only one recognized species, *Ignatzschineria larvae*. This species was isolated from the first and second larval stages of an obligate parasitic fly, *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), responsible for myiasis (Tóth *et al.*, 2001, 2006). The genus is characterized by aerobic, Gram-negative, non-spore-forming, non-motile and regular rod-shaped bacteria. The main fatty acids are C_{18:1}, C_{16:0} and C_{14:0}; the main polar lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylserine (PS), the

predominant respiratory quinone is Q-8 and the G+C content of the DNA is 42 mol%. A recently described gammaproteobacterium, *Wohlfahrtiimonas chitiniclastica*, was originally isolated from the same species of fly but belongs to a distinct lineage close to *I. larvae* (Tóth *et al.*, 2008). Other bacterial species belonging to the genus *Ignatzschineria* have not been documented, although close relatives have been isolated from a maggot-infested wound (Maurin *et al.*, 2007) and a diabetic foot ulcer (Roudiere *et al.*, 2007) in a human patient suffering from myiasis and might induce bacteraemia in humans. The presence of *Ignatzschineria* sp. isolates and clones has been reported from diverse environmental and animal sources (Juteau *et al.*, 2004; De Luna *et al.*, 2009; Valiente Moro *et al.*, 2009).

In this study, the results of an investigation using a polyphasic taxonomic approach are presented. Two bacterial strains, FFA1^T and FFA3^T, were isolated from the gut contents of adult flesh flies (*Sarcophaga* sp., Diptera: Sarcophagidae). The strains were recovered by serial dilution plating of the gut contents on Luria–Bertani agar (HiMedia) and plates were incubated at 37 °C. Gram staining of both strains was performed using a Gram-stain kit (HiMedia) according to the manufacturer's instructions. The cell morphology of the strains was observed in the lag, exponential and stationary phases of growth. The motility of the cells was examined by the hanging drop method using phase-contrast microscopy (Eclipse E600; Nikon) and the semi-solid agar method. Ultrastructural investigations of strains FFA1^T and FFA3^T were carried out as described by Spurr (1969) and samples were examined in a transmission electron microscope (TEM910; Zeiss) at an acceleration voltage of 80 kV. Images were recorded digitally with a Slow-Scan CCD-Camera (1024 × 1024; ProScan) with ITEM-Software (Olympus Soft Imaging Solutions).

For comparative analyses, strains *I. larvae* L1/68^T and *W. chitiniclastica* S5^T were obtained from Dr Erika M. Tóth (Tóth *et al.*, 2001, 2008). All of the phenotypic tests described below were performed on strains FFA1^T, FFA3^T and its closest phylogenetic neighbour, *I. larvae* L1/68^T, under the same experimental conditions. The growth of the strains was tested on nutrient agar, Müller–Hinton agar (MH agar), MacConkey agar, Salmonella Shigella agar, brain heart infusion agar and King's medium A base agar (HiMedia) at 37 °C for 2 days. Subcultivation, colony characteristics and the determination of optimal growth parameters (temperature, pH, NaCl and oxygen requirement) were performed on MH agar at 37 °C because of the luxuriant growth in this medium. Oxidase, catalase and Voges–Proskauer reactions were tested according to the methods of Tarrand & Gröschel (1982) and Cowan & Steel (1974). Growth at different temperatures (0, 4, 10, 15, 20, 28, 37, 45 and 48 °C), NaCl tolerance (0, 0.5, 2, 4, 6, 8, 10, 12 and 15 %, w/v) and pH values (pH 5, 6, 7, 7.5, 8, 8.5, 9, 10 and 11) were determined using MH agar or broth. Anaerobic jars with AnaeroHiGas Packs (LE002B;

HiMedia) were used to assess growth under anaerobic conditions. Urease activity, reduction of NO₃⁻, indole production from tryptophan, H₂S production from peptone, phosphatase activity, starch hydrolysis, caseinase, gelatinase, cellulase, pectinase, xylanase and carboxymethylcellulase (CMCase) activities were studied according to Smibert & Krieg (1994) and Gerhardt *et al.* (1994). Haemolysin production was tested on ox-blood agar plates (Elek & Levy, 1954). Utilization of citrate was checked according to the method of Simmons (1926). Chitinase activity was studied as described by Holding & Collee (1971) and modified by Tóth *et al.* (2001) and O'Brien & Colwell (1987). The classic medium of Koser (1923), as modified by Ventosa *et al.* (1982), was used for determination of the range of substrates used as carbon and energy or as carbon, nitrogen and energy sources. The basal medium contained: 5 g NaCl l⁻¹, 2 g KCl l⁻¹, 0.2 g MgSO₄ · 7H₂O l⁻¹, 1 g KNO₃ l⁻¹, 1 g (NH₄)₂HPO₄ l⁻¹, 0.5 g KH₂PO₄ l⁻¹ and 0.05 g yeast extract l⁻¹ (HiMedia). To this basal medium, 0.1 % (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2 % (w/v). For the testing of amino acids as substrates, neither KNO₃ nor (NH₄)₂HPO₄ were used. The organic compounds used as sole source of carbon and energy, or carbon, nitrogen and energy were: L-arabinose, D-fructose, D-glucose, α-lactose, maltose, sucrose, D-mannitol, sodium acetate, sodium citrate, sodium butyrate, L-maleic acid, citric acid, L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine-free base, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine hydrochloride, 4-hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. Results were interpreted according to Ventosa *et al.* (1982) after incubation at 37 °C for 3 days under aerobic conditions. The metabolism of glucose, lactose and sucrose was checked by the O/F test according to Hugh & Leifson (1953) and was also tested on stabs of triple-sugar-iron agar (HiMedia). Fermentation of various carbohydrates was tested with carbohydrate differentiation discs (adonitol, arabinose, cellobiose, glucose, dulcitol, fructose, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose; HiMedia) with basal media which contained phenol red as an indicator. Acid production from carbohydrates (L-arabinose, D-fructose, D-glucose, α-lactose, maltose, sucrose and D-mannitol) was determined using phenol red base supplemented with 1 % (w/v) filter-sterilized carbohydrate. Acid production from different carbohydrates was also studied using API ID 32 GN and API 50 CHE test strips (bioMérieux) according to the manufacturer's instructions. Phenylalanine deaminase activity was determined by flooding phenylalanine agar (HiMedia) after 24 h incubation with 1 % (w/v) ferric chloride solution. Decarboxylase activity of amino acids (L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine-free base, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine hydrochloride,

4-hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine) were detected using decarboxylase broth base (Møller; HiMedia) as described by Møller (1954). Biochemical characteristics were determined using the Hi25 *Enterobacteriaceae* identification kit (HiMedia) and parts A, B and C of the HiCarbohydrate kit (HiMedia), according to the manufacturer's instructions. The oxidation pattern of 95 carbon compounds was studied on Biolog GN2 plates. Susceptibility to antimicrobial compounds was determined according to the conventional Kirby-Bauer method (Bauer *et al.*, 1966) using standard commercial antibiotic-susceptibility octadiscs (HiMedia) with varying amounts of antibiotics. Plates were incubated at 37 °C for 72 h under aerobic conditions and susceptibility was scored on the basis of the distance from the edge of the clear zone to the disc. The strain was considered susceptible when the diameter of the inhibition zone was >14 mm, intermediate at 10–13 mm and resistant at <10 mm.

Genomic DNA extraction, PCR amplification of the 16S rRNA gene and sequencing of amplicons were carried out according to the method of Rainey *et al.* (1996). The 23S rRNA and *gyrB* genes from strains FFA1^T, FFA3^T, *I. larvae* L1/68^T and *W. chitiniclastica* S5^T were amplified according to Van Camp *et al.* (1993) and Yamamoto & Harayama (1995), respectively. The 16S rRNA, 23S rRNA and *gyrB* nucleotide sequences were determined using an automated DNA sequencer (3730XL; Applied Biosystems). The 16S rRNA gene sequences of strains FFA1^T (1525 bp) and FFA3^T (1463 bp) were analysed by the NCBI-BLAST (Altschul *et al.*, 1997) and megaBLAST (Zhang *et al.*, 2000) programs for the identification of closely related type strains with validly published names (Chun *et al.*, 2007). The 23S rRNA and *gyrB* gene sequences of strain FFA1^T (2572 bp for 23S rRNA and 1024 bp for *gyrB*), strain FFA3^T (2467 bp for 23S rRNA and 1024 bp for *gyrB*), *I. larvae* L1/68^T (2555 bp for 23S rRNA and 1024 bp for *gyrB*) and *W. chitiniclastica* S5^T (2487 bp for 23S rRNA and 1024 bp for *gyrB*) were compared with respective reference gene sequences retrieved from the GenBank and EMBL databases. The 16S rRNA gene sequences with the highest scores were selected for the calculation of pairwise sequence similarity using a global alignment algorithm, which was implemented at the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The 16S rRNA and 23S rRNA gene sequences and *gyrB* amino acid sequences of the closest relatives were downloaded and aligned with the CLUSTAL_X program (Thompson *et al.*, 1997). The multiple sequence alignment was edited and corrected manually using DAMBE (Xia & Xie, 2001) and a degapped alignment of 1380 bp each for 16S rRNA gene sequences, 2450 bp each for 23S rRNA gene sequences and 352 amino acid sequences each for the *gyrB* gene were used to obtain an unambiguous sequence alignment. Evolutionary genetic distances were corrected according to the algorithm of the Kimura two-parameter model (Kimura, 1980). Tree

topologies were inferred with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971), using MEGA 3.1 (Kumar *et al.*, 2004) with 1000 bootstrap replicates to obtain a strict consensus tree.

Cell biomass from strains FFA1^T and FFA3^T for quinones and polar lipids analyses was obtained from cultures grown in MH broth for 72 h at 37 °C. Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and the profile was investigated using HPLC (9001; HP) (Groth *et al.*, 1997). Polar lipids were extracted from 100 mg freeze-dried cell material using a chloroform:methanol:0.3 % aqueous NaCl mixture 1:2:0.8 (by vol.) modified after Bligh & Dyer (1959). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform:methanol:0.3 % aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.). Polar lipids were separated by two dimensional silica gel TLC (Art. No. 818 135; Macherey-Nagel) according to the method of Tindall *et al.* (2007). Analysis of respiratory quinones and polar lipids was carried out by the Identification Service of the DSMZ and Dr Brian Tindall, Braunschweig, Germany. For fatty acid analysis, strains FFA1^T, FFA3^T and *I. larvae* L1/68^T were cultivated on MH agar media for 48 h at 37 °C. Cellular fatty acid analysis was conducted by the identification service of Royal Life Sciences, Pvt., Ltd. Briefly, cellular fatty acids were saponified, methylated and extracted according to standard protocols provided by the MIDI/Sherlock Microbial Identification System (MIDI; Microbial ID). Fatty acids were separated by GC and identified by the Microbial Identification software package (version 6; MIDI/Sherlock). The DNA base composition was determined by disrupting the bacterial cells with a French pressure cell and purification on hydroxyapatite, according to the procedure of Cashion *et al.* (1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated using bovine alkaline phosphatase and separated by reversed-phase HPLC (HP 9001) as described by Tamaoka & Komagata (1984). The DNA G + C content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al.* (1989). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multi-cell changer and a temperature controller with *in situ* temperature probe (Varian).

The distribution of repetitive DNA sequences in the newly isolated strains, FFA1^T and FFA3^T, and the closest phylogenetic relative *I. larvae* L1/68^T was examined and compared by analysis of the enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) element PCR as described by Versalovic *et al.* (1991). PCR amplification of bacterial genomic DNA by ERIC oligonucleotide primers, ERIC1R (5'-ATGTAGATCC-TGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACT-

GGGGTGAGCG-3') and with REP primers, REP1R-I (5'-IIICGICGICATCIGGC-3') and REP2-I (5'-ICGICTTAT-CIGGCCTAC-3') were performed in an automated thermal cycler (Perkin-Elmer) with an initial denaturation (90 °C, 3 min) followed by 30 cycles of denaturation (90 °C, 30 s), annealing (ERIC, 45 °C, 1 min; REP, 40 °C, 1 min), and extension (72 °C, 4 min) with a single final extension (72 °C, 16 min). Amplicons were analysed by horizontal agarose gel electrophoresis and ethidium bromide staining. Stained gels were photographed on a GelDoc UV transilluminator (SynGene) and fingerprints were compared visually.

The morphological, physiological and biochemical characteristics of strains FFA1^T and FFA3^T are given in the species description and in Table 1. Transmission electron micrographs of longitudinal sections of strains FFA1^T and FFA3^T showed no characteristic invagination of the inner membrane or the ring-like structure as seen in *I. larvae* L1/68^T (Tóth *et al.*, 2001) (see Supplementary Figs S1 and S2, available in IJSEM Online). Strain FFA1^T gave a negative result in tests for urease activity, H₂S production, nitrate reductase activity, acid production from D-fructose, fermentation of fructose and maltose and decarboxylation of L-lysine hydrochloride, but strain FFA3^T gave positive results. Characteristics that served to distinguish strains FFA1^T and FFA3^T from *I. larvae* L1/68^T are shown in Table 1.

