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# *Clostridium amylolyticum* sp. nov., isolated from $H_2$-producing UASB granules

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A Gram-stain-positive, strictly anaerobic, mesophilic, amylolytic, rod-shaped bacterium, designated strain SW408$^T$, was isolated from a laboratory-scale $H_2$-producing upflow anaerobic sludge blanket reactor. The strain grew at 24–45 °C (no growth at or below 22 °C or at or above 47 °C), with optimum growth at 37 °C. The pH range for growth was 4.0–9.0 (no growth at or below pH 3.6 or at or above pH 9.3), with optimum growth at pH 7.0. Starch, cellobiose, glucose, fructose, galactose, lactose, maltose, mannose, ribose and sucrose supported growth. The major end products from glucose fermentation were ethanol, acetate, hydrogen and carbon dioxide. Abundant $H_2$ was produced from starch fermentation. The DNA G+C content was 33.1 mol% ($T_m$ method). Phylogenetic analysis based on 16S rRNA gene sequence analysis showed that the bacterium represents a previously unrecognized species within *Clostridium* rRNA cluster I and is most closely related to the type strain of *Clostridium frigidicarnis* (94.9 % similarity). On the basis of phenotypic, genotypic and phylogenetic characteristics, strain SW408$^T$ was identified as a representative of a novel species of the genus *Clostridium*, for which the name *Clostridium amylolyticum* sp. nov. is proposed. The type strain is SW408$^T$ (=JCM 14823$^T$=AS 1.5069$^T$).

During a survey of the microbial community in hydrogen-producing granules of a laboratory-scale upflow anaerobic sludge blanket (UASB) reactor for treating sucrose-rich synthetic wastewater, a novel Gram-positive anaerobic bacterium, strain SW408$^T$, was isolated that generated a large amount of $H_2$ from the fermentation of starch and other saccharides. Phylogenetically, the strain was affiliated to *Clostridium* rRNA cluster I; however, it was distantly related to recognized members of this cluster. The characterization and taxonomic position of the strain was studied.

Strain SW408$^T$ was isolated in pre-reduced basal medium, described by McInerney *et al.* (1979), supplemented with 1 % glucose as substrate by serial dilution and the Hungate roll-tube technique (Hungate, 1969). Routine cultivation was in the same medium in anaerobic tubes sealed with butyl rubber stoppers under a gaseous atmosphere of 100 % $N_2$ (100 kPa) at 37 °C. A single colony was picked and transferred to the same broth and incubated at 37 °C for 24 h. Culture purity was checked by microscopic examination. Colonies of strain SW408$^T$ were irregular, grey, flat to raised, translucent and measured 1.2 mm in diameter after cultivation at 37 °C for 48 h.

Gram-staining was carried out according to Johnson *et al.* (1995). Cell morphology was examined by light microscopy (BH-2; Olympus) as well as by electron microscopy (H-600A; Hitachi) using cells that were negatively stained with uranyl acetate. Cells of strain SW408$^T$ were Gram-stain-positive rods of 0.5–0.7 × 2.0–7.5 μm, occurring singly or in short chains and motile by peritrichous flagella (see Supplementary Fig. S1 available in IJSEM Online). The Gram-positive reaction of the cell wall was confirmed by the KOH lysis test (Smibert & Krieg, 1994). Strain SW408$^T$ grew exclusively in pre-reduced media and growth was completely inhibited by air. Spores were not detected either by microscopy or after heat treatment at 80 °C for 10 min.

The growth temperature range of strain SW408$^T$ was determined using a water bath (Guangming Medical Instrument Plant) at 25–45 °C, with optimum growth at 37 °C. No growth was detected at or below 22 °C or at or above 47 °C. The pH range for growth of strain SW408$^T$ was 4.0–9.0, with an optimum of pH 7.0, in medium adjusted to different pH values with HCl or NaOH (1 mol $l^{-1}$). No growth was detected at or below pH 3.6

Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Clostridium amylolyticum* SW408$^T$ is EU037903.

An electron micrograph of a negatively stained cell of strain SW408$^T$ (Fig. S1) and a table showing the cellular fatty acid composition of strain SW408$^T$ and phylogenetically related clostridia (Table S1) are available with the online version of this paper.

or at or above pH 9.3. Biochemical properties of strain SW408$^T$ were determined using both conventional methods (Kämpfer *et al.*, 1991) and the API 50CH system (bioMérieux). Strain SW408$^T$ utilized several kinds of mono- and di-saccharides and exhibited identical biochemical properties using both test methods, although weak positive rather than positive results were obtained using the API 50CH system for aesculin hydrolysis and acid production from galactose and rhamnose. A detailed profile of fermentable sugars is given in the species description. Strain SW408$^T$ required yeast extract and peptone for growth and inorganic nitrogen compounds such as $NH_4Cl$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $KNO_3$ could not serve as nitrogen sources.

The fermentation products of strain SW408$^T$ from glucose and starch were measured by GC (GC-14B; Shimadzu) according to Chen & Dong (2004); acetic acid, ethanol, $H_2$ and $CO_2$ were detected. Abundant hydrