Reclassification of the members of the genus *Tetrathiobacter* Ghosh *et al.* 2005 to the genus *Advenella* Coenye *et al.* 2005

A. Gibello,[1] A. I. Vela,[1,2] M. Martín,[3] A. Barra-Caracciolo,[4] P. Grenni[4] and J. F. Fernández-Garayzábal[1,2]

Correspondence
J. F. Fernández-Garayzábal
garayzab@vet.ucm.es

[1]Dpto Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

[2]Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

[3]Dpto Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

[4]Water Research Institute – National Research Council, Via Reno 1, 00198 Rome, Italy

The taxonomic position of the genera *Advenella* and *Tetrathiobacter* was examined. 16S rRNA gene sequence analysis revealed that the two genera are closely related, representing a monophyletic cluster with high sequence similarity (98.1–99.7 %) within the family *Alcaligenaceae*. The phenotypic characteristics of the type strains of *Advenella incenata*, *Tetrathiobacter kashmirensis* and *Tetrathiobacter mimigardefordensis* were re-examined using the API 20NE, API ZYM and API 50CH systems. Phylogenetic data together with similarities in phenotypic characteristics, G+C content and cellular acid composition suggest that they should be classified in the same genus. On the basis of the data presented, the two species of the genus *Tetrathiobacter* should be transferred to the genus *Advenella*, since this genus has nomenclatural priority. Therefore, *Tetrathiobacter kashmirensis* and *Tetrathiobacter mimigardefordensis* should be transferred to the genus *Advenella* as *Advenella kashmirensis* comb. nov. (type strain WT001^T =LMG 22695^T =MTCC7002^T) and *Advenella mimigardefordensis* comb. nov. (type strain DPN7^T =DSM 17166^T =LMG 22922^T). Emended descriptions of *Advenella incenata* and the genus *Advenella* are also presented.

In the course of a study of environmental bacteria able to use n-triazines, we isolated several strains from groundwater contaminated by terbutylazine. Preliminary phylogenetic analysis of the 16S rRNA gene sequences of these strains (about 700 nt) revealed that their closest relatives were members of the genera *Advenella* and *Tetrathiobacter*. The genus *Advenella* was proposed by Coenye *et al.* (2005) to accommodate Gram-negative, rod-shaped to coccoid, oxidase-positive bacteria isolated from various human and veterinary clinical samples. *Advenella incenata* is the type and single species of this genus. The genus *Tetrathiobacter*, with the type species *Tetrathiobacter kashmirensis*, was created by Ghosh *et al.* (2005) to describe Gram-negative, non-flagellated, oval to coccoid-shaped bacteria occurring singly or in pairs, chains, branched chains or clusters isolated from bulk soils of a temperate orchard in Srinagar, Jammu and Kashmir, India. Another species, *Tetrathiobacter mimigardefordensis*, was described soon after by Wübbeler *et al.* (2006). Both genera are members of the family *Alcaligenaceae* (De Ley *et al.*, 1986). In this study, we present the results of the phenotypic and phylogenetic characterization of the environmental bacterial isolates and a critical taxonomic evaluation of the members of the two genera, and propose the combination of the genus *Tetrathiobacter* with the genus *Advenella*, since the latter has nomenclatural priority.

The environmental isolates 4GA-2008, 6GA-2008 and 7GA-2008 were isolated from groundwater contaminated by terbutylazine, located in Assisi, in central Italy. Primary isolation was achieved on minimal medium (MM) [l^{-1}; 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g $CaSO_4.2H_2O$, 1 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4.7H_2O$, 2 g $(NH_4)_2SO_4$, 15 g agar], supplemented with 2 p.p.m. terbutylazine and 0.03 % Casamino acids, after incubation at 30 °C for 48 h under aerobic conditions. After primary isolation and further subculture on Luria–Bertani (LB) agar plates at 30 °C for 48 h, isolates were stored at −20 °C as glycerol suspensions (20 % v/v). *A. incenata* CCUG 45225^T, *T.*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *A. incenata* strains CCUG 45225^T and 4GA-2008 are AM944734 and AM944735.

kashmirensis LMG 22695^T and LMG 22696 and *T. mimigardefordensis* LMG 22922^T were obtained from the respective culture collections, grown aerobically at 30 °C on LB agar plates for 48 h and stored at −20 °C as glycerol suspensions (20 % v/v).

Phylogenetic analysis of the environmental isolates was performed by comparative 16S rRNA gene sequence analysis as described previously (Vela *et al.*, 2006). The sequence of a large fragment of the 16S rRNA gene of the three isolates (approx. 1440 bases) as well as that of *A. incenata* CCUG 45225^T (1341 bp; the sequence available previously in GenBank had only 734 nucleotides) was obtained bidirectionally. 16S rRNA gene sequence analysis revealed 100 % similarity among the three environmental strains. Sequence searches of GenBank using the program FASTA (Pearson, 1994) confirmed the preliminary sequencing results and confirmed that the environmental isolates were phylogenetically most closely related to *T. kashmirensis* WT001^T (99.7 % sequence similarity), *A. incenata* CCUG 45225^T (99.4 % to the new, longer sequence) and *T. mimigardefordensis* DPN7^T (99.0 %). These sequences and those of other representative members of the family *Alcaligenaceae* were retrieved from GenBank and aligned with the newly determined sequence by using the program DNATools (Rasmussen, 1995). Phylogenetic trees were constructed according to three different methods, a neighbour-joining algorithm (Saitou & Nei, 1987), performed with the programs DNATools and TreeView (Page, 1996), maximum-likelihood analysis done using the PHYML software (Guindon & Gascuel, 2003) and maximum-parsimony method carried out using the MEGA software package version 3.1 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by Kimura's two-parameter model (Kimura, 1980) and close-neighbour interchange (search level=2, random additions=100) was applied in maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). The members of the genera *Advenella* and *Tetrathiobacter* formed a monophyletic cluster with 100 % bootstrap support and were readily differentiated from other genera of the family *Alcaligenaceae* (Fig. 1). Within this cluster, *T. mimigardefordensis* DPN7^T formed a distinct subline from that formed by *A. incenata* CCUG 45225^T and *T. kashmirensis* WT001^T with 99 % bootstrap support. This tree topology was confirmed by the three phylogenetic algorithms. Pairwise 16S rRNA gene sequence similarity values within this cluster ranged between 98.1 and 99.7 %; these values are typical of members of the same genus. The G+C contents of members of the genera *Advenella* (53.5–58.0 mol%) and *Tetrathiobacter* (54.0–55.2 mol%) and their cellular fatty acid compositions, with 16 : 0 and 18 : 1ω7*c* as the predominant fatty acids, are very similar (Coenye *et al.*, 2005; Ghosh *et al.*, 2005; Wübbeler *et al.*, 2006).

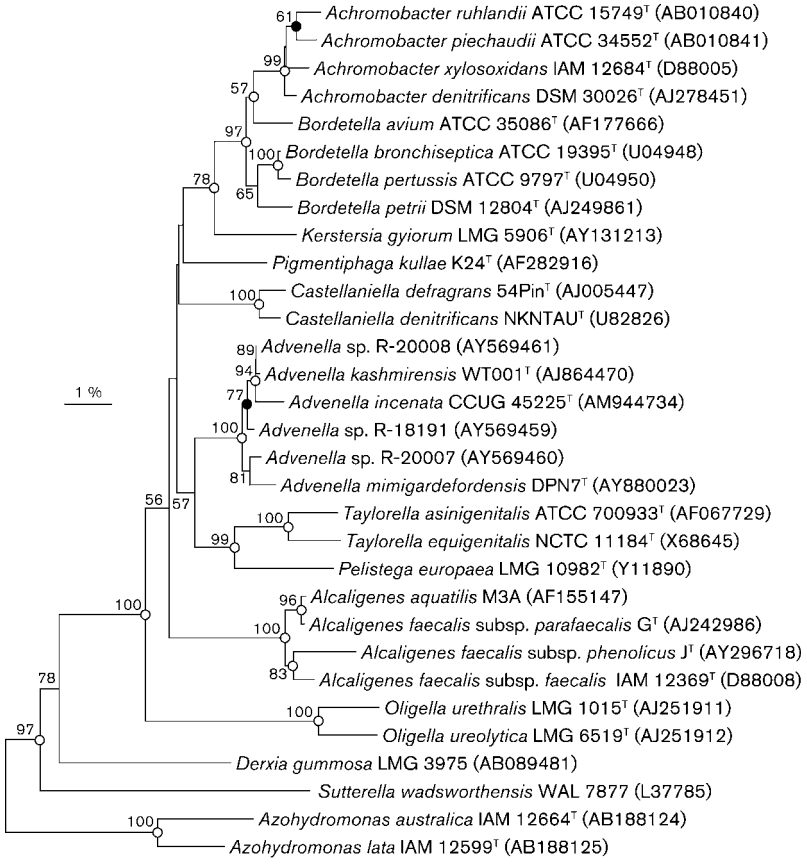

Fig. 1. Phylogenetic tree inferred from 16S rRNA gene sequence comparison using the neighbour-joining method, showing the relationships of the members of the genus *Advenella* with other taxa of the family *Alcaligenaceae*. The sequence of *Zoogloea ramigera* ATCC 19324 (GenBank accession no. D14257) was used as an outgroup (not shown). Bootstrap values (expressed as percentages of 1000 replications) higher than 50 % are given at branching points. Filled circles indicate that the corresponding nodes (groupings) were also obtained in maximum-likelihood trees. Open circles indicate that the corresponding nodes (groupings) were also obtained in maximum-likelihood and parsimony trees. Bar, 1 % sequence divergence.

