

Rheinheimera chironomi sp. nov., isolated from a chironomid (Diptera; Chironomidae) egg mass

Malka Halpern,¹ Yigal Senderovich¹ and Sagi Snir²

Correspondence

Malka Halpern

mhalpern@research.haifa.ac.il

¹Department of Biology, Faculty of Science and Science Education, University of Haifa, Oranim, Tivon 36006, Israel

²Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel

A Gram-negative, rod-shaped bacterial strain, designated K19414^T, was isolated from a chironomid (Diptera; Chironomidae) egg mass which was sampled from Kishon River in northern Israel. Phylogenetic analysis based on the 16S rRNA gene sequence positioned the novel strain among the genus *Rheinheimera*, with closest similarity to *Rheinheimera pacifica* KMM 1406^T. The levels of similarity to type strains of *Rheinheimera* species were lower than 96.5%. Isolate K19414^T is aerobic, motile by means of a single polar flagellum, catalase-negative and oxidase-positive; growth was observed at salinities of 0–2% NaCl and the temperature for growth ranged from 4 to 40 °C. The major cellular fatty acids are 16:0 (14.8%) and 16:1 ω 7c and/or 15:0 iso 2-OH (25.76%). The DNA G+C content is 49.9 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain K19414^T (=LMG 23818^T =DSM 18694^T) was classified in the genus *Rheinheimera* as the type strain of a novel species, for which the name *Rheinheimera chironomi* sp. nov. is proposed.

The genus *Rheinheimera* was created by Brettar *et al.* (2002) while screening blue-coloured bacterial isolates from different depth stations in the central Baltic Sea. At present, the genus comprises three species: *Rheinheimera baltica* (Brettar *et al.*, 2002), *Rheinheimera pacifica* (Romanenko *et al.*, 2003) and *Rheinheimera perlucida* (Brettar *et al.*, 2006). All three species were isolated from marine environments. In this study, a new member of the genus *Rheinheimera* that was isolated from a freshwater insect egg mass is proposed on the basis of polyphasic studies.

Strain K19414^T was isolated from a chironomid (non-biting midge) egg mass while the diversity of the culturable bacteria in chironomid egg masses was under study (Halpern *et al.*, 2007). Chironomid egg masses were sampled in September 2004, as described previously (Halpern *et al.*, 2007). Egg masses were washed thoroughly with sterile saline water and then their homogenates were diluted and cultured directly on bacteriological media (Halpern *et al.*, 2007). Strain K19414^T was isolated from an egg mass that was sampled from Kishon River in northern Israel, cultured on LB agar and incubated at 30 °C for 48 h in the dark. Another isolate from the above-described

study was recently characterized as the type strain of *Oceanobacillus chironomi* (Raats & Halpern, 2007).

Strain K19414^T is a motile Gram-negative rod, psychrotolerant and strictly aerobic. Its exact taxonomic position was determined by means of a polyphasic approach that included phenotypic properties and phylogenetic analysis based on the 16S rRNA gene sequence.

For electron microscopy, bacteria in LB agar medium were suspended in saline. The samples were adhered to a carbon-coated grid and stained with 2% uranyl acetate and photographed under a JEM-1200EX electron microscope (JEOL). Electron microscopy showed that the cells were rods with a polar flagellum (0.3–0.7 × 1.0–2.4 μm) (Supplementary Fig. S1 in IJSEM Online).

The 16S rRNA gene was analysed to determine its phylogenetic position. Universal bacterial primers 8f and 1512r (Felske *et al.*, 1997) were used to amplify internal fragments of the 16S rRNA gene. The amplified PCR product was purified with the Wizard PCR product purification kit (Promega). Purified PCR products were sequenced with primers 8f, 534r, 968f and 1512r as described in detail by Raats & Halpern (2007). This resulted in a sequence of 1499 bp.

For identification of closest relatives, the 16S rRNA gene of strain K19414^T was compared with those of previously reported strains available in EMBL (<http://www.ebi.ac.uk/>). Using the BLAST program (version 2.0), the closest sequences obtained were those of *R. baltica* OS140 (96.9%),

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K19414^T is DQ298025.

An electron micrograph of cells of strain K19414^T and details of its fatty acid profile in comparison with those of related type strains are available as supplementary material with the online version of this paper.

