Rheinheimera texasensis sp. nov., a halointolerant freshwater oligotroph

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A Gram-negative, rod-shaped, motile, non-spore-forming bacterium, designated strain A62-14B^T, was isolated from a constant-temperature, spring-fed, freshwater lake. On the basis of the complete 16S rRNA gene sequence, strain A62-14B^T was shown to belong to the class *Gammaproteobacteria*, being most closely related to *Rheinheimera* sp. HTB082 (96.2% sequence similarity), *Rheinheimera baltica* (95.01%), *Rheinheimera pacifica* (96.35%), *Rheinheimera perlucida* and *Alishewanella fetalis* (95.9%). The major fatty acids (C_{16:1}ω7c, 38.56%; C_{16:0}, 19.04%; C_{12:0} 3-OH, 12.83%; C_{18:1}ω7c, 7.70%) and the motility of strain A62-14B^T support its affiliation to the genus *Rheinheimera*. The salt intolerance of strain A62-14B^T, together with the results of other physiological and biochemical tests, allowed the differentiation of this strain from the three species of the genus *Rheinheimera* with validly published names. Therefore strain A62-14B^T represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera texasensis* sp. nov. is proposed. The type strain is A62-14B^T (=ATCC BAA-1235^T=DSM 17496^T). The description of the genus *Rheinheimera* is emended to reflect the halointolerance and freshwater origin of strain A62-14B^T.

The genus *Rheinheimera* was first proposed by Brettar *et al.* (2002) and, at the time of writing, contains three species, *Rheinheimera baltica* (Brettar *et al.*, 2002), *Rheinheimera pacifica* (Romanenko *et al.*, 2003) and *Rheinheimera perlucida* (Brettar *et al.*, 2006), all of which are of marine origin. On the basis of 16S rRNA gene sequence comparisons, members of the genus *Rheinheimera* are related to a clinical isolate, *Alishewanella fetalis* (Fonnesbech Vogel *et al.*, 2000), and an alkaliphilic psychrophile, '*Arsukibacterium ikkense*' (Schmidt *et al.*, 2007). A new isolate, strain A62-14B^T, is described in this paper and is proposed as a novel member of the genus *Rheinheimera* on the basis of the recommendations for the description of a novel species given by Kämpfer *et al.* (2003).

Strain A62-14B^T was isolated from Spring Lake in San Marcos, Texas, USA (29° 53' 26" N 97° 56' 1" W), during a study of freshwater bacteria capable of colonizing dialysis tubing in response to acylated homoserine lactones (McLean *et al.*, 2005). The strain was isolated on R2A agar (Difco) after incubation for 48 h at 30 °C and produced white, semi-transparent colonies. Subculturing was performed on R2A agar incubated for 48 h at 25 °C

and 7 days at 20 °C. On R2A agar, strain A62-14B^T was able to grow at temperatures in the range 20–37 °C. No growth was seen at 4, 42 or 55 °C. Limited growth was seen on dilute (10%, w/v) tryptone soy agar after 48 h at 30 °C. No other media tested [brain-heart infusion agar, full-strength tryptone soy agar, Luria–Bertani agar, nutrient agar (all from Difco) and Biolog universal growth media (Biolog)] (Amy *et al.*, 1992) could support growth of strain A62-14B^T. The organism grew between pH 6.5 and 9.6, with optimal growth occurring at pH 7.5–8.0. No growth was observed at pH 5.0. The novel strain could not grow in the presence of NaCl at concentrations greater than 1% (w/v). Gram staining was performed using a standard protocol (Doetsch, 1981).

Cell morphology was observed using a Zeiss microscope at a magnification of ×1000, using cells grown overnight on R2A agar at 30 °C. Motility was observed by examining a wet-mount slide preparation of strain A62-14B^T with a Zeiss microscope at ×800, using both phase-contrast and dark-field optics. Dilute suspensions of strain A62-14B^T and related organisms, namely *R. pacifica* KMM 1406^T [obtained from the Culture Collection of the University of Göteborg, Sweden, and cultured overnight at 30 °C on brain-heart infusion agar (Difco)] and *Alishewanella fetalis* ATCC BAA-284^T (obtained from the American Type Culture Collection, USA, and cultured overnight at 37 °C on brain-heart infusion agar), were examined for the presence of flagella and pili by using negative staining and transmission electron microscopy (Tolson *et al.*, 1995). The

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain A62-14B^T is AY701891.

An expanded phylogenetic tree for strain A62-14B^T and related taxa is available with the online version of this paper.

