

Advenella incenata gen. nov., sp. nov., a novel member of the *Alcaligenaceae*, isolated from various clinical samples

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A polyphasic taxonomic study of 14 isolates recovered from various human and veterinary clinical samples was performed. Phenotypically these isolates shared several characteristics with members of the *Alcaligenaceae* and related genera. Random amplified polymorphic DNA fingerprinting and whole-cell protein analysis suggested the presence of multiple genomic groups, which was confirmed by DNA–DNA hybridization experiments. 16S rRNA gene sequence analysis indicated that these isolates were related to the genera *Pelistega*, *Taylorella*, *Oligella*, *Pigmentiphaga*, *Alcaligenes*, *Kerstersia*, *Achromobacter* and *Bordetella* and belonged to the family *Alcaligenaceae*. Based on the results of the present study the organisms were classified in a novel genus, *Advenella* gen. nov. This genus comprises one named species, *Advenella incenata* sp. nov. (type strain LMG 22250^T = CCUG 45225^T) and five currently unnamed genomic species. The DNA G+C content of members of the novel genus *Advenella* is between 54.0 and 57.7 mol%.

INTRODUCTION

The family *Alcaligenaceae* belongs to the β -subclass of the *Proteobacteria* and was proposed by De Ley *et al.* (1986) to accommodate the genera *Bordetella* and *Alcaligenes*. Subsequently, the genera *Achromobacter*, *Pigmentiphaga* and *Kerstersia* were also assigned to the *Alcaligenaceae* (Yabuuchi *et al.*, 1998; Blümel *et al.*, 2001; Coenye *et al.*, 2003b). The genera *Oligella*, *Taylorella*, *Pelistega* and *Brackiella* are closely related to the *Alcaligenaceae* but have not been formally assigned to this family (Rossau *et al.*, 1987; Vandamme *et al.*, 1998; Willems *et al.*, 2002). Members of most of the above-mentioned genera have been isolated from various human and veterinary clinical samples, including respiratory secretions of cystic fibrosis (CF) patients; however, some (e.g. *Pigmentiphaga kullae*)

appear to be truly environmental organisms, which have not been implicated in human or animal diseases.

We performed a polyphasic taxonomic study to elucidate the taxonomic position of 14 isolates recovered from various human and veterinary clinical samples. Phenotypically these organisms shared some characteristics with members of the *Alcaligenaceae* and related genera, but identification to the species level proved to be impossible using conventional biochemical analysis.

METHODS

Bacterial strains and growth conditions. The 14 strains used in this study are listed in Table 1. All strains were grown aerobically on trypticase soy agar (TSA) (BBL) at 37 °C unless otherwise indicated.

Randomly amplified polymorphic DNA (RAPD) fingerprinting. DNA was prepared by heating one or two colonies at 95 °C for 15 min in 20 μ l lysis buffer containing 0.25% (w/v) SDS and 0.05 M NaOH. Following lysis, 180 μ l distilled water was added to the lysate. Isolate genotyping by means of RAPD fingerprinting and numerical analysis were performed as described by Coenye *et al.* (2002).

SDS-PAGE of whole-cell proteins. Strains were grown on TSA for 48 h at 37 °C. Preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of the protein profiles, and

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Abbreviations: CF, cystic fibrosis; RAPD, randomly amplified polymorphic DNA; SF, summed feature.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 22250^T, R-16599, R-18191, R-20007 and R-20008 are AY569457–AY569461, respectively.

A figure showing the protein profiles and a table giving the fatty acid composition of the strains studied are available as supplementary material in IJSEM Online.

Table 1. List of strains studied

CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium.

Strain	Other strain designation	Depositor	Source
<i>Advenella incenata</i> gen. nov., sp. nov.			
LMG 22250 ^T	CCUG 45225 ^T	PHLS Göteborg	Human sputum, 69-year-old woman (Sweden)
R-12612, R-20001	CCUG 46453	J. Gigi	CF patient 1 (Belgium)
R-16574, R-20002	CCUG 46454	H. Franckx	CF patient 2 (Belgium)
R-16599		J. Van Eldere	CF patient 2 (Belgium)
LMG 8437	CCUG 22619	B. Bochner	
LMG 8439	CCUG 22621	B. Bochner	
R-20004	CCUG 12437	A. Malmborg	Contaminated horse blood (Göteborg, Sweden)
R-1086		G. Funke	Human blood (Switzerland, 1996)
<i>Advenella</i> sp.			
LMG 16602	CCUG 30713	R. Priwin	Human sputum, 16-year-old male (Göteborg, Sweden)
R-18191	CCUG 30155	R. Priwin	Human sputum, 16-year-old male (Göteborg, Sweden)
R-20008	CCUG 39400	A. Nordius	Human wound, 21-year-old male (Skövde, Sweden)
R-20007	CCUG 46954	PHLS Göteborg	Human sputum, 12-year-old male with CF (Göteborg, Sweden)
R-20003	CCUG 17517	PHLS Göteborg	Air in piggery (Sweden)
R-20005	CCUG 24018	U. B. Stolt	Human wound, 67-year-old male (Uppsala, Sweden)

numerical analysis using Pearson's product-moment correlation coefficient were performed using the GELCOMPAR 4.2 software package (Applied Maths).

16S rRNA gene sequencing. DNA was prepared as described above. The sequence of the 16S rRNA gene of strains LMG 22250^T, R-16599, R-20008, R-18191 and R-20007 was determined as described by Coenye *et al.* (1999). Phylogenetic analyses and bootstrap analysis (1000 replicates) were performed using the KODON 2.0 software package (Applied Maths); a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987).

Preparation of high-molecular-mass DNA and DNA–DNA hybridization experiments. High-molecular-mass DNA was prepared as described by Pitcher *et al.* (1989), and DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 45 °C. Reciprocal experiments were performed for every pair of strains.

Determination of DNA base composition. DNA (prepared as described above) was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% acetonitrile. The detector used was a Waters model 484 UV-VIS absorbance detector set at 270 nm. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

Fatty acid methyl ester analysis. After an incubation period of 24 h at 28 °C, a loopful of well-grown cells was harvested and fatty acid methyl esters were prepared as described by Vandamme *et al.* (1992); these were then separated and identified using the Sherlock Microbial Identification System (version 3.0; MIDI Inc.).

Phenotypic characterization. Classical phenotypic tests were performed as described by Vandamme *et al.* (1993). API20NE and APIZYM tests were performed according to the recommendations of the manufacturer (bioMérieux).

RESULTS AND DISCUSSION

Phylogenetic position and structure

Reproducibility of RAPD fingerprinting was checked by generating RAPD patterns in duplicate. The level of correlation between these patterns was more than 87% (data not shown). After numerical analysis of the RAPD patterns, eight and two isolates grouped together in clusters I and II, respectively, whereas the other isolates occupied separate positions in the dendrogram (Fig. 1). Reproducibility of SDS-PAGE of whole-cell proteins was checked by preparing protein extracts in duplicate. The level of correlation between these patterns was more than 93% (data not shown). Visual comparison of the protein profiles confirmed the grouping as observed following numerical analysis of the RAPD patterns (shown in Supplementary Fig. A in IJSEM Online). 16S rRNA gene sequences of five isolates were determined and compared with available 16S rRNA gene sequences of other *β-Proteobacteria* (Fig. 2). The sequences of these five isolates were very similar to each other (similarities ranging from 99.9 to 96.5%). Similarity levels to representatives of the genera *Pelistega* (95.4–94.6%), *Taylorella* (95.9–92.9%), *Oligella* (93.8–89.7%), *Brackiella* (94.8–91.6%), *Pigmentiphaga* (96.3–94.3%), *Alcaligenes* (95.8–92.8%), *Kerstesia* (96.6–93.5%), *Achromobacter* (95.7–92.6%) and *Bordetella* (96.2–92.9%) were generally lower. Bootstrap analysis indicated that strains LMG 22250^T, R-16599, R-20008, R-18191 and R-20007 formed a stable phylogenetic group (bootstrap value was 100%). DNA was prepared from strains LMG 22250^T, R-12612, R-18191, R-20008 and R-20007. The levels of DNA–DNA relatedness are given in Table 2. The G+C content of the isolates was between 53.5 and 58.0 mol%. The cellular fatty acid

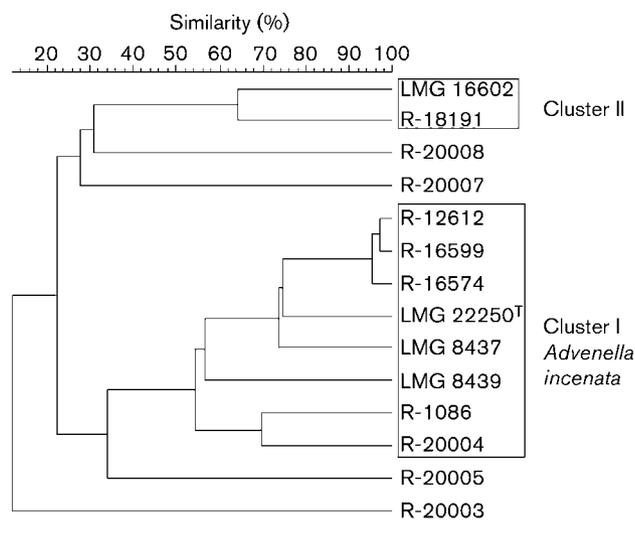


Fig. 1. Dendrogram derived from UPGMA linkage of correlation coefficients between the RAPD patterns of the strains studied. The correlation coefficient is expressed as percentage similarity for convenience.

composition of all 14 isolates was also determined (shown in Supplementary Table A in IJSEM Online). The 14 isolates formed a homogeneous group: the predominant fatty acids were 16:0, 18:1 ω 7c, summed feature 2 (SF2) (comprising 14:0 3-OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length of 10.928 or 12:0 ALDE, or any combination of these fatty acids) and summed feature 3 (SF3) (comprising 16:1 ω 7c and/or 15:0 iso 2-OH). All 14 isolates examined showed oxidase, catalase and leucine arylamidase, but no amylase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, gelatinase, DNase,

C₁₄-lipase, valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosidase, α -mannosidase or α -fucosidase activity. Haemolysis, hydrolysis of Tween 80 and aesculin, denitrication, indole production and production of acid or H₂S from triple-sugar-iron agar were not observed. Growth was observed at 30 and 37 °C on Drigalski agar (Vahlne, 1945) and at NaCl concentrations of 0–3 %. Growth was not observed on acetamide or on oxidation–fermentation (OF) medium supplemented with maltose, adonitol or D-fructose. No assimilation of DL-norleucine, phenylacetate, sucrose, mannose or maltose was observed. All strains examined assimilated citrate and DL-lactate. The following characteristics were strain dependent: growth at 42 °C, growth on OF medium supplemented with D-glucose or D-xylose, growth on cetrimide agar, growth with 10 % lactose, growth with 4.5 and 6.0 % NaCl, reduction of nitrate and nitrite, assimilation of D-glucose, trehalose, L-arginine, D-mannitol, *N*-acetylglucosamine, L-arabinose, L-malate and caprate and urease, alkaline and acid phosphatase, C₄-lipase, C₈-lipase, cysteine arylamidase and phosphoamidase activities.

Comparative 16S rRNA gene sequence analysis indicated that isolates LMG 22250^T, R-16599, R-20008, R-18191 and R-20007 formed a single and stable phylogenetic lineage. The relatively low similarity to their closest relatives and the stability of the cluster (as indicated by a bootstrap value of 100 %) warrant the inclusion of these isolates in a novel genus, for which we propose the name *Advenella*. Analysis of RAPD patterns and protein profiles suggested the presence of multiple species, sharing more than 97 % 16S rRNA gene sequence similarity. In order to clarify the relationships between these isolates, DNA–DNA hybridization was performed (Stackebrandt & Goebel, 1994). The results of