R. baltica OSBAC5 (96.7%), *R. pacifica* KMM 1406^T (96.5%), *R. perlucida* BA131^T (96.0%), *R. baltica* OSBAC1^T (95.8%), *Alishewanella fetalis* CCUG 30811^T (95.8%), *Brenneria salicis* DSM 30166^T (91.5%) and *Colwellia piezophila* Y223G^T (89.0%). The sequences were aligned by the multiple alignment package CLUSTAL W. Phylogenetic trees were generated by neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). NJ and MP trees were obtained using the MEGA software package (Kumar *et al.*, 2004), while ML was computed by PHYLIP (Felsenstein, 1993). For NJ, distance correction was performed using the Kimura two-parameter model (K2ST) using rate variation across sites. The bootstrap values obtained were from 1000 iterations. The NJ tree was drawn by the MEGA software (Fig. 1). In contrast to the highest sequence similarity to *R. baltica* OS140 as found by BLAST, all phylogenetic inference methods located the novel strain as a sibling to *R. pacifica* KMM 1406^T.

For phenotypic characterization, LB agar was used as the basal medium with a final concentration of 0.5% NaCl. Salt tolerance was determined in LB agar containing varying concentrations of NaCl at 37 °C. Growth at various temperatures (4, 6, 8, 10, 15, 18, 25, 30, 32, 35, 37, 39, 40, 41 and 43 °C) was measured on LB agar. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on LB agar supplemented with glucose or nitrate. Strain K19414^T had optimal growth between 0.5 and 1% NaCl and at 30–37 °C (Table 1).

Biochemical tests were performed by means of API 20E, API 20NE and API ZYM identification systems (bioMérieux) at 37 °C. Carbon assimilation was analysed using Biolog GN microwell plates according to the manufacturer's instructions (release 3.50, version DE; Biolog). The plates were incubated for 48 h at 37 °C. Wells that changed to purple were marked as positive for metabolic activity. Sensitivity of the strain to antibiotics was tested by means of LB agar and Sensi-discs (BBL). Tests were incubated for 48 h.

For the cellular fatty acid analysis, cells were cultured on a tryptic soy agar (Difco) at 28 °C for 24 h and then the fatty acids were extracted. The fatty acid profile was analysed by means of the MIDI/Hewlett Packard Microbial Identification system (Analytical Services Inc.), which uses GC

profiles of fatty acid methyl esters. The major fatty acid components (exceeding 10%) of strain K19414^T are 16:0 (14.80%) and summed feature 3 (16:1 ω 7c and/or 15:0 iso 2-OH; 25.76%) (Supplementary Table S1).

For determination of the DNA G+C content, genomic DNA of strain K19414^T was prepared according to a modification of the procedure of Wilson (1987). The DNA G+C content was determined using HPLC analysis of hydrolysed DNA according to Mesbah *et al.* (1989). The analysis was performed by the BCCM/LMG Bacteria Collection Identification Service (Laboratory of Microbiology, Ghent University, Belgium). The G+C content of strain K19414^T was 49.9 mol%.

The morphological, physiological and biochemical traits of strain K19414^T are summarized in the species description and in Table 1. API ZYM tests of strain K19414^T revealed a broad set of 12 enzyme activities (Table 1). Phylogenetic relationships between strain K19414^T and some related taxa are shown in Fig. 1. Comparative 16S rRNA gene sequence analysis showed that the new isolate was phylogenetically most closely related to *Rheinheimera* species, with 95.8–96.5% sequence similarity to the type strains. The new isolate shared its main characteristics with *Rheinheimera* species, such as being aerobic and oxidase-positive, motile rods by means of polar flagella, assimilating *N*-acetylglucosamine and hydrolysing gelatin. Its DNA G+C content was close to those of the other described *Rheinheimera* species (48.9–49.9 mol%) (Table 1). Unsaturated fatty acids formed a major fraction of the total fatty acids in strain K19414^T, as in the other described strains of the genus *Rheinheimera* (Supplementary Table S1). Together with the findings mentioned above, strain K19414^T showed significant characteristics that allowed clear differentiation from all three described *Rheinheimera* species. It lacked catalase activity, grew at relatively low NaCl concentrations (0–2%), grew at 40 °C, which is the highest growth temperature described for species of this genus, and was positive for malate assimilation and for valine arylamidase, cystine arylamidase and α -mannosidase (Table 1). On the basis of phenotypic characterization and phylogenetic analysis (Stackebrandt & Goebel, 1994), we propose that strain K19414^T should be classified as the type strain of a novel species, *Rheinheimera chironomi* sp. nov.