Fig. 1. Transmission electron micrographs of cells of strain A62-14B^T showing a single polar flagellum (a) and multiple polar and lateral flagella (b) (indicated by arrows in both pictures). Filaments (F) are also seen between some adjacent cells. Contrast was optimized using Adobe Photoshop, version 7.0.1 (Adobe Systems). Bars, 1 μm.

transmission electron microscopy examination revealed that cells of strain A62-14B^T have either single polar flagella (Fig. 1a) or multiple polar and lateral flagella; occasionally,

filaments were seen between cells lying in close proximity (Fig. 1b). Cells of *R. pacifica* KMM 1406^T had single polar flagella, but no flagella were seen on cells of *Alishewanella fetalis* ATCC BAA-284^T (data not shown).

For 16S rRNA gene sequencing, cultures were grown for 16 h at 30 °C in R2A broth. DNA was extracted using the DNeasy tissue kit (Qiagen). The 16S rRNA gene was amplified using primers EUB7F and 1492R, as described elsewhere (Lane, 1991). The PCR products were purified using a minElute gel extraction kit (Qiagen). Sequencing was performed using a DNA sequencer (model 377; Applied Biosystems) and a BigDye cycle sequencing kit (version 3.1; Perkin-Elmer Biosystems); the 1447 bp sequence was aligned with related sequences obtained from GenBank and EMBL by using BLAST and FASTA searches (Altschul *et al.*, 1997; Pearson & Lipman, 1988) using Sequencher 4.2.2 (Gene Codes Corporation) and CLUSTAL_X (Thompson *et al.*, 1997). Using the MrModeltest program, version 2.2 (Nylander, 2004), the GTR+I+G model of sequence evolution was found to best fit this dataset. Phylogenetic analyses (Fig. 2) were performed using PAUP 4.0b10 with distance-based neighbour-joining, maximum-parsimony and maximum-likelihood methods (Swofford, 2002). Confidence in the topology obtained from these analyses was gauged using bootstrap resampling methods in PAUP and included 10 000 replications (1000 replications for the maximum-likelihood tree) and a full heuristic search. Only those bootstrap percentages of at least 70 % were retained, as they demonstrate good support (Hillis & Bull, 1993). Bayesian analysis was completed using MRBAYES (version 3.0) and a 90 % majority rule consensus tree was generated from this treefile in PAUP

Fig. 2. Maximum-likelihood phylogenetic tree of strain A62-14B^T and the most closely related cultured organisms, created using PAUP 4.0b10 and a GTR+I+G model of sequence evolution (Swofford, 2002). Numbers reflect bootstrap support percentages (of 1000 replications) generated in PAUP; numbers in parentheses are Bayesian posterior probabilities created using MRBAYES, version 3.0 (Huelsenbeck & Ronquist, 2001). Asterisks indicate that the branch could not be resolved by neighbour-joining analysis. *Vibrio mytili* DSM 19137^T (GenBank accession number X99761) was used as an out-group. An expanded phylogenetic analysis which includes sequences from cultured and uncultured organisms is available with the online version of this paper (see Supplementary Fig. S1).

(Huelsenbeck & Ronquist, 2001). Fig. 2 shows a representative analysis of closely related cultured species (see Supplementary Fig. S1, available at IJSEM Online, for a full analysis with cultured and uncultured bacterial sequences).

Strain A62-14B^T most closely resembled an unclassified, filament-producing, gammaproteobacterium designated strain F8 (GenBank accession number AY077611) that had also been isolated from a freshwater environment (Böckelmann *et al.*, 2006). Here, the three recognized species of the genus *Rheinheimera* and strain A62-14B^T partitioned into four distinct clades. Only the Bayesian analyses partially resolved the relationships between the recognized species of the genus *Rheinheimera* and their close relatives. As noted by Brettar *et al.* (2006), the phylogenetic position of strain A62-14B^T was unstable with respect to the genus *Alishewanella*, as demonstrated by a lack of bootstrap support in the neighbour-joining, maximum-parsimony and maximum-likelihood analyses.

Metabolic profiles for preferred carbon, nitrogen, phosphorous and sulfur sources were obtained by the use of the phenotype microarray technique (Bochner *et al.*, 2001). An isolate of strain A62-14B^T was cultured on R2A agar and shipped to Biolog (USA) for phenotype microarray testing of approximately 1000 characteristics. Duplicate phenotype microarray readings were recorded for 24 h and kinetic data were analysed with proprietary software. The cellular fatty acids were determined at MIDI Laboratories (USA) by using fatty acid methyl ester analysis (Norton & LeChevallier, 2000). Strain A62-14B^T grew on R2A agar, but, unlike *R. baltica*, *R. pacifica*, *R. perlucida* and *Alishewanella fetalis*, did not grow on marine agar or Luria–Bertani agar. As a result, comparison of the fatty acid profiles of these species is limited by differences in growth conditions. The metabolic profiles are summarized in Table 1 and in the species description. As strain A62-14B^T is generally salt-intolerant, we provide an emended description for the genus *Rheinheimera* based on the

Table 1. Phenotypic characteristics used to distinguish strain A62-14B^T from related species

Taxa: 1, strain A62-14B^T (data from this study); 2, *R. baltica* OSBAC1^T (Brettar *et al.*, 2002); 3, *R. pacifica* KMM 1406^T (Romanenko *et al.*, 2003); 4, *R. perlucida* BA131^T (Brettar *et al.*, 2006); 5, *Alishewanella fetalis* CCUG 30811^T (Fonnesbech Vogel *et al.*, 2000; Romanenko *et al.*, 2003). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
O ₂ requirement	Facultative	Aerobic	Aerobic	Facultative	Facultative
Pigmentation	None	Blue	None	None	None
Cell size (µm):					
Length	1.25–2.5	1.8–2.0	0.9–4.5	0.9–2.4	2.0
Width	0.7–0.8	0.5–1.5	0.6–0.8	0.6–1.2	0.5–1.0
Motility	+	+	+	+	–
Flagella	Single polar or multiple polar and lateral	Single polar	Multiple polar and lateral	Single polar	None
Temperature for growth (°C):					
Range	25–37	4–30	4–37	4–37	25–41
Optimum	30–37	20–25	ND	20–30	ND
pH for growth:					
Range	6.5–9.6	5.7–10	ND	5.7–10	ND
Optimum	7.5–8.0	7	ND	7	ND
NaCl concentration for growth (% w/v):					
Range	0–1	0–6	0–8	0–8	3–8
Optimum	0	1–3	ND	1–3	ND
Starch hydrolysis	+	+	+	+	–
Reduction of nitrate to nitrite	+	–	–	+	+
Assimilation of:					
<i>N</i> -Acetylglucosamine	+	+	+	+	–
Arabinose	+	–	+	–	ND
Cellobiose	+	–	–	–	ND
Citrate, glycerol	–	–	+	–	ND
Maltose	–	+	+	–	ND
DNA G+C content (mol%)	48.2	48.9	49.6	48.9	50.6
Major fatty acids (>5%)	C _{16:1} ω7c, C _{16:0} , C _{12:0} 3-OH, C _{18:1} ω7c, C _{17:1} ω8c	C _{16:1} ω7c, C _{16:0} , C _{18:1} ω7c	C _{16:0} , C _{17:0} , C _{16:1} ω7c, C _{18:1} ω7c, C _{17:1} ω8c	C _{16:1} ω7c, C _{16:0} , C _{17:1} ω8c, C _{18:1} ω7c	C _{17:1} ω8c, C _{16:1} ω7c, C _{17:0} , C _{16:0} , C _{18:1} ω7c

current study and that of Brettar *et al.* (2002). On the basis of the data from this study, strain A62-14B^T represents a novel species of the genus *Rheinheimera*, for which the name *Rheinheimera texasensis* sp. nov. is proposed.

Emended description of the genus *Rheinheimera*

Rheinheimera (Rhein.hei'me.ra. N.L. n. *Rheinheimera* named after the German marine microbiologist Gerhard Rheinheimer, in recognition of his work on marine and estuarine bacteria).

The description of the genus *Rheinheimera* is as given by Brettar *et al.* (2002) but with the following changes. Of freshwater, estuarine or marine origin. NaCl supports growth, except in the case of one species, but strains are not tolerant of high salinities (>6 %).

Description of *Rheinheimera texasensis* sp. nov.

Rheinheimera texasensis (tex.as.en'sis. N.L. fem. adj. *texasensis* pertaining to Texas, the location from which the organism was first isolated).

Cells are Gram-negative, rod-shaped, 1.2–2.5 µm long and 0.7–0.8 µm wide, with single polar flagella or several polar and lateral flagella. Facultatively anaerobic. Network of filaments noted among cells lying in close proximity. After 24 h growth on R2A agar at 37 °C, colonies are 1–2 mm in diameter, smooth, non-pigmented, transparent, raised and circular with entire margins. Optimal growth occurs between 30 and 37 °C, with slow growth occurring at 20–25 °C and no growth above 40 °C. Withstands pH levels from 6.5 to 9.6, with optimal growth occurring at pH 7.5–8.0. No growth occurs at NaCl concentrations above 1 % (w/v). Carbon sources utilized include *N*-acetylglucosamine, Tweens 20, 40 and 80, D-glucose, D-lactose, D-galactose, sucrose, trehalose, methyl pyruvate, β-cyclodextrin, laminarin, glycogen and palatinose; citrate is not utilized. The type strain is positive in tests for starch and gelatin hydrolysis, nitrate reduction and oxidase and catalase activities. Indole production is not detected. The DNA G+C content of the type strain is 48.2 mol%. The predominant fatty acids are C_{16:1}ω7c (38.6 %), C_{16:0} (19.0 %), C_{12:0} 3-OH (12.8 %), C_{18:1}ω7c (7.7 %), C_{17:1}ω8c (3.8 %), C_{12:0} (2.0 %) and C_{18:0} (2.0 %).

The type strain, A62-14B^T (=ATCC BAA-1235^T=DSM 17496^T), was isolated from Spring Lake, a freshwater lake in San Marcos, Texas, USA.

Note added in proof

Since this paper was accepted for publication, two further novel species of the genus *Rheinheimera* have been described, *Rheinheimera aquimaris* (Yoon *et al.*, 2007) and *Rheinheimera chironomi* (Halpern *et al.*, 2007).

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References

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Amy, P. S., Haldeman, D. L., Ringelberg, D., Hall, D. H. & Russell, C. (1992). Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. *Appl Environ Microbiol* **58**, 3367–3373.
- Bochner, B. R., Gadzinski, P. & Panomitros, E. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* **11**, 1246–1255.
- Böckelmann, U., Janke, A., Kuhn, R., Neu, T. R., Wecke, J., Lawrence, J. R. & Szewzyk, U. (2006). Bacterial extracellular DNA forming a defined network-like structure. *FEMS Microbiol Lett* **262**, 31–38.
- 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.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Fonnesbech Vogel, B., Venkateswaran, K., Christensen, H., Falsen, E., Christiansen, G. & Gram, L. (2000). Polyphasic taxonomic approach in the description of *Alishewanella fetalis* gen. nov., sp. nov., isolated from a human foetus. *Int J Syst Evol Microbiol* **50**, 1133–1142.
- Halpern, M., Senderovich, Y. & Snir, S. (2007). *Rheinheimera chironomi* sp. nov., isolated from a chironomid (Diptera; Chironomidae) egg mass. *Int J Syst Evol Microbiol* **57**, 1872–1875.
- Hillis, D. M. & Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol* **42**, 182–192.
- Huelsenbeck, J. P. & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.
- Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J. & Stackebrandt, E. (2003). Towards a standardized format for the description of a novel species (of an established genus): *Ochrobactrum gallinifacis* sp. nov. *Int J Syst Evol Microbiol* **53**, 893–896.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- McLean, R. J. C., Barnes, M. B., Windham, M. K., Merchant, M. M., Forstner, M. R. J. & Fuqua, C. (2005). Cell-cell influences on bacterial community development in aquatic biofilms. *Appl Environ Microbiol* **71**, 8987–8990.
- Norton, C. D. & LeChevallier, M. W. (2000). A pilot study of bacteriological population changes through potable water treatment and distribution. *Appl Environ Microbiol* **66**, 268–276.

- Nylander, J. A. A. (2004).** MrModeltest 2.2. Distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden. <http://people.scs.fsu.edu/~nylander/mrmodeltest2/mrmodeltest2.html>
- Pearson, W. R. & Lipman, D. J. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- 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.
- Schmidt, M., Prieme, A. & Stougaard, P. (2007).** *Arsukibacterium ikkense* gen. nov., sp. nov, a novel alkaliphilic, enzyme-producing γ -Proteobacterium isolated from a cold and alkaline environment in Greenland. *Syst Appl Microbiol* **30**, 197–201.
- Swofford, D. L. (2002).** PAUP*: Phylogenetic analysis using parsimony (* and other methods), version 4. Sunderland, MA: Sinauer Associates.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tolson, D. L., Barrigar, D. L., McLean, R. J. C. & Altman, E. (1995).** Expression of a non-agglutinating fimbria by *Proteus mirabilis*. *Infect Immun* **63**, 1127–1129.
- Yoon, J.-H., Park, S. E., Kang, S.-J. & Oh, T.-K. (2007).** *Rheinheimera aquimaris* sp. nov., isolated from seawater of the East Sea in Korea. *Int J Syst Evol Microbiol* **57**, 1386–1390.