The level of 16S rRNA and 23S rRNA gene sequence similarity between strains FFA1^T and FFA3^T was 99 % and 97.15 %, respectively. The highest 16S rRNA gene sequence similarity values for strains FFA1^T (98.6 %) and FFA3^T (99.3 %) were found with *I. larvae* L1/68^T. The 16S rRNA gene sequence similarity of strains FFA1^T and FFA3^T was very low when compared with other representatives of the class *Gammaproteobacteria* (92.1–92.3 % with *W. chitiniclastica* S5^T; 87.5–87.8 % with *Pseudomonas tremae* CFBP 6111; 87.3–87.6 % with *Halothiobacillus neapolitanus* DSM 581). The 23S rRNA gene sequence similarity of strains FFA1^T and FFA3^T with *I. larvae* L1/68^T were 97.33 % and 99.31 %, respectively. In a result that was similar to the 16S rRNA gene sequence analysis, the 23S rRNA gene sequences of the three strains showed a high degree of similarity. The 23S rRNA gene sequence similarity of strains FFA1^T, FFA3^T and *I. larvae* L1/68^T with *W. chitiniclastica* S5^T was less than 93 %. However, the level of 23S rRNA gene sequence similarity with respect to other closely related species found in the GenBank and EMBL databases was very low. The closest relatives of both new strains were from the genus *Pseudomonas* (*Pseudomonas putida* W619, 87 %) with ≤87 % similarity. The results of the 23S rRNA gene sequence comparison confirmed those obtained from the 16S rRNA gene sequence analyses and clearly supported the conclusion that strains FFA1^T and FFA3^T constituted a stable cluster separate from the other closely related species that could represent two separate species within a single genus. Based on *gyrB* gene sequences, the level of similarity between strains FFA1^T

Table 1. Differential characteristics of strains FFA1^T and FFA3^T compared with *Ignatzschineria larvae* L1/68^T

Taxa: 1, FFA1^T; 2, FFA3^T; 3, *I. larvae* L1/68^T. +, Positive; –, negative. All data are from the present study, except for DNA G+C contents which were carried out using the same experimental conditions. Data in parentheses were reported in the original description of the type species by Tóth *et al.* (2001). All taxa were able to grow on nutrient agar, MacConkey agar, Salmonella Shigella agar, brain heart infusion agar and King's medium A base agar. All were positive in tests for catalase, oxidase, phosphatase and phenylalanine deaminase and negative for Simmons' citrate, Methyl red, Voges–Proskauer, indole production, aesculin, caseinase, gelatinase, amylase, pectinase, xylanase and CMCase activities. Growth was not observed with any organic compounds (L-arabinose, D-fructose, D-glucose, α-lactose, maltose, sucrose, D-mannitol, sodium acetate, sodium citrate, sodium butyrate, L-maleic acid, citric acid, L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine-free base, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine hydrochloride, 4-hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine) used as sole carbon and energy or carbon, nitrogen and energy sources. Unable to metabolize glucose, lactose and sucrose for growth and also negative for ONPG (β-galactosidase) and malonate utilization. Negative in tests for fermentation of adonitol, arabinose, cellobiose, glucose, dulcitol, galactose, inositol, inulin, lactose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol and sucrose and for trehalose carbohydrate differentiation discs. No acid production from the carbohydrates (L-arabinose, D-glucose, α-lactose, maltose, sucrose and D-mannitol) tested. All three taxa were negative for the acid production test as determined by API ID 32 GN and API 50 CHE test strips and parts A, B and C of the HiCarbohydrate kit. No decarboxylase activity of amino acids (L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cysteine-free base, L-cystine, L-glutamic acid, L-glutamine, L-glycine, L-histidine hydrochloride, 4-hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine or L-valine) was seen for any of the three taxa. Oxidation of different carbon sources (Biolog GN2) and response to various antimicrobials are given in the species description and in Supplementary Tables S1 and S2.

