Wohlfahrtiimonas chitiniclastica gen. nov., sp. nov., a new gammaproteobacterium isolated from *Wohlfahrtia magnifica* (Diptera: Sarcophagidae)

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New *Gammaproteobacteria* were isolated from 3rd stage fly larvae of the parasitic fly *Wohlfahrtia magnifica*. Phylogenetic analysis of the new isolates showed that these bacteria belong to a distinct lineage close to *Ignatzschineria larvae*, which was originally isolated from the same species of fly. The low similarity values in 16S rRNA gene sequences (93.8–94.8 %), and differences in fatty acid profiles, RiboPrint patterns, MALDI-TOF mass spectra of cell extracts, and physiological and biochemical characteristics differentiate the isolates from the type strain of *Ignatzschineria larvae* (DSM 13226^T), and indicate that our isolates represent a new genus within the *Gammaproteobacteria*. The major isoprenoid quinone of the strains is Q8, the major fatty acids are C_{18:1} and C_{14:0}, and the predominant polar lipids are phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine. The G + C content of the DNA of the type strain is 44.3 mol%. The name *Wohlfahrtiimonas chitiniclastica* gen. nov., sp. nov., is proposed for this novel genus and species. The type strain is S5^T (=DSM 18708^T=CCM 7401^T).

Myiasis is an infestation of vertebrate animals with dipterous larvae. The obligate parasitic fly *Wohlfahrtia magnifica* develops only in live vertebrates and is a serious pest of livestock of Eastern Europe, the Mediterranean and Middle Asia (Nedelchev, 1988; Ruiz-Martinez *et al.*, 1991; Hall & Wall, 1995; Farkas *et al.*, 1997; Tóth *et al.*, 1998). *Ignatzschineria larvae* (Tóth *et al.*, 2001, 2007) was isolated from a homogenate sample of 1st and 2nd stage larvae of this fly in 1997. Studies to identify other bacterial partners of *W. magnifica* have continued, and we have isolated two novel bacterial strains, S5^T and E43, from 3rd stage larvae. These strains belong to the same lineage as *Ignatzschineria larvae*, although their DNA relatedness to the type strain of *Ignatzschineria larvae* (DSM 13226^T) is only 93.8 and 94.3 %, respectively. Until now, bacteria belonging to the same lineage have not been isolated, although close

relatives have been found in a clone library generated from *Eudiptomus gracilis* (Crustacea: Copepoda) (Homonnay, 2006). The aim of the present work was to determine the taxonomic position of strains S5^T and E43 and characterize them by using a polyphasic approach.

Strain S5^T was isolated from the homogenate of 3rd stage larvae of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), collected from a primary laboratory culture of the fly. Three larvae were taken out of the cultivation medium, washed three times in sterile distilled water and homogenized in a sterile glass mortar. Strain E43 was isolated from the aseptically excised foregut of a 3rd stage maggot of *Wohlfahrtia magnifica*, collected from the infested vulval wound of a Romney breed sheep at Mezőfalva State Farm, Hungary (18° 40' E, 46° 50' N), in 2002. It was washed three times in sterile 0.025 M sodium phosphate buffer solution (pH 6.8) and the foregut was dissected under a preparatory microscope under aseptic conditions. Bacterial strains S5^T and E43 were isolated and maintained on King B agar medium (King *et al.*, 1954).

Colony morphology of strains was tested on King B agar medium by direct observation of single colonies. Cell morphology and motility were studied in native preparations

Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S5^T is AM397063.

A transmission electron micrograph, a dendrogram based on MALDI-TOF data and the polar lipid pattern of strain S5^T are available as supplementary figures with the online version of this paper.

and by Gram staining according to Claus (1992). Electron-microscopic investigation was carried out as indicated by Tóth *et al.* (2001) using a JEM100CX II microscope (JEOL).

Growth in the absence of oxygen was tested using King B slant agar cultures incubated in an anaerobic chamber (Forma Scientific). Oxidase activity was studied by the method of Tarrand & Gröschel (1982). Catalase production and the Voges–Proskauer reaction were demonstrated by the methods of Cowan & Steel (1974). Acid production from D-glucose was checked by the OF test according to Hugh & Leifson (1953). Growth at different temperatures (4, 28, 38 and 45 °C), NaCl tolerance (0, 2.5, 5, 10 %, w/v) and pH tolerance (pH 4–12 in increments of 0.5 units) were determined using King B agar medium or broth. Urease activity, reduction of NO₃⁻, starch hydrolysis, indole production from tryptophan, caseinase, gelatinase, phosphatase activity, hydrolysis of Tween 80 and H₂S production from cysteine were studied according to Smibert & Krieg (1994). Haemolysin production was tested on ox-blood agar plates (Elek & Levy, 1954). Chitinase activity was studied by the method of Holding & Collee (1971), modified by Tóth *et al.* (2001). Oxidation of 95 different carbon sources as sole source of carbon was studied on Biolog GN plates, using an Elisa Reader (Sunrise Remote; Tecan). To evaluate the results, System 3.5 was used, following the instructions of the manufacturer (Biolog).

The cell wall diamino acids of the strains were determined from whole-cell hydrolysates as described by Hasegawa *et al.* (1983). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and the profile was analysed by HPLC (HP 9001) (Groth *et al.*, 1997) after cells were cultivated in liquid rich medium (Yamada & Komagata, 1972). For polar lipid and fatty acid analyses, the strains were cultivated on Tryptic Soy Agar medium (Difco) for 24 h. Cellular fatty acids were extracted by the method of Stead *et al.* (1992) and were analysed by GC (Groth *et al.*, 1996). Polar lipids were determined according to the method described by Minnikin *et al.*, (1979). The DNA base composition was determined from bacterial cells disrupted by using a French press. After purification of the DNA on hydroxyapatite, according to the procedure of Cashion *et al.* (1977), the DNA was degraded to nucleosides using P1 nuclease and bovine intestinal mucosa alkaline phosphatase as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC (HP 9001) by the method described by Tamaoka & Komagata (1984). The G + C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine. For DNA–DNA hybridization, DNA was isolated and purified as described above. Hybridization was carried out in 2 × SSC buffer at 67 °C 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 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

Extraction of genomic DNA and PCR-mediated amplification of the 16S rRNA gene of bacterial strains S5^T and E43 were carried out by the method of Rainey *et al.* (1996). PCR products were purified by the Prep-A-Gene kit (Bio-Rad). Cycling sequencing was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's protocol, and analysed in an Applied Biosystems model 310 Genetic Analyzer. The 16S rRNA gene sequences of the bacteria were aligned against the ARB-formatted RDP Database Release 7.1 (Maidak *et al.*, 1997) using the ARB program package (Strunk & Ludwig, 1995). To check for more recent updates, an additional BLAST search (Altschul *et al.*, 1997) in the GenBank database (www.ncbi.nlm.nih.gov/) was also performed. Sequences were aligned and a phylogenetic tree was constructed using the ARB software package with the Kimura two-parameter model (Kimura, 1980) and the neighbour-joining algorithm (Saitou & Nei, 1987). Fully automated ribotyping of *EcoRI*-digested samples was performed with a RiboPrinter system (DuPont Qualicon) as described by Bruce (1996).

Sample preparation for matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein analysis was carried out according to the ethanol/formic acid extraction protocol recommended by Bruker Daltonics (<http://clinprot.bdal.de>). First, about 10 mg biomass from agar cultures was resuspended in 300 µl water by careful mixing. Then the suspension was mixed with 900 µl ethanol. The biomass was collected by centrifugation and the pellet was resuspended in 50 µl 70 % formic acid. The suspension was mixed carefully with 50 µl acetonitrile. After centrifugation, the supernatant was removed immediately and aliquots of 1.5 µl were placed on each spot of a stainless-steel target plate. After air-drying, 1.5 µl matrix solution (saturated solution of α-cyanohydroxycinnaminic acid in 50 % aqueous acetonitrile containing 2.5 % trifluoroacetic acid) per spot was applied. MALDI-TOF MS was conducted using a Microflex L20 mass spectrometer (Bruker Daltonics) equipped with an N₂ laser. All spectra were recorded in linear, positive ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 500 shots across a spot. A mass range of 2000–20 000 *m/z* was used for analysis.

On King B agar medium, colonies of strains S5^T and E43 grown for 24 h were small, convex, entire, smooth and glistening. Colonies were spreading, and composed of small (1.5–2.2 × 0.5–1 µm), regular rods, occurring singly. In ageing cultures, no characteristic shape shifts could be detected. The cells stained Gram-negative. Endospores were not formed. Transmission electron micrographs of longitudinal sections of S5^T and E43 strains (see Fig. S1, available as supplementary material with the online version of this paper) showed no characteristic invagination as seen in *Ignatzschineria larvae* DSM 13226^T.

Strains S5^T and E43 grew under strictly aerobic conditions; no anaerobic growth was observed even in the presence of

NO₃⁻. Catalase and oxidase reactions were positive. Glucose was not utilized. The temperature range of growth on King B agar medium was between 4 and 45 °C, with an optimum between 28 and 37 °C. Growth occurred between pH 5 and 10.5. Both strains were negative in Voges–Proskauer, aesculin, urease, indole, caseinase, gelatinase, amylase and H₂S production tests, as well as for Tween 80 utilization. Both strains had similarly strong chitinase activity compared to *Ignatzschineria larvae* DSM 13226^T. They did not produce haemolysin. Results for the oxidation of different carbon sources as sole sources of carbon (Biolog) are given in the species description. Phenotypic differences between strains S5^T and E43 and *Ignatzschineria larvae* are shown in Table 1. It is clear that there are phenotypic differences between *Ignatzschineria larvae* and our isolates; strains S5^T and E43 are very similar to one another.