The phenotypic characteristics described for *A. incenata*, *T. kashmirensis* and *T. mimigardefordensis* are not directly comparable, because the same characteristics were not determined for the three species (Coenye *et al.*, 2005; Ghosh *et al.*, 2005; Wübbeler *et al.*, 2006). In this study, the environmental isolates were characterized phenotypically and the phenotypic characteristics of *A. incenata* CCUG 45225^T, *T. kashmirensis* LMG 22695^T and *T. mimigardefordensis* LMG 22922^T were re-examined using commercial kits. Biochemical and enzyme characteristics were determined using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Carbohydrate assimilation was essayed by using API 50CH strips (bioMérieux) which were inoculated with a 0.5 McFarland suspension of bacterial cells in AUX medium (bioMérieux). The API 50CH strips were read for up to 4 days of incubation at 30 °C. Assimilation of DL-lactate was determined in MM broth containing 1 % (w/v) DL-lactate (Sigma). The environmental isolates exhibited almost identical phenotypic characteristics, which matched those exhibited by *A. incenata* CCUG 45225^T except that they did not hydrolyse urea (*A. incenata* CCUG 45225^T was positive). *A. incenata* CCUG 45225^T, *T. kashmirensis* LMG 22695^T and *T. mimigardefordensis* LMG 22922^T also exhibited many common characteristics, although several tests can be used for their differentiation. The results are given in the species descriptions and in Table 1.

The distinct phylogenetic position of *T. mimigardefordensis* and its separate species status with respect to *T. kashmirensis* were supported by DNA–DNA hybridization experiments and by differences in biochemical and chemotaxonomic characteristics (Wübbeler *et al.*, 2006). Therefore, genomic relatedness was examined to determine the species status of the environmental strains with respect to *A. incenata* and *T. kashmirensis* and between *A. incenata* and both species of *Tetrathiobacter*. DNA–DNA hybridization experiments were carried out between isolate 4GA-2008 and isolates 6GA-2008 and 7GA-2008, between isolate 4GA-2008 and its nearest phylogenetic neighbours *A. incenata* CCUG 45225^T and *T. kashmirensis* LMG 22695^T, between the two latter strains and between *A. incenata* CCUG 45225^T and *T. kashmirensis* LMG 22696 and *T. mimigardefordensis* LMG 22922^T. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Preparation of high-molecular-mass DNA and DNA–DNA hybridization experiments were performed by the DSMZ Identification Service (Braunschweig, Germany). DNA–DNA hybridization between isolate 4GA-2008 and isolates 6GA-2008 and 7GA-2008 showed DNA relatedness values of 95.1 and 84.9 %, respectively. The DNA–DNA reassociation values between isolate 4GA-2008 and *A. incenata* CCUG 45225^T, *T. kashmirensis* LMG 22695^T and *T. kashmirensis* LMG 22696 were 93.9, 44.8 and 31.4 %, respectively, demonstrating that the environmental isolates are members of the species *A. incenata* (Wayne *et al.*, 1987). DNA–DNA reassociation values between *A. incenata* CCUG 45225^T and *T. kashmirensis* LMG 22695^T, *T. kashmirensis* LMG 22696 and *T. mimigardefordensis* LMG 22922^T were 48.3, 44.0 and 27.7 %, respectively. These values are below the recommended threshold value of 70 %, confirming that they merit their separate species status (Wayne *et al.*, 1987).

Considering the phenotypic similarities, phylogenetic position and genetic and chemotaxonomic data, *A. incenata*, *T. kashmirensis* and *T. mimigardefordensis* should be members of the same genus. The names *Advenella* and *Tetrathiobacter* were published in the same year but, according to the Bacteriological Code (Rule 24b), the genus *Advenella* has priority and, consequently, the two species of the genus *Tetrathiobacter* should be reclassified as members of the genus *Advenella*.