Characteristic	1	2	3
Oxidation of (Biolog GN2):			
Acetic acid	+	–	–
cis-Aconitic acid	+	–	– (+)
L-Alaninamide	+	–	–
D-Alanine	+	–	–
L-Alanine	+	–	–
L-Aspartic acid	+	–	–
Bromosuccinic acid	+	–	+
Citric acid	+	–	– (+)
Formic acid	+	+	–
D-Fructose	–	+	+
Glycerol	+	–	–
DL-α-Glycerol phosphate	+	–	–
Glycyl-L-aspartic acid	+	–	–

Table 1. cont.

Characteristic	1	2	3
α -Hydroxybutyric acid	+	+	-
γ -Hydroxybutyric acid	+	-	-(+)
Hydroxy-L-proline	+	-	-
<i>myo</i> -Inositol	-	-	+
α -Ketobutyric acid	+	-	-
DL-Lactic acid	+	+	-
D-Psicose	-	-	+
L-Pyrogutamic acid	-	-	+
Pyruvic acid methyl ester	+	-	-
Succinamic acid	+	-	-
Succinic acid monomethyl ester	+	-	+
Uridine	+	-	+
Urocanic acid	-	-	+
Activities of:			
Chitinase	-	-	+
H ₂ S from peptone	-	+	+
Nitrate reductase	-	+	-
Urease	-	+	+
Acid production from:			
D-Fructose	-	+	+
Fermentation of (carbohydrate discs):			
Maltose	-	+	-
Fructose	-	+	+
Xylose	-	-	+
Decarboxylation of (decarboxylase broth, Möller):			
L-Lysine hydrochloride	-	+	-
Sensitivity to antimicrobials (μ g per disc):			
Amoxicillin/clavulanate (30)	-	+	+
Cephotaxime (30)	+	+	-
Ciprofloxacin (30)	+	-	+
Kanamycin (30)	+	-	+
Nalidixic acid (30)	+	-	+
Ofloxacin (5)	+	-	+
Sparfloxacin (5)	+	-	+
Tetracycline (50)	+	-	+
DNA G + C content (mol%)	41.1	40.1	42*

*Data from Tóth *et al.* (2001).

and FFA3^T was 78.1 %. With respect to *I. larvae* L1/68^T, the *gyrB* gene sequence similarity values were 78.9 % and 90.6 %, respectively, for strains FFA1^T and FFA3^T. The analysis of the additional phylogenetic marker, *gyrB*, showed that strains FFA1^T and FFA3^T could represent two separate novel species of the genus *Ignatzschineria*.

Phylogenetic analysis based on 16S rRNA and 23S rRNA gene sequences and *gyrB* amino acid sequences among members of the class *Gammaproteobacteria* showed that strains FFA1^T and FFA3^T formed a coherent cluster with *I. larvae* L1/68^T (Figs 1, 2 and 3) with a high bootstrap-resampling value. The strains were clearly separated from other species belonging to related genera. The topologies of the phylogenetic trees built using the maximum-parsimony algorithm also supported the notion that strains FFA1^T

and FFA3^T could represent novel species that were phylogenetically distinct from the closely related species, *I. larvae* L1/68^T (see Supplementary Figs S3a, b and c, available in IJSEM Online).

The respiratory quinone was Q-8 for both strains, which confirmed their membership of the class *Gammaproteobacteria* (Collins & Jones, 1981). Q-8 was also reported as a predominant respiratory quinone for species of the genus *Ignatzschineria* by Tóth *et al.* (2001). Both strains showed similar polar lipid patterns and contained PG, PE, phospholipids (PL1–PL2) and aminophospholipid (PN) in their cell membrane (see Supplementary Figs S4a and b). The main polar lipids were essentially the same as those reported for the type species of the genus *Ignatzschineria* by Tóth *et al.* (2001). The major cellular fatty acids for both strains were C_{16:0}, summed feature 8 (C_{18:1 ω 7c} and/or C_{18:1 ω 6c}), CyC_{19:0 ω 8c} and C_{14:0}. The composition of all cellular fatty acid components of strains FFA1^T and FFA3^T were similar. The detailed fatty acid compositions of both strains and the closest phylogenetic neighbour, *I. larvae* L1/68^T, are presented in Table 2. The fatty acid profiles of both new strains were similar to that of *I. larvae* L1/68^T, which contained predominantly the same fatty acid components with similar ratios between the different fatty acids (Table 2). The DNA G + C contents of strains FFA1^T (41.1 mol%) and FFA3^T (40.1 mol%) were very close to that of *I. larvae* L1/68^T (42 mol%). All of the chemotaxonomic data supported the affiliation of both new strains to the genus *Ignatzschineria*.