The results of phylogenetic analysis are shown in Fig. 1 (Kimura two-parameter model phylogenetic distance calculations, neighbour-joining tree). The length of the 16S rRNA gene sequence used for this analysis was 1393 and 1343 bp for S5^T and E43, respectively. The similarity of the two isolates to each other was 98.4%. The highest similarity values of S5^T (93.8%) and E43 (94.3%) were found with *Ignatzschineria larvae* (DSM 13226^T). Similarities of sequences to other representatives of the *Gammaproteobacteria* were low (e.g. to *Suttonella indologenes* ATCC 25869^T, 84.9%; *Cardiobacterium hominis* ATCC 15826^T, 85.3%; *Pseudomonas pachastrellae* KMM 330^T, 87.4%; *Shewanella surugensis* DSM 17177^T, 87.5%; *Proteus vulgaris* IFAM 1737, 85.6%; *Escherichia coli* K-12, 85.9%; *Xanthomonas campestris* LMG 568^T, 87.7%; *Stenotrophomonas maltophilia* LMG 958^T, 87.3%; *Xylella*

Table 1. Differences in the phenotypic characteristics of *Wohlfahrtiimonas chitiniclastica* (strains S5^T and E43) compared to strains of *Ignatzschineria larvae*.

Symbols: +, weak positive reaction; ++, positive reaction; +++, strong positive reaction; –, negative reaction; v, variable between strains.

Characteristic	<i>Wohlfahrtiimonas chitiniclastica</i>	<i>Ignatzschineria larvae</i>
Oxidation of:		
Glycogen	++	–
<i>cis</i> -Aconitic acid	–	+++
Citric acid	–	+++
Glycyl-L-aspartic acid	–	+++
Glycyl-L-glutamic acid	–	+++
L-Histidine	–	+++
L-Pyrogutamic acid	–	+++
D-Psicose	+++	v
Methylpyruvate	v	v
Formic acid	+++	v
D-Fructose	+++	v
γ-Hydroxybutyric acid	–	+++
Propionic acid	+++	–
γ-Aminobutyric acid	v	+++
Glycerol	++	v
Oxidase reaction	+	v
Phosphatase reaction	v	+++
Urease activity	–	+++
H ₂ S from peptone	–	+++
Growth at pH 5	+++	–

fastidiosa ATCC 35871, 87.9%; *Lysobacter antibioticus* DSM 2044^T, 86.2%; *Rhodanobacter lindanoclasticus* LMG

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains S5^T and E43 among *Gammaproteobacteria*. GenBank accession numbers are given in parentheses. Only bootstrap values above 50% are shown (1000 replications). Bar, 10 substitutions per 100 nt.

Fig. 2. RiboPrint patterns of strains S5^T, E43 and *Ignatzschineria larvae* DSM 13226^T. Cluster analysis was performed by UPGMA based on the Pearson correlation coefficient (opt. 1.20 %) [0.0–100.0 %]. VCA indicates a standard *EcoRI* batch.

18385^T, 87.7 %; *Frateriura aurantia* IFO3245^T, 86.4 %). The tree topology based on De Soete algorithm and Jukes–Cantor distance calculations was similar (data not shown). The G + C contents of the strains S5^T and E43 were 44.3 and 43.4 mol%, respectively. The RiboPrint band patterns (Fig. 2) show that the pattern similarity of the strains S5^T and E43 is high (82.5 %) while both strains are clearly separated from the type strain of *Ignatzschineria larvae* (DSM 13226^T) by a pattern similarity of only 54 %. MALDI-TOF mass spectra of cell extracts confirmed the differentiation of strains S5^T and E43 from the type strain of *Ignatzschineria larvae* (see Fig. S2, available as supplementary material with the online version of this paper). While strains S5^T and E43 could be distinguished by their RiboPrint band patterns (Fig. 2), the MALDI-TOF mass spectra of both strains were identical. A DNA–DNA hybridization value of 78.5 % (repetition 86.9 %) indicates that strains S5^T and E43 belong to the same species.