Table 1. Phenotypic characteristics that differentiate type strains of the genus *Advenella*

Strains: 1, *A. incenata* CCUG 45225^T; 2, *A. kashmirensis* comb. nov. LMG 22695^T; 3, *A. mimigardefordensis* comb. nov. LMG 22922^T. Data were obtained in this study.

Characteristic	1	2	3
Nitrate reduction	−	+	−
Assimilation of:			
Glycerol	+	−	+
L-Rhamnose	+	+	−
D-Fructose	−	+	−
Melezitose	−	−	+
2-Ketogluconate	+	−	−
DL-Lactate	+	−	+
Esterase (C4) activity	+	+	−

Emended description of the genus *Advenella* Coenye *et al.* 2005

Advenella (Ad.ven.el′la. L. n. *advena* a stranger, a foreigner; L. dim. ending *-ella*; N.L. fem. n. *Advenella* the little stranger, referring to the fact that the source of the first strains was unknown).

The description is as given by Coenye *et al.* (2005) with the following modifications. Some members of the genus do not assimilate DL-lactate, D-mannose or maltose. The type species is *Advenella incenata*.

Emended description of *Advenella incenata* Coenye *et al.* 2005

Advenella incenata (in.ce.na′ta. L. fem. adj. *incenata* that has not dined, fasting, referring to the fact that this organism shows little biochemical activity).

The description remains that given by Coenye *et al.* (2005) with the following additions. Assimilates adipate, glycerol, D- and L-xylose, D-arabinose, galactose, ribose, rhamnose, D- and L-fucose, gluconate and 2-ketogluconate, but does not assimilate erythritol, D-adonitol, turanose, tagatose, D-lyxose, xylitol, gentiobiose, glycogen, sucrose, melibiose, D- or L-arabitol, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, sorbitol, starch, raffinose, inulin, dulcitol, inositol, L-sorbose, methyl β-D-xylopyranoside, D-fructose, trehalose or melezitose.

The type strain is CCUG 45225^T =LMG 22250^T, isolated from human sputum in Sweden.

Description of *Advenella kashmirensis* comb. nov.

Advenella kashmirensis (kash.mir.en′sis. N.L. masc. adj. *kashmirensis* of Kashmir, after the name of the province from where the original strains of the species were isolated).

Basonym: *Tetrathiobacter kashmirensis* Ghosh *et al.* 2005.

The description is as given for *Tetrathiobacter kashmirensis* by Ghosh *et al.* (2005) with the following additions or modifications. Assimilates adipate, D- and L-xylose, D-arabinose, galactose, ribose, rhamnose, D- and L-fucose, gluconate and fructose, but does not assimilate erythritol, D-adonitol, turanose, tagatose, D-lyxose, xylitol, gentiobiose, glycogen, sucrose, melibiose, D- or L-arabitol, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, sorbitol, starch, raffinose, inulin, dulcitol, inositol, L-sorbose, methyl β-D-xylopyranoside, glycerol, trehalose, 2-ketogluconate or melezitose. Esterase (C4), acid phosphatase (weak reaction) and alkaline phosphatase activities are detected. No activity is detected for esterase lipase (C8) and cystine arylamidase.

The type strain is WT001^T (=LMG 22695^T =MTCC7002^T), isolated from bulk soil of a temperate orchard in Srinagar, Jammu and Kashmir, India.

Description of *Advenella mimigardefordensis* comb. nov.

Advenella mimigardefordensis (mi.mi.gar.de.for.den′sis. M.L. masc. adj. *mimigardefordensis* of Mimegardefordum, a medieval name of Münster, where the type strain was isolated).

Basonym: *Tetrathiobacter mimigardefordensis* Wübbeler *et al.* 2006.

The description remains that given for *Tetrathiobacter mimigardefordensis* by Wübbeler *et al.* (2006) with the following additions. Assimilates glycerol, DL-lactate, adipate, D- and L-xylose, D-arabinose, D-galactose, ribose, D- and L-fucose, gluconate and melezitose, but does not assimilate erythritol, D-adonitol, turanose, tagatose, D-lyxose, xylitol, gentiobiose, glycogen, sucrose, melibiose, starch, D- or L-arabitol, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, sorbitol, raffinose, inulin, dulcitol, inositol, L-sorbose, methyl β-D-xylopyranoside, D-fructose, D-rhamnose, trehalose or 2-ketogluconate. Alkaline phosphatase (weak reaction), acid phosphatase and esterase lipase (C8) are detected. No activity is detected for esterase (C4) or cystine arylamidase.

The type strain is DPN7^T (=DSM 17166^T =LMG 22922^T), isolated from a sample of matured compost from a compost plant in Münster (Germany).

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