A visual inspection of the ERIC and REP PCR amplicon fingerprint profiles showed the marked differences in the banding patterns between strains FFA1^T, FFA3^T and *I. larvae* L1/68^T (see Supplementary Figs S5a and b). The distinct banding patterns found among strains FFA1^T, FFA3^T and *I. larvae* L1/68^T suggested that the ERIC and REP PCRs yielded a species-specific banding pattern for the genus *Ignatzschineria*. Phenotypically, strain FFA1^T could be differentiated from *I. larvae* L1/68^T on the basis of its inability to produce H₂S from peptone, acid from fructose, fermentation of fructose and xylose, absence of urease and chitinase activity, ability to oxidize pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, α -ketobutyric acid, DL-lactic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-aspartic acid, glycyl-L-aspartic acid, hydroxy-L-proline, glycerol and DL- α -glycerol phosphate, inability to oxidize D-fructose, *myo*-inositol, D-psicose, L-pyrogutamic acid and urocanic acid, inability to decarboxylate L-lysine hydrochloride and sensitivity to cephotaxime (30 μ g), but resistance to amoxicillin/clavulanate (30 μ g). In addition, strain FFA3^T differed from *I. larvae* L1/68^T in having nitrate reductase activity, lacking chitinase activity, being unable to ferment xylose, able to ferment maltose, able to oxidize formic acid, α -hydroxybutyric acid and DL-lactic acid, being unable to oxidize *myo*-inositol, D-psicose, succinic acid monomethyl ester, bromosuccinic acid, L-pyrogutamic acid, urocanic

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of strains FFA1^T and FFA3^T among members of the class *Gammaproteobacteria*. GenBank accession numbers are given in parentheses. Numbers at nodes indicate percentages of bootstrap support, based on 1000 resampled datasets. *Wolinella succinogenes* ATCC 29543^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

acid and uridine and being sensitive to cephotaxime (30 µg), but resistant to ciprofloxacin (30 µg), kanamycin (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg), sparfloxacin (5 µg) and tetracycline (50 µg). It was apparent that there were also phenotypic differences between the two novel strains (Table 1). All these differences were in agreement with the very low levels of DNA–DNA hybridization found between strains FFA1^T and FFA3^T (24 %) and with *I. larvae* L1/68^T (22 % and 44 %, respectively). These similarity values were well below the value of 70 % generally accepted as the threshold level for the delineation of a novel bacterial species (Wayne *et al.*, 1987). This further supported the proposal of strains FFA1^T and FFA3^T as two separate species of the genus *Ignatzschineria*.

Therefore, the results of DNA–DNA hybridization experiments in combination with phylogenetic (16S rRNA, 23S rRNA and *gyrB* gene sequences), chemotaxonomic (fatty acid composition, DNA G+C contents and polar lipid patterns), biochemical and electron microscopy data demonstrated that the new strains represented two novel species of the genus *Ignatzschineria*, for which the names *Ignatzschineria indica* sp. nov. and *Ignatzschineria ureiclastica* sp. nov. are proposed.

Description of *Ignatzschineria indica* sp. nov.

Ignatzschineria indica (in'di.ca. L. fem. adj. *indica* Indian, named after source of isolation of the strain).

Cells are Gram-negative-staining, non-motile, aerobic, regular short rods (0.8–1.8 × 0.3–0.8 µm) and non-haemolytic. Colonies grown on MH agar at 37 °C for 48 h are small (0.5–2 mm in diameter), non-pigmented, entire, convex, translucent, occur singly and are able to grow on nutrient agar, MacConkey agar, Salmonella Shigella agar, brain heart infusion agar and King's medium A base agar. Catalase and oxidase reactions are positive. The temperature, NaCl and pH ranges for growth are 4–45 °C, 0–6 % NaCl (w/v) and pH 6–9, respectively, with optima of 37 °C, 2 % NaCl (w/v) and 7.5, respectively. Positive for phosphatase activity and the phenylalanine deaminase reaction. Negative results in tests for urease, H₂S production, nitrate reductase, aesculin, caseinase, gelatinase, amylase, pectinase, xylanase, CMCase and chitinase activity. Indole production, Methyl red, Voges–Proskauer and Simmons' citrate tests are negative. Based on Biolog tests, gives a positive result for oxidation of L-histidine, L-leucine, formic acid, α-ketoglutaric acid, L-phenylalanine, DL-lactic acid, L-asparagine, α-hydroxybutyric acid, L-glutamic acid, succinic acid, glycyl L-glutamic acid, acetic acid, bromosuccinic acid, *cis*-aconitic acid, succinamic acid, hydroxy-L-proline, citric acid, α-ketobutyric acid, uridine, L-alaninamide, D-alanine, L-alanine, L-proline, L-aspartic acid, glycerol, DL-α-glycerol phosphate, pyruvic acid methyl ester, glycyl L-aspartic acid, succinic acid monomethyl ester and γ-hydroxybutyric acid, but negative for α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80,

Fig. 2. Neighbour-joining phylogenetic tree based on 23S rRNA gene sequences showing the phylogenetic position of strains FFA1^T and FFA3^T among members of the class Gammaproteobacteria. GenBank accession numbers are given in parentheses. Numbers at nodes indicate percentages of bootstrap support, based on analysis of 1000 resampled datasets. *Pseudomonas aeruginosa* ATCC 10145^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

N-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, *myo*-inositol, α -lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β -hydroxybutyric acid, *p*-hydroxyphenylacetic

acid, itaconic acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-alanylglycine, L-ornithine, L-pyrogutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, α -D-glucose 1-phosphate and α -D-glucose 6-phosphate. No acid production from API ID 32 GN or API 50 CHE test strips or parts A, B and C of the HiCarbohydrate kit. The

Fig. 3. Neighbour-joining phylogenetic tree based on *gyrB* amino acid sequences showing the phylogenetic position of strains FFA1^T and FFA3^T among members of the class Gammaproteobacteria. GenBank accession numbers are given in parentheses. Numbers at nodes indicate percentages of bootstrap support, based on 1000 resampled datasets. Bar, 0.05 substitutions per amino acid position.

Table 2. Fatty acid composition of strains FFA1^T, FFA3^T and *I. larvae* L1/68^T

Taxa: 1, FFA1^T; 2, FFA3^T; 3, *I. larvae* L1/68^T. Data are from the present study and represent percentages of total fatty acids. Fatty acids amounting to <1 % of the total fatty acids in all strains are omitted. Major fatty acids (>10 %) are shown in bold. The cellular fatty acids for strains FFA1^T, FFA3^T and *I. larvae* L1/68^T were determined after cultivation on MH agar at 37 °C for 2 days.

Fatty acid	1	2	3
C _{14:0}	11.8	14.8	11.6
C _{16:0}	33.8	31.6	26.9
C _{12:0} 3-OH	3.6	3.0	3.2
CyC _{19:0} ω8c	15.9	19.5	14.9
Summed features*:			
2	2.4	2.1	2.2
3	2.3	2.4	1.9
8	25.0	21.6	33.8

*Summed features are groups of two or three fatty acids that could not be separated by GLC using the MIDI system. Summed feature 2 comprised C_{16:1} iso I and/or C_{14:0} 3-OH; summed feature 3 comprised C_{16:1}ω7c and/or C_{16:1}ω6c and summed feature 8 comprised C_{18:1}ω7c and/or C_{18:1}ω6c.

major cellular fatty acids are C_{16:0}, summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c), CyC_{19:0}ω8c and C_{14:0}. The respiratory quinone is Q-8. Polar lipids are PG, PE, PL1, PL2 and PN. Susceptible to (μg): amikacin (30), azithromycin (15), cefaclor (30), ceftriaxone (30), cephotaxime (30), chloramphenicol (30), ciprofloxacin (30), clindamycin (2), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), netillin (30), nitrofurantoin (300), norfloxacin (10), ofloxacin (5), piperacillin/tazobactam (100/10), sparfloxacin (5), streptomycin (25), tetracycline (50) and tobramycin (10), but resistant to ampicillin (10), amoxicillin/clavulanate (30), aztreonam (30), cephalothin (5), colistin (50), mecillinam (25), metronidazole (5), penicillin (10 units), teicoplanin (30), ticarcillin (75) and vancomycin (30). Shows intermediate resistance to carbencillin (30), ceftazidime (100), cephalixin (30), cephoxitin (30), colistin methane sulphate (25), co-trimoxazole (25), doxycycline hydrochloride (30), gatifloxacin (30), sulphamethoxazole (25), sulphatriad (200) and trimethoprim (1.25).