The main fatty acid components of strains S5^T and E43 were the same (C_{18:1} and C_{14:0}); even their whole profiles were rather similar. Their main fatty acids and those of *Ignatzschineria larvae* DSM 13226^T were also the same, although their ratios differed strongly: strains S5^T and E43 have remarkably high contents of C_{16:0}, C_{14:0} 3-OH and C_{18:0}, and lack C_{12:0} and C_{19:0} (Table 2). The predominant respiratory quinone of strains S5^T and E43 was Q-8, while Q-7 and Q-9 could be detected as minor components. Both strains contained phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS), two unknown glycolipids and three unknown polar lipids (see Fig. S3, available as supplementary material with the online version of this paper).

The low similarity values in 16S rRNA gene sequences (93.8–94.8 %), the clear separation by RiboPrint patterns (54 % similarity), differentiation by MALDI-TOF mass spectra, as well as differences in fatty acid profiles and in physiological

and biochemical characters indicate that these two strains are distinct from *Ignatzschineria larvae* and represent a novel genus and species within the *Gammaproteobacteria* for which the name *Wohlfahrtiimonas chitiniclastica* gen. nov., sp. nov. is proposed.

Description of *Wohlfahrtiimonas* gen. nov.

Wohlfahrtiimonas (Wohl'fahr.ti.i.mo'nas. N.L. n. *Wohlfahrtia*, the generic name of a sarcophagid fly; Gr. fem. n. *monas*, a unit, monad; N.L. fem. n. *Wohlfahrtiimonas*, a monad from a fly of the genus *Wohlfahrtia*).

Cells are Gram-negative, regular, non-motile rods. Endospores are not formed. Strictly aerobic. Catalase and oxidase reactions are positive. Contains *meso*-diaminopimelic acid in the cell wall. Main fatty acids are C_{18:1} and C_{14:0}; main polar lipids are PG, PE and PS. Predominant respiratory quinone is Q-8. G + C content of DNA of the type strain is 44.3 mol%. Phylogenetically a member of the *Gammaproteobacteria*.

The type species is *Wohlfahrtiimonas chitiniclastica*.

Description of *Wohlfahrtiimonas chitiniclastica* sp. nov.

Wohlfahrtiimonas chitiniclastica (chi.ti.ni.clas'ti.ca. N.L. neut. n. *chitinum*, chitin; Gr. adj. *klastos*, broken in pieces; N.L. adj. *clasticus*, breaking; N.L. fem. adj. *chitiniclastica*, chitin-cleaving).

Cells are straight, short rods (1.5–2.0 × 0.5–1 µm), non-motile, occurring singly at all ages. On King B agar medium colonies are non-pigmented, small, convex, entire, smooth and glistening. Strictly aerobic. Optimum growth occurs between pH 5.0 and 10.5, temperature optimum is at 28–37 °C. Catalase- and oxidase-positive. Negative in Voges–Proskauer, aesculin, urease, indole, caseinase,

Table 2. Fatty acid composition of strains S5^T, E43 and *Ignatzschineria larvae* DSM 13226^T

For fatty acid analysis, the three strains were cultivated under similar conditions.

Strain	Fatty acid composition (%)									
	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{16:1}	C _{18:1}	C _{12:0} 3-OH	C _{14:0} 3-OH	Cy C _{18:0}	Cy C _{19:0}
S5 ^T	5.24	21.82	7.67	–	–	47.24	2.45	–	–	7.32
E43	5.69	24.52	7.11	–	–	44.61	2.58	–	–	6.77
DSM 13226 ^T	–	20.1	23.1	0.6	2.8	30.7	2.8	6.0	8.9	–

gelatinase, amylase and H₂S production tests, as well as for Tween 80 utilization. Strong chitinase activity. Based on Biolog test results, both strains were positive for oxidation of glycogen, Tween 80, D-fructose, D-mannose, D-psicose, mono-methylsuccinate, acetic acid, formic acid, α -hydroxybutyric acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-ornithine, L-phenylalanine, L-threonine, glycerol, α -keto-butylric acid, α -ketoglutaric acid and DL-lactic acid, but negative for α -cyclodextrin, dextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, L-fucose, D-galactose, gentiobiose, α -D-glucose, *m*-inositol, α -D-lactose, lactulose, maltose, D-mannitol, β -methyl-D-glucoside, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, quinic acid, D-saccharic acid, sebatic acid, glucuronamide, alaninamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-pyroglutamic acid, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, DL- α -glycerol phosphate, glucose 1-phosphate, glucose 6-phosphate, malonic acid, α -ketovaleric acid and itaconic acid. A varied reaction between the strains was observed for Tween 40, melibiose, methylpyruvate, L-leucine, L-proline, D-serine, L-serine, γ -aminobutyric acid and 2,3-butanediol.

The type strain, isolated from 3rd stage larvae of *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), is S5^T (=DSM 18708^T=CCM 7401^T).

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