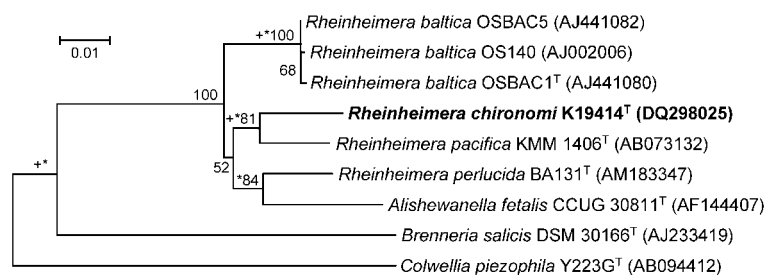


Fig. 1. Unrooted NJ tree (MEGA3, based on alignment from CLUSTAL W) of strain K19414^T and its closest relatives from the genus *Rheinheimera* and related taxa based on 16S rRNA gene sequences. Bootstrap values are indicated along branches. Branches found by ML with *P* values < 0.01 are marked by an asterisk. Branches marked by a plus sign are in consensus among all reconstruction methods. Bar, 0.01 accumulated changes per nucleotide position.

Table 1. Differential properties of strain K19414^T and *Rheinheimera* type strains

Strains: 1, strain K19414^T; 2, *R. perlucida* BA131^T (data from Brettar *et al.*, 2006); 3, *R. baltica* OSBAC1^T (Brettar *et al.*, 2002); 4, *R. pacifica* KMM 1406^T (Romanenko *et al.*, 2003). +, Positive; w, weakly positive; –, negative; ND, no data available.

Feature	1	2	3	4
Cell size (µm)				
Width	0.3–0.7	0.6–1.2	0.4–1.5	0.6–0.8
Length	1.0–2.4	0.9–2.4	0.9–4.5	1.8–2.0
Flagellar arrangement	Polar	Polar	Polar	Bipolar, lateral
Pigmentation	No pigment	No pigment	Blue	No pigment
DNA G + C content (mol%)	49.9	48.9	48.9	49.6
Catalase	–	+	+	+
Hydrolysis of DNA	–	ND	ND	+
Reduction of nitrate to nitrite	+	+	–	–
NaCl concentration for growth (%)				
Range	0–2	0–8	0–3*	0–8
Optimum	0.5–1	1–3	1–3	ND
Temperature for growth (°C)				
Range	4–40	4–37	4–30	4–37
Optimum	30–37	20–30	20–25	ND
Enzyme activities				
Esterase (C4)	+	+	–	+
Chymotrypsin	+	+	–	+
Acid phosphatase	+	+	–	–
<i>N</i> -Acetyl- β -glucosaminidase	+	+	–	+
Valine arylamidase	w	–	–	–
Cystine arylamidase	w	–	–	–
α -Mannosidase	+	–	–	–
Acid production from glucose	–	–	+	–
Assimilation of:				
Arabinose, citrate	–	–	–	+
Glucose	+	–	+	–
Maltose	+	–	+	+
Malate	+	ND	–	–
Utilization of:				
α -Cyclodextrin	–	w	–	ND
Tween 40	+	–	+	ND
Tween 80	+	–	–	ND
β -Hydroxybutyric acid	–	+	–	ND
<p><i>p</i>-Hydroxyphenylacetic acid</p>	–	–	+	ND
L-Alanine	–	+	–	ND
L-Alanyl glycine, L-threonine	–	w	–	ND
Glucose, maltose, sucrose	w	–	–	ND
Asparagine, arginine, lysine	–	–	–	+
Pyruvic acid methyl ester, succinic acid monomethyl ester, succinic acid, glyceryl L-glutamic acid	+	–	ND	ND
Glycyl L-aspartic acid	w	–	ND	ND
Acetic acid, glycerol	–	–	–	+

**R. baltica* OS550 grew in the presence of 6% NaCl (Brettar *et al.*, 2002).

Description of *Rheinheimera chironomi* sp. nov.

Rheinheimera chironomi [chi.ro'no.mi. N.L. gen. n. *chironomi* of *Chironomus*, named after the non-biting midge insect from the genus *Chironomus* (Diptera; Chironomidae) from which the type strain was isolated].