The type strain, FFA1^T (=DSM 22309^T=KCTC 22643^T=NCIM 5325^T), was isolated from the gut contents of the adult flesh fly (Diptera: Sarcophagidae). DNA G + C content of the type strain is 41.1 mol%.

Description of *Ignatzschineria ureiclastica* sp. nov.

Ignatzschineria ureiclastica [u.re.i.clas'ti.ca. N.L. n. *urea*, *urea*; N.L. adj. *clasticus* -a -um (from Gr. adj. *klastos* -ê -on) broken in pieces, breaking; N.L. fem. adj. *ureiclastica* breaking *urea*].

Cells are Gram-negative-staining, non-motile, aerobic, regular short rods (1–2 × 0.5–1 μm) and non-haemolytic. Colonies grown on MH agar at 37 °C for 48 h are small (0.5–2.5 mm in diameter), non-pigmented, entire, convex, translucent, occur singly and are able to grow on nutrient agar, MacConkey agar, Salmonella Shigella agar, brain heart infusion agar and King's medium A base agar. Catalase and oxidase reactions are positive. The temperature, NaCl and pH ranges for growth are 4–45 °C, 0–6 % NaCl (w/v) and pH 6–9, respectively, with optima of 37 °C, 2 % NaCl (w/v) and pH 7.5, respectively. Positive for urease, H₂S production, nitrate reductase, phosphatase activity and phenylalanine deaminase reaction. Negative for aesculin, caseinase, gelatinase, amylase, pectinase, xylanase, CMCase and chitinase activity. Tests for indole production, Methyl red, Voges–Proskauer and Simmons' citrate are negative. Based on Biolog tests, gives a positive result for oxidation of L-histidine, L-leucine, formic acid, α-ketoglutaric acid, L-phenylalanine, DL-lactic acid, D-fructose, L-asparagine, α-hydroxybutyric acid, L-glutamic acid, succinic acid and glycyl L-glutamic acid, but negative reaction for acetic acid, bromosuccinic acid, *cis*-aconitic acid, succinamic acid, hydroxy-L-proline, citric acid, α-ketobutyric acid, uridine, L-alaninamide, D-alanine, L-alanine, L-proline, L-aspartic acid, glycerol, DL-α-glycerol phosphate, pyruvic acid methyl ester, glycyl L-aspartic acid, succinic acid monomethyl ester, γ-hydroxybutyric acid, α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-alanyl-glycine, L-ornithine, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, α-D-glucose 1-phosphate and α-D-glucose 6-phosphate. No acid production from API ID 32 GN or API 50 CHE test strips or parts A, B and C of the HiCarbohydrate kit. Able to decarboxylate L-lysine hydrochloride amino acid (decarboxylase broth Base, Møller) and ferment fructose and maltose (carbohydrate differentiation discs). The major cellular fatty acids are C_{16:0}, summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c), CyC_{19:0}ω8c and C_{14:0}. The respiratory quinone is Q-8. Polar lipids are PG, PE, PL1, PL2 and PN. Susceptible to (μg): amikacin (30), amoxicillin/clavulanate (30), ceftriaxone (30), cephotaxime (30), chloramphenicol (30), clindamycin (2), netillin (30), nitrofurantoin (300), streptomycin (25) and tobramycin (10), but resistant to ampicillin (10), aztreonam (30), ceftazidime (100), ciprofloxacin (30), colistin (50), doxycycline hydrochloride (30), kanamycin (30), mecillinam (25),

metronidazole (5), nalidixic acid (30), ofloxacin (5), penicillin (10 U), piperacillin/tazobactam (100/10), sparfloxacin (5), sulphamethoxazole (25), sulphatriad (200), teicoplanin (30), ticarcillin (75), tetracycline (50) and vancomycin (30). It shows intermediate resistance to azithromycin (15), carbenicillin (30), cefaclor (30), cephalixin (30), cephalothin (5), cephoxitin (30), colistin methane sulphate (25), co-trimoxazole (25), erythromycin (15), gatifloxacin (30), gentamicin (100), norfloxacin (10) and trimethoprim (1.25).

The type strain, FFA3^T (=DSM 22310^T=KCTC 22644^T=NCIM 5326^T), was isolated from the gut contents of an adult flesh fly (Diptera: Sarcophagidae). The DNA G+C content of the type strain is 40.1 mol%.

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