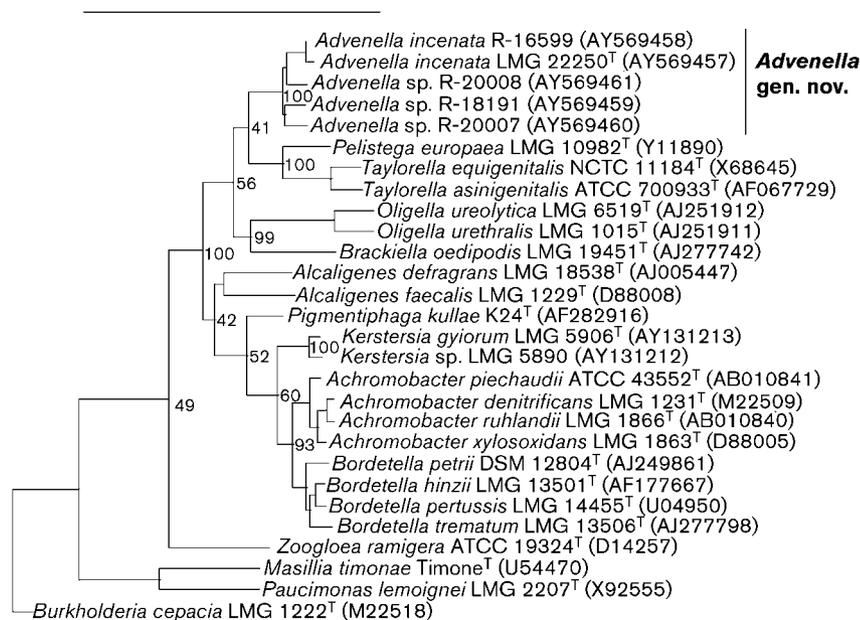


Fig. 2. Phylogenetic tree (based on 16S rRNA gene sequences) showing the position of *Advenella* gen. nov. Bar, 10 % sequence dissimilarity.

Table 2. DNA G+C content and levels of DNA–DNA relatedness of all strains examined

DNA–DNA binding values represent means of reciprocal experiments. ND, Not determined.

Strain	G+C content (mol%)	DNA binding values (%) with strain:				
		1	2	3	4	5
1. <i>Advenella incenata</i> LMG 22250 ^T	54.2	100				
2. <i>Advenella incenata</i> R-12612	54.5	97	100			
3. <i>Advenella</i> sp. R-18191	54.0	ND	37	100		
4. <i>Advenella</i> sp. R-20008	53.6	9	36	21	100	
5. <i>Advenella</i> sp. R-20007	57.7	18	11	17	22	100

these hybridizations, combined with the results of the RAPD fingerprinting and the protein analysis, showed (i) that isolates LMG 22250^T, R-12612, R-16599, R-16574, LMG 8437, LMG 8439, R-1086 and R-20004 comprise a single species, and (ii) that the other isolates listed in Table 1 represent several additional genomic species. We propose the formal name *Advenella incenata* gen. nov., sp. nov. for the eight isolates that grouped together in RAPD cluster I. Pending the availability of similar isolates, cluster II isolates LMG 16602 and R-18191 and isolates R-20008, R-20007, R-20003 and R-20005 are classified as *Advenella* spp.

Of the eight cluster I isolates, three (R-12612, R-16599 and R-16574) have virtually identical RAPD patterns. These isolates were recovered from two different CF patients, attending different treatment centres in Belgium. The two cluster II isolates (LMG 16602 and R-18191) also had similar RAPD patterns. These isolates were recovered from a single Swedish CF patient on different occasions. Although we did not perform detailed epidemiological investigations, our data suggest that these strains might be capable of prolonged infection.

Identification of members of the genus *Advenella*

Based on a limited number of conventional phenotypic tests, all the genomic species described here (including *A. incenata*) can be separated from each other (Table 3). However, members of the genus *Advenella* cannot easily be separated from members of the genera *Pelistega*, *Taylorella*, *Oligella*, *Brackiella*, *Alcaligenes*, *Achromobacter*, *Kerstersia*, *Bordetella* and *Pigmentiphaga*. A number of phenotypic characteristics useful for differentiation of the genus *Advenella* from related genera is shown in Table 4. In addition, in contrast to *Pelistega* species, *Brackiella* and several *Bordetella* species, *Advenella* spp. isolates grow well on standard media under aerobic conditions, without the need for addition of blood or specific growth factors. *Advenella* species can also be separated from related genera by means of whole-cell protein and fatty acid analysis. Finally, the DNA G+C content of members of the genus *Advenella* is considerably different from that of several related taxa (Table 4).