Cells are aerobic, Gram-negative, non-pigmented rods, 1.0–2.4 µm long and 0.3–0.7 µm wide, occurring as single cells or in pairs, and they are motile by means of a single polar flagellum. Colonies are circular, non-pigmented, smooth and convex. Oxidase-positive, catalase-negative and able to reduce nitrate to nitrite. Sodium ions are not required for growth. Growth is observed in 0–2% (w/v) NaCl, but not in 3% NaCl. Grows at 4–40 °C, but not at 41 °C. Hydrolyses gelatin and casein but not DNA. Grows on LB and half-strength marine agar. Does not haemolyse bovine blood and does not grow on MacConkey agar. The major measurable fatty acid components (exceeding 5%) of the type strain are 11:0 3-OH (5.53%), 12:0 3-OH (8.54%), 15:0 (6.86%), 16:0 (14.80%), summed feature 3 (16:1 ω 7c and/or 15:0 iso 2-OH; 25.76%), 17:0 (5.76%), 17:1 ω 8c (7.56%) and 18:1 ω 7c (6.68%). The following fatty acids are detected in the type strain as minor components: 9:0 (0.2%), 10:0 (1.2%), 11:0 (1.35%), 10:0 3-OH (0.53%), unknown ECL 11.799 (2.63%), 12:0 (1.06%), 13:0 (0.62%), 12:0 iso 3-OH (0.41%), 14:0 iso (0.19%), 14:0 (0.89%), summed feature 1 (one or more of 13:0 3-OH, 15:1 iso I and 15:1 iso H; 1.9%), 15:0 anteiso (0.28%), 15:1 ω 8c (2.75%), 15:1 ω 6c (1.04%), summed feature 2 (14:0 3-OH and/or 16:1 iso I; 0.28%), 16:0 iso (1.45%), 17:0 iso (0.19%), 17:0 anteiso (0.41%), 17:1 ω 6c (0.87%) and 18:0 (0.25%). Cells are resistant to neomycin, penicillin G and bacitracin but susceptible to tetracycline, ampicillin, vancomycin, streptomycin, chloramphenicol, novobiocin and SXT (sulfamethoxazole and trimethoprim). In the API 20NE test system, nitrate is reduced to nitrite, glucose, *N*-acetylglucosamine, maltose and malate are assimilated and enzyme activities of protease (gelatinase) and β -galactosidase are detected. In the API ZYM test system, 12 enzyme activities are detected: alkaline and acid phosphatases, esterase (C4 and C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, *N*-acetyl- β -glucosaminidase and α -mannosidase. In the Biolog GN2 test system, Tweens 40 and 80, D-galactose, D-glucose, maltose, sucrose, pyruvic acid methyl ester, succinic acid

monomethyl ester, L-alanine, L-alanyl glycine, glycyl L-aspartic acid and glycyl L-glutamic acid are utilized as substrates; all other Biolog GN2 substrates are not utilized. The G+C content of the type strain is 49.9 mol%.

The type strain is strain K19414^T (=DSM 18694^T =LMG 23818^T), which is of freshwater origin.

References

- Brettar, I., Christen, R. & Höfle, M. G. (2002). *Rheinheimera baltica* gen. nov., sp. nov., a blue-coloured bacterium isolated from the central Baltic Sea. *Int J Syst Evol Microbiol* **52**, 1851–1857.
- Brettar, I., Christen, R. & Höfle, M. G. (2006). *Rheinheimera perlucida* sp. nov., a marine bacterium of the *Gammaproteobacteria* isolated from surface water of the central Baltic Sea. *Int J Syst Evol Microbiol* **56**, 2177–2183.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A. D. L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology* **143**, 2983–2989.
- Halpern, M., Landsberg, O., Raats, D. & Rosenberg, E. (2007). Culturable and VBNC *Vibrio cholerae*: interactions with chironomid egg masses and their bacterial population. *Microb Ecol* **53**, 285–293.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Raats, D. & Halpern, M. (2007). *Oceanobacillus chironomi* sp. nov., a halotolerant and facultatively alkaliphilic species isolated from a chironomid egg mass. *Int J Syst Evol Microbiol* **57**, 255–259.
- Romanenko, L. A., Uchino, M., Falsen, E., Zhukova, N. V., Mikhailov, V. V. & Uchimura, T. (2003). *Rheinheimera pacifica* sp. nov., a novel halotolerant bacterium isolated from deep sea water of the Pacific. *Int J Syst Evol Microbiol* **53**, 1973–1977.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Wilson, K. (1987). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith & K. Struhl. New York: Greene Publishing and Wiley-Interscience.