NOTE

***Cellulomonas persica* sp. nov. and *Cellulomonas iranensis* sp. nov., mesophilic cellulose-degrading bacteria isolated from forest soils**

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Two newly described species of mesophilic, cellulose-degrading, aerobic bacteria were isolated from forest humus soils along the southern border of the Caspian Sea. *Cellulomonas persica* and *Cellulomonas iranensis* are proposed as new specific epithets based on comparative sequence analyses of 16S rDNA, DNA–DNA hybridization and phenotypic characteristics. Formal species descriptions are provided.

Keywords: *Cellulomonas persica*, *Cellulomonas iranensis*, cellulase, soil, 16S rRNA

Three strains of cellulolytic bacteria were isolated from forest humus soil as described previously (Malekzadeh *et al.*, 1993). Two of the isolates, strains GG and I^T, were characterized as *Cellulomonas* spp. based on selected phenotypic characteristics, including coryneform morphology, the ability to hydrolyse cellulose and the presence of ornithine in the cell wall (Malekzadeh *et al.*, 1993). An additional isolate not previously described, strain O^T, was isolated concurrently with strains GG and I^T and has phenotypic characteristics similar to those of strain I^T (Table 1).

Phylogeny of the isolated *Cellulomonas* spp. was established by comparative analysis of 16S rDNA and by DNA–DNA hybridization. DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Ausubel *et al.*, 1995). Residual RNA was removed by RNase A treatment. The resultant high-

molecular-weight DNA was precipitated by adding 0.6 vol. 2-propanol, spooled onto a glass rod, washed twice with cold 70% (v/v) ethanol and allowed to air dry. The DNA recovered was dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). The purity of each DNA preparation was such that the A_{260}/A_{280} ratio was 1.8–1.9. The gene encoding 16S rRNA was amplified between positions 29 and 1541 (*Escherichia coli* positions) by PCR using the primer combination 5'-GAGTTTGATCCTGGCTCAG-3' (forward primer) and 5'-AAGGAGGTGATCCAGCC-3' (reverse primer) (Achenbach & Woese, 1995; Rainey *et al.*, 1995). Reaction mixtures contained the following components in 20 µl (final volume) in a 200 µl dome top reaction tube: 1× PCR buffer (Perkin Elmer), 200 µmol of each deoxyribonucleoside triphosphate, 15 mM MgCl₂, 0.025% (v/v) formamide, 20 pmol of each primer, 100 ng chromosomal DNA and 0.25 U *Taq* (Ampli*Taq*; Perkin Elmer). PCR reactions were controlled with a Peltier thermocycler (PTC 200; MJ Research), programmed as follows: initial hold at 95 °C for 15 s; 30 cycles at

The GenBank/EMBL accession numbers for the 16S rDNA sequences of strains GG, I^T and O^T are AF140036, AF064701 and AF064702, respectively.

Table 1. Phenotypic characteristics that differentiate strains of *Cellulomonas*

Strains: 1, strain GG; 2, strain I^T; 3, strain O^T; 4, *C. biazotea* (type strain); 5, *C. cellasea* (type strain); 6, *C. cellulans* (type strain); 7, *C. flavigena* (type strain); 8, *C. fimi* (type strain); 9, *C. gelida* (type strain); 10, *C. uda* (type strain). ND, Not determined; +, positive; ++, strongly positive; w⁺, weakly positive; −, negative.

Phenotypic character*	Strain									
	1	2	3	4	5	6	7	8	9	10
Peptidoglycan amino acid†	OG	OG	OG	OG	OG	LSA	OA	OG	OG	OG
Cell wall composition										
Glucose	+	+	+	−	−	−	+	−	+	+
Glucosamine	++	++	++	++	−	−	++	++	++	+
Mannose	+	+	+	−	+	−	+	−	−	−
Rhamnose	++	++	++	++	++	++	−	++	−	−
Growth substrates										
Acetate	w ⁺	+	+	+	+	+	+	−	+	+
Dextrin	+	+	+	−	−	ND	+	+	+	+
Gluconate	−	−	−	−	−	+	+	−	−	−
Lactate	−	−	−	+	+	+	−	+	−	−
Lactose	+	−	−	+	ND	+	+	+	+	+
Raffinose	−	−	−	+	−	−	−	−	−	−
Mannose	−	+	+	+	ND	+	+	+	+	+
D-Ribose	−	−	−	−	−	+	+	−	−	−
Gelatin hydrolysis	w ⁺	w ⁺	w ⁺	+	+	ND	+	+	+	+
Urea hydrolysis	−	+	+	−	ND	ND	−	−	−	−
Motility	+	+	+	+	−	ND	−	+	+	−
Colony pigment‡	Y	Y-W	Y-W	Y-W	Y	Y-W	Y	Y-W	Y-W	W

* Phenotypic characteristics of described species are from Malekzadeh *et al.* (1993), Stackebrandt & Kandler (1979), Stackebrandt & Kiedde (1986) and Yamada & Komagata (1972).

† OG, L-Ornithine-D-glutamyl; OA, L-ornithine-D-aspartyl; LSA, L-lysine-D-serine-D-aspartyl.

‡ Y, yellow; Y-W, yellow-white; W, white.

94 °C for 15 s, 53 °C for 30 s, 72 °C for 60 s; and final hold at 72 °C for 5 min. Plasmid libraries were generated by directly ligating PCR fragments into plasmid pCRII (Invitrogen) according to the manufacturer's recommendations. The plasmid libraries were screened for clones containing 16S rDNA inserts by direct PCR of colonies (Elbertson & Sowers, 1997). For sequencing, plasmid DNA from selected clones was purified with a Qiagen Plasmid Mini kit and amplified with an ABI Prism Dye Terminator Cycle Sequencing reaction kit (Perkin Elmer) according to the manufacturer's recommendations. Both strands of 16S rDNA (1511 bp) were sequenced using flanking and nested primers. Flanking primers included T7 and SP6. Nested primers were universal for *E. coli* 16S rRNA positions 519–536f, 549–531r, 907–926f, 926–907r and 1406–1392r (Achenbach & Woese, 1995; Lane *et al.*, 1985). The reaction mixture was loaded onto an ABI 373 automated sequencer and the sequence was analysed using ABI Prism version 2.1.1. Levels of genomic relatedness were determined by DNA–DNA dot blot hybridization (Marteisson *et al.*, 1995). Duplicate aliquots containing 100 and 200 ng of each genomic DNA were denatured by boiling for

10 min in 6 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and transferred onto Nylon membranes (MagnaGraph) using a dot blot apparatus (Bio-Rad). Prehybridization was carried out for 30 min at 60 °C and hybridization was performed at the same temperature in a buffer solution containing 5 × SSC and formamide (35%, v/v). Chromosomal DNA of the test strains was denatured by boiling and labelled using DIG High Prime DNA Labelling and Detection Starter kit II (Boehringer Mannheim). The resultant DNA was added (10 ng ml^{−1}) to the pre-hybridized membranes in hybridization buffer and the preparations were incubated for 16 h. Hybridization was performed at 60 °C overnight and the hybridized membranes were washed according to the manufacturer's instructions. Chemoluminescence was detected using a detection kit (Boehringer Mannheim) and exposed to Hyperfilm (Eastman Kodak). The chemoluminescent intensity of each blot was quantified using a densitometer. The signal produced by self-hybridization of the probe with homologous target DNA was taken as 100% and the percentage hybridization values were calculated for the duplicated slots.

Fig. 1. Comparative sequence analyses of 16S rDNA from *Cellulomonas flavigena* GG, *Cellulomonas persica* I^T, *Cellulomonas iranensis* O^T and representative strains from GenBank. The dendrogram was constructed from evolutionary distance matrices with TREECON software using parameters indicated in the text (Van de Peer & De Wachter, 1994). The significance of each branch is indicated by a bootstrap value calculated for 100 subsets. Bar, 0.02 substitutions/site. The GenBank accession numbers for the sequences used to generate the phylogenetic tree are as follows: *Cellulomonas biazotea* DSM 20112^T, X83802; *Cellulomonas cellasea* DSM 20118^T, X83804; *Cellulomonas fermentans* DSM 3133^T, X83805; *Cellulomonas fimi* DSM 20113^T, X83803; *Cellulomonas flavigena* DSM 20109^T, X83799; *Cellulomonas gelida* DSM 20111^T, X83800; *Cellulomonas uda* DSM 20107^T, X83801; *Cellulomonas turbata* DSM 20577^T, X83806; and *Cellulomonas* sp. CE40, X82598.

The 16S rDNA sequences were aligned with representative 16S rDNA sequences in the GenBank/EMBL database using PILEUP (Genetics Computer Group, V.8). Based on comparison of a 1450 bp segment of rDNA (*E. coli* positions 41–1512), isolate GG showed a high level of similarity (99.7%) to *Cellulomonas flavigena*. Evolutionary distances expressed as estimated changes per 100 nucleotides were calculated from the percentage similarities by the correction of Jukes & Cantor (1969). A dendrogram based on the unweighted pair group method using arithmetic means (UPGMA) showed significant phylogenetic relatedness between strains GG and *Cellulomonas flavigena*, indicated by a bootstrap value of 100% (Fig. 1). Comparative analysis of 1450 bp rDNA segments from isolates I^T and O^T showed 97.2% similarity with a bootstrap value of 100%. Isolate I^T showed phylogenetic relatedness to *C. flavigena*, with bootstrap values of 90% and a similarity value of 97.6%; isolate O^T showed phylogenetic relatedness to *Cellulomonas uda* and *Cellulomonas gelida*, with a bootstrap value of 80% and similarity values of 98.0 and 98.4%, respectively (Fig. 1).