Description of *Advenella* gen. nov.

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 these unusual organisms is unknown).

Gram-negative, small (1–2 µm), rod-shaped or coccoid cells that occur alone, in pairs or in short chains. Motility is strain dependent. On nutrient agar, colonies are flat or slightly convex with smooth margins. Colour is light brown. Oxidase and catalase activity but no β-galactosidase activity is observed. Additional biochemical characteristics are given above (see Results and Discussion). The following fatty acid components are present in significant amounts: 12:0, 16:0, 17:0 cyclo, 18:1ω7c, SF2 and SF3. The DNA G+C content ranges from 53.6 to 57.7 mol%. Strains of this genus have been isolated from various human and veterinary clinical samples. The type species is *Advenella incenata*.

Table 3. Phenotypic characteristics useful in the differentiation of genomic groups within the genus *Advenella*

1, *A. incenata* (n=6); 2, *Advenella* sp. R-18191; 3, *Advenella* sp. R-20008; 4, *Advenella* sp. R-20007; 5, *Advenella* sp. R-20003; 6, *Advenella* sp. R-20005. +, Present; –, absent.

Characteristic	1	2	3	4	5	6
Growth on cetrimide	–	–	–	–	–	+
Reduction of:						
Nitrate	–	–	+	–	–	–
Nitrite	–	–	–	+	–	–
Assimilation of:						
Trehalose	–	–	–	–	+	+
L-Arginine	–	–	–	–	+	–
D-Mannitol	–	–	–	–	+	–
N-Acetylglucosamine	–	–	–	–	+	–
L-Malate	+	–	+	+	+	+
Caprate	–	–	–	–	–	+
Alkaline phosphatase activity	+	+	+	+	–	+

Table 4. Characteristics useful in the differentiation of the genus *Advenella* from related genera

Genera: 1, *Advenella* gen. nov.; 2, *Pelistega*; 3, *Taylorella*; 4, *Oligella*; 5, *Brackiella*; 6, *Alcaligenes*; 7, *Achromobacter*; 8, *Kerstersia*; 9, *Bordetella*; 10, *Pigmentiphaga*. +, Present; -, absent; v, strain-dependent; ND, not determined. Data for reference taxa were taken from Vandamme *et al.* (1998), Kilian & Biberstein (1984), Jang *et al.* (2001), Rossau *et al.* (1987), Willems *et al.* (2002), Kersters & De Ley (1984), Yabuuchi *et al.* (1998), Coenye *et al.* (2003a, b), Pittman (1984), von Witzingerode *et al.* (2001) and Blümel *et al.* (2001).

Characteristic	1	2	3	4	5	6	7	8	9	10
Oxidase	+	+	+	+	+	+	+	-	+	+
Growth in 3% NaCl	+	ND	-	+	ND	+	+	+	ND	ND
Assimilation of:										
Citrate	+	v	-	v	ND	+	v	+	+	+
Phenylacetate	-	-	ND	ND	ND	+	+	+	v	-
DNA G+C content (mol%)	53–58	42–43	36–38	46–48	ND	55–60	63–69	61–63	66–70	68–69

Description of *Advenella incenata* sp. nov.

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 is the same as for the genus. In addition, it does not reduce nitrate or nitrite, does not assimilate trehalose, L-arginine, D-mannitol, N-acetylglucosamine or caprate and does not grow on cetrinamide agar. Assimilates L-malate and shows alkaline phosphatase activity. The DNA G+C content ranges from 54.0 to 54.5 mol%.

The type strain, LMG 22250^T (=CCUG 45225^T), was isolated from human sputum in Sweden in 2001. Characteristics for the type strain are as described above for the species. Its DNA G+C content is 54.2 mol%. Strains LMG 22250^T, LMG 8437 and LMG 8439 have been deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Gent, Belgium).

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