Plasticicumulans acidivorans gen. nov., sp. nov., a polyhydroxyalkanoate-accumulating gammaproteobacterium from a sequencing-batch bioreactor

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Here, we describe a novel bacterium, strain TUD-YJ37^T, which can accumulate polyhydroxybutyrate (PHB) to more than 85 % (w/w) dry cell weight. The bacterium was isolated from a mixed-culture bioreactor by using a feast–famine regime and its properties were characterized. Phylogenetic analysis based on full 16S rRNA gene sequences revealed that the isolate is a member of the *Gammaproteobacteria*, forming an independent, deep phylogenetic lineage. It is most closely related to members of the genera *Methylocaldum*, *Methylococcus* and *Natronocella*, with sequence similarities below 91 %. Strain TUD-YJ37^T was an obligately aerobic, ovoid, Gram-negative bacterium, motile by means of a polar flagellum. It utilized C₂–C₁₀ fatty acids as carbon and energy sources. The temperature range for growth was 20–35 °C, with an optimum of 30 °C; the pH range was 6.0–8.0, without a clear optimum. The major respiratory quinone was Q-8. Polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids, an unidentified aminolipid and another unidentified lipid. The predominant fatty acids in the membrane polar lipids were C_{16:1ω7c}, C_{16:0} and C_{18:1ω7c}. The G + C content of the genomic DNA was 67.4 mol%. On the basis of phenotypic, chemotaxonomic and molecular data, the isolate is proposed to represent a novel genus and species, for which the name *Plasticicumulans acidivorans* gen. nov., sp. nov. is proposed. The type strain of *Plasticicumulans acidivorans* is TUD-YJ37^T (=DSM 23606^T =CBS 122990^T).

The intracellular accumulation of polyhydroxyalkanoates (PHAs) is an important ecological feature of bacteria that gives them a selective advantage under varying environmental conditions, especially feast–famine conditions (van Loosdrecht *et al.*, 1997). PHA is also of substantial commercial interest as a raw material for biodegradable plastic (Braunegg *et al.*, 1998; Steinbüchel, 2001). Since genetically engineered bacteria are usually used for the production of PHAs under sterile conditions with defined growth substrates, bioplastics are relatively expensive. An alternative approach could be eco-biotechnology, which aims to produce products by employing mixed cultures under non-sterile conditions, waste streams as substrates

and ecological selection principles to select for the organisms of interest (Kleerebezem & van Loosdrecht, 2007). In this way, the costs of the processes could be reduced substantially. The often-assumed disadvantage of the use of mixed cultures for PHA production is a relatively low production yield. At the time of writing, the highest reported PHA yields were 90 % (w/w) of cell dry weight from a genetically modified strain of *Escherichia coli* (Slater *et al.*, 1988) and only 65 % (w/w) of dry weight in a mixed culture (Serafim *et al.*, 2004).

In this paper, we describe a novel PHA-producing bacterium that dominated a mixed-culture bioreactor and which represents a new genus and species within the *Gammaproteobacteria* on the basis of its distinct phenotypic properties and phylogeny.

A 2.5 l bioreactor was inoculated with active sludge from a second aerobic stage in the Dokhaven wastewater

Abbreviations: PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S-rRNA gene sequence of strain TUD-YJ37^T is GU206550.

A supplementary figure is available with the online version of this paper.

treatment plant in Rotterdam, The Netherlands (September 2004), and was maintained under non-sterile conditions. It was fed with sodium acetate as sole carbon and energy source in a sequencing-batch mode. The reactor was operated at 30 °C and maintained at pH 7.0 ± 0.05 by using 1 M HCl and 1 M NaOH (Johnson *et al.*, 2009). The concentration of dissolved oxygen (DO) was measured with a DO electrode (Mettler Toledo) and the pH was monitored with a pH electrode (Mettler Toledo). DO varied between 30 and 100 % during operation of the reactor. One of the mixed cultures enriched under these conditions was able to generate PHA to 89 % (w/w) of dry cell weight (Johnson *et al.*, 2009). It was dominated by relatively large ovoid bacteria with a very high content of storage material.

In an attempt to isolate the dominant bacterium, several traditional methods, such as serial dilution-to-extinction and plating, were used, without much success. Therefore, a more sophisticated approach was tried that relied on the physical separation of single cells from a mixed culture using alginate gel beads (Sarma *et al.*, 1998). For this, the reactor biomass was homogenized in a potter tube and serially diluted and cells were mixed with 10 ml sterile sodium alginate solution (1.5 % w/v). The mixture was then dropped through a syringe needle into 1 % (w/v) CaCl_2 solution kept on ice. Beads were kept in this cold solution for about 1 h to harden and then washed with sterile mineral medium. One hundred of the roundest beads were selected randomly for further sequencing-batch cultivation consisting of alternating incubations in the mineral medium (2 h) and acetate-free mineral medium (12 h) for 3 days. Individual beads showing formation of microcolonies were harvested, washed in sterile mineral medium and placed in wells of a microtitre plate containing mineral medium. The plate was subsequently incubated at 30 °C. Eventually, individual beads with colonies were homogenized and cells were plated onto mineral agar plates.

The composition of the mineral medium used for isolation was as follows: 12.5 mM sodium acetate trihydrate, 5 mM NH_4Cl , 0.1 mM KH_2PO_4 , 0.56 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.075 % (v/v) trace element solution (Vishniac & Santer, 1957). The acetate-free mineral medium contained the same components as mineral medium but without sodium acetate. All media were autoclaved at 121 °C for 20 min before use.

A microcolony from one of the incubated beads resulted in a positive culture dominated by a morphotype similar to the reactor biomass. These cells formed very distinctive, round, dome-like, white-yellowish colonies on mineral agar plates. They were purified by repetitive streaking on solid medium. The resulting isolate was designated strain TUD-YJ37^T. When freshly isolated, the strain exhibited two types of colony after repetitive plating from liquid culture. One was similar to the original wild-type colony type, and the other was smoother and flat (Fig. 1a, b). These two

Fig. 1. Micrographs of colonies and cells of strain TUD-YJ37^T. (a) Dome-like rough-type colony; (b) flat smooth-type colony; (c) aggregated coccoid cells from dome-like rough-type colony; (d) elongated cells from a flat smooth-type colony. (e) Cells from bioreactor containing PHB granules; (f) electron micrograph of cells containing PHB granules. Bars, 10 µm (c–e) and 0.5 µm (f).

colony types also corresponded to different modes of growth in liquid culture; the dome-like colonies were mostly formed from aggregated coccoid cells and the flat, smooth type were mostly formed from free elongated cells (Fig. 1c, d). After prolonged maintenance in liquid culture, the culture shifted entirely to the latter variant. The cell morphology of strain TUD-YJ37^T varied from coccoid in the reactor biomass to ovoid in freshly isolated cultures; cells were $1.5\text{--}1.8 \times 2\text{--}3.5$ µm, rarely motile and generally contained large globules of polyhydroxybutyrate (PHB) material (Fig. 1e, f). The alkali lysis test and electron microscopy of thin-sectioned cells revealed a Gram-negative cell-wall type.

The phylogenetic position of the isolate was studied using 16S rRNA gene sequence analysis. Genomic DNA was extracted from strain TUD-YJ37^T using the Ultra Clean soil DNA extraction kit (MoBio Laboratories) following the manufacturer's instructions. Nearly 1400 bp of the 16S

rRNA gene was amplified by using the universal bacteria primers GM3 (5'-AGAGTTTGATCMTGGC-3') and GM4 (5'-TACCTTGTACGACTT-3'). The DNA fragment obtained was sequenced by Macrogen (Seoul, South Korea) by using primers GM3, GM4, 341F (5'-CCTACGGGAGGCAGCAG-3'), 907F (5'-AAACTCAAAGGAATTGACGG-3') and 907RM (5'-CCGTCAATTCMTTGTAGTTT-3'). Sequence similarity was initially analysed using BLAST. The highest similarity was obtained with uncultured bacterial clones from active sludge in Australia (Schroeder *et al.*, 2009). In addition, a similarity analysis was performed against sequences of type strains on the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The nearest cultured relatives were members of the genera *Methylocaldum* (highest sequence similarity 91.0%), *Methylococcus* (90.6%), *Natronocella* (90.5%), *Thiocapsa* (90.2%), *Thioalkalivibrio* (90.1%), *Nitrosococcus* (90.1%) and *Ectothiorhodospira* (90.1%). A phylogenetic tree was reconstructed from the alignment using the ARB software program (Ludwig *et al.*, 2004) and the neighbour-joining algorithm with Felsenstein's correction (Fig. 2).

Respiratory quinones and polar lipids were analysed according to the method described by Tindall (1990a, b) by the Identification Service of the DSMZ and Dr B. J. Tindall (DSMZ, Braunschweig, Germany). The main quinone component was Q-8 (>96%), which is characteristic of many gammaproteobacteria (Yokota *et al.*, 1992). Major polar lipids were phosphatidylglycerol and phosphatidylethanolamine, with diphosphatidylglycerol, three unidentified phospholipids, an unidentified aminolipid and another unidentified lipid present in smaller amounts (Supplementary Fig. S1, available in IJSEM Online).

For analysis of fatty acid methyl esters, polar lipids were extracted from dry cell material with acidic methanol. The resulting methyl esters were analysed by GC-MS using the Sherlock Microbial Identification system (MIDI Inc.) according to Zhilina *et al.* (1997). The major cellular fatty

acids of strain TUD-YJ37^T were C_{16:1}ω7c (43%), C_{16:0} (30%) and C_{18:1}ω7c (18%), typical of proteobacteria. Table 1 shows the detailed fatty acid composition of strain TUD-YJ37^T.

The G + C content of genomic DNA extracted from 1 g wet cell biomass according to Marmur (1961) was determined by the thermal denaturation technique (Marmur & Doty 1962). The value obtained was 67.4 mol%.

Physiological characteristics of strain TUD-YJ37^T were examined in batch liquid culture. The organism grew on acetate as substrate at 20–35 °C (optimum 30 °C) and at pH 6–8. Growth and respiration of cells were inhibited completely by NaCl and KCl at concentrations above 150 mM. The bacterium could not grow anaerobically with nitrate, but could reduce nitrate to nitrite. Substrate utilization tests were performed in liquid acetate-free mineral medium mixed with carbon sources listed in Table 2. The results demonstrated that strain TUD-YJ37^T was specialized for use of simple fatty acids (C₂–C₁₀) and was able to use pyruvate, succinate, lactate, fumarate and ethanol as carbon sources. PHA granules in cells could be observed with different tested fatty acids. Maximum PHB content (89% w/w) was obtained when acetate was the sole carbon source and ammonia was deficient. With acetate as carbon source, strain TUD-YJ37^T could use ammonia, nitrate and complex organic nitrogen (yeast extract) as nitrogen sources. Oxidase [1% (w/v) tetramethyl *p*-phenylenediamine] and catalase [3% (v/v) H₂O₂] tests were positive. The most important phenotypic characteristics of strain TUD-YJ37^T are compared with those of its closest relatives in Table 2.

The overall results of phenotypic and phylogenetic analyses suggest that the novel PHA-accumulating bacterium from a bioreactor is substantially different from other gamma-proteobacteria described so far. Therefore, it is proposed to be accommodated in a novel genus and species, *Plasticicumulans acidivorans* gen. nov., sp. nov.

Fig. 2. Neighbour-joining tree reconstructed from 16S rRNA gene sequences showing phylogenetic relationships between strain TUD-YJ37^T and selected members of the Gammaproteobacteria. Bootstrap values >50% are indicated at branch-points. Bar, 0.1 substitutions per nucleotide position.

Table 1. Cellular fatty acids of strain TUD-YJ37^T

Values are percentages of total fatty acids. Fatty acids in bold represent predominant types.

Fatty acid	Proportion (%)
C _{12:0}	1.2
C _{12:0} 3-OH	0.2
iso-C _{14:0}	0.1
C _{14:1} ω6	1.4
C _{14:0}	2.7
C _{15:0}	0.2
iso-C _{16:0}	0.3
C_{16:1}ω7c	43.3
C _{16:1} ω7t	0.4
C_{16:0}	29.9
C _{18:1} ω9	0.2
C_{18:1}ω7c	18.0
C _{18:1} ω5t	0.1
C _{18:0}	1.7
C _{18:1} 3-OH	0.1
Total	100.0

Description of *Plasticicumulans* gen. nov.

Plasticicumulans (Plas'ti.ci.cu'mu.lans. N.L. n. *plasticum* plastic; L. part. adj. *cumulans* accumulating; N.L. part. adj. used as a masc. n. *Plasticicumulans* accumulating plastic).

Gram-negative ovoids, obligately aerobic and heterotrophic, with preference for simple fatty acids (C₂–C₁₀) as carbon and energy sources. High capacity to store PHAs. Mesophilic and neutrophilic. Identified membrane polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids, an unidentified aminolipid and another unidentified lipid. The major cellular fatty acids are C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. The DNA G+C content of the type strain of the type species is 67.4 mol%. Belongs to the *Gammaproteobacteria*, with the genera *Methylocaldum*, *Methylococcus* and *Natronocella* as closest phylogenetic neighbours. The type species is *Plasticicumulans acidivorans*.

Description of *Plasticicumulans acidivorans* sp. nov.

Plasticicumulans acidivorans (a.ci.di.vo'rans. N.L. n. *acidum* an acid; L. part. adj. *vorans* eating, devouring; N.L. part. adj. *acidivorans* acid-devouring).

Table 2. Comparison of the properties of strain TUD-YJ37^T and its nearest phylogenetic relatives

Taxa: 1, strain TUD-YJ37^T; 2, *Methylocaldum* (data for three species from Bodrossy *et al.*, 1997); 3, *Methylococcus* (eight species; Hazeu *et al.*, 1980; Bowman *et al.*, 1993); 4, *Natronocella acetinitrilica* (Sorokin *et al.*, 2007); 5, *Thiocapsa* (six species; Imhoff *et al.*, 1982; Imhoff, 1984; Asao *et al.*, 2007); 6, *Thioalkalivibrio* (nine species; Sorokin *et al.*, 2001); 7, *Nitrosococcus* (two species; Watson, 1971); 8, *Ectothiorhodospira* (ten species; Asselineau & Trüper, 1982; Imhoff, 2006; Gorlenko *et al.*, 2009). Numbers in parentheses represent numbers of species giving the result shown. ND, No data available.

Characteristic	1	2	3	4	5	6	7	8
16S rRNA gene sequence similarity to strain TUD-YJ37 ^T (%)*	(100)	91.0	90.6	90.5	90.2	90.1	90.1	90.1
Cell shape(s)†	CD, OD	C, PR	C, R	R	C	R, SP	C, E	O, R
Nutritional type‡	COH§	OM	OM	COH	ANP	OCLA	OCLA	ANP
Halophily	—	ND	+	+	—	+	+	+
Alkaliphily	—	ND	+	+	+	+	—	+
Thermophily	—	+	+	ND	—	+	—	—
Denitrification	—	ND	—	—	ND	+	ND	ND
Reduction of nitrate to nitrite	+	ND	+	+	ND	+	ND	ND
Major quinone(s)¶	Q-8	ND	MQ-8	ND	Q-8	Q-8	ND	MK-7, Q-7 (or Q-8)
Dominant phospholipid fatty acids	C _{16:1} , C _{16:0} , C _{18:1}	C _{16:0} , C _{16:1}	C _{16:0} , C _{16:1}	C _{18:1} , C _{16:0}	ND	ND	ND	C _{18:1} , C _{16:0} , C _{16:1}
Major polar lipids¶	DPG, PG, PE, 3PL, AL, L	ND	ND	ND	PG, CL, PE	ND	ND	CL, PG, PE, PC
DNA G+C content (mol%)	67.4	56.5–59	56.3–66	50.6–51.5	62.7–66.3	61.0–65.6	50.5–51.0	61.4–68.4

*Similarity between strain TUD-YJ37^T and the type strain of the type species.

†C, Coccus; CD, coccoid; E, ellipsoid; O, oval; OD, ovoid; PR, pleomorphic rod; R, rod; SP, spirillum.

‡ANP, Anaerobic phototroph; COH, chemo-organoheterotroph; OCLA, obligate chemolithoautotroph; OM, obligate methanotroph.

§See species description for carbon and energy sources.

¶MK, Menaquinone; MQ, methyleneubiquinone; Q, ubiquinone.

¶CL, Cardiolipin; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; PL, unidentified phospholipid; L, unidentified lipid.

Displays the following properties in addition to those given for the genus. Cell shape varies from coccoid to ovoid, $1.5\text{--}1.8 \times 3\text{--}5\text{ }\mu\text{m}$. Forms two colony variants, dome-like rough and flat smooth, whitish-yellowish, up to 3 mm in diameter after 72 h of incubation at $30\text{ }^{\circ}\text{C}$. Accumulates up to 89 % (w/w) PHA, with storage polymers in the form of large intracellular granules. Utilizes the following organic compounds as carbon and energy sources: acetate, propionate, butyrate, valerate, caproate, heptanoate, octanoate, nonanoate, decanoate, pyruvate, succinate, lactate, fumarate, ethanol, malate and yeast extract. The following compounds are not utilized: hexoses (including glucose), pentoses (including arabinose), methanol, formate and H_2 . Ammonium and nitrate serve as nitrogen sources. Oxidase and catalase are positive. Hydrolysis of aesculin and gelatin is negative. Grows at $20\text{--}35\text{ }^{\circ}\text{C}$ (optimum $30\text{ }^{\circ}\text{C}$) and pH 6–8. Salt-sensitive; growth and respiration are inhibited in the presence of 150 mM NaCl or KCl. Nitrate is reduced to nitrite. The major respiratory quinone is Q-8.

The type strain is TUD-YJ37^T (=DSM 23606^T =CBS 122990^T), isolated from a sequencing-batch bioreactor fed with acetate.

Acknowledgements

These investigations were supported by the Netherlands Organization for Scientific Research (NWO) in the NWO-ACTS research programme B-Basic and by the Foundation for Technical Sciences (STW). We thank our co-workers, Ben Abbas, Kees van Sluis and Samira Mohamad, for their help in this study.

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