It is proposed that strains GG, I^T and O^T be placed in the genus *Cellulomonas*, based on their coryneform morphology, cellulolytic activity and the presence of ornithine in the cell wall (Malekzadeh *et al.*, 1993). Significant phylogenetic relatedness, derived from comparative 16S rDNA sequence analysis, indicates that isolates GG and I^T group with *C. flavigena*. A DNA–DNA hybridization value of 97% supports designation of strain GG as *C. flavigena* (Table 2). Phenotypic characteristics exhibited by strain GG that differentiate it from the type strain of *C. flavigena* include motility and lactose fermentation (Table 1). In contrast, strain I^T has a DNA–DNA hybridization value of only 62% with *C. flavigena*. Phenotypic characteristics exhibited by strain I^T that differentiate

Table 2. DNA hybridization among strains of *Cellulomonas*

Strain	DNA hybridization (%) with DIG-labelled reference DNA from strain:		
	<i>C. flavigena</i>	<i>C. uda</i>	<i>C. gelida</i>
<i>C. flavigena</i>	100	56	17
<i>C. uda</i>	30	100	24
<i>C. gelida</i>	41	66	100
<i>Cellulomonas</i> sp. GG	97	37	18
<i>Cellulomonas</i> sp. I ^T	62	54	48
<i>Cellulomonas</i> sp. O ^T	67	39	19

it from the type strain of *C. flavigena* include colony colour, urea hydrolysis, and fermentation of mannose and trehalose. Comparative 16S rDNA sequence analysis indicates that isolate O^T groups with *C. uda* and *C. gelida*. However, DNA–DNA hybridization values of 39 and 19% with *C. uda* and *C. gelida*, respectively, differentiate strain O^T from these species. Phenotypic characteristics exhibited by strain O^T that differentiate it from the type strains of *C. uda* and *C. gelida* include colony colour, urea hydrolysis, fermentation of mannose and the presence of aspartate in the cell wall. It is proposed that strains I^T and O^T be placed in the newly described species of *Cellulomonas*, *Cellulomonas persica* and *Cellulomonas iranensis*, respectively. I^T and O^T are type strains for these species; descriptions are provided below.

Description of *Cellulomonas persica* sp. nov.

Cellulomonas persica (per'si.ca. L. fem. adj. *Persica* Persian, from Persia, classical name of Iran, where the organism was isolated).

Irregular, straight or slightly curved rods 0.6×2.0 – $4.0 \mu\text{m}$, which occasionally exhibit typical coryneform branching. Cells are motile, with one to four peritrichous flagella. Gram-positive, but readily decolorized. Colonies are light yellow, ca. 1 mm in diameter, circular, convex and glossy. Optimal growth temperature is 30°C . Optimal pH for growth is 7.2–7.4. Additional characteristics that differentiate this species include the ability to produce acid from dextrin and the inability to utilize D-ribose or raffinose. Starch and gelatin are hydrolysed, the latter weakly. Nitrate is reduced to nitrite; DNase produced. Urea hydrolysed as N source in cellulose fermentation. Cell wall is of the L-Orn-D-Asp type and contains the sugars rhamnose, glucosamine and minor amounts of mannose. Source of the isolate is forest humus soil located under walnut, fig, hornbeam, mimosa and box trees in Ramsar Forest, 1.6 km south of Ramsar, Iran, along the border of the Caspian Sea. Type strain I^T has been deposited as ATCC 700642^T.

Description of *Cellulomonas iranensis* sp. nov.

Cellulomonas iranensis (i.ran.en'sis. M.L. adj. *iranensis* from Iran, where the organism was isolated).

Irregular, straight or slightly curved rods 0.4×2.5 – $4.0 \mu\text{m}$, which occasionally exhibit typical coryneform branching. Cells are motile, with one to four peritrichous flagella. Gram-positive, but readily decolorized. Colonies are light yellow, ca. 1 mm in diameter, circular, convex and glossy. Optimal growth temperature is 30°C . Optimal pH for growth is 7.2–7.4. Additional characteristics that differentiate this species include the ability to produce acid from dextrin and the inability to utilize D-ribose or raffinose. Starch and gelatin are hydrolysed, the latter weakly. Nitrate is reduced to nitrite; DNase produced. Urea hydrolysed as N source in cellulose fermentation. Cell wall is of the L-Orn-D-Asp type and contains the sugars rhamnose, glucosamine and minor amounts of mannose. Source of the isolate is forest humus soil located under walnut, fig, hornbeam, mimosa and box trees in Ramsar Forest, 1.6 km south of Ramsar, Iran, along the border of the Caspian Sea. Type strain O^T has been deposited as ATCC 700643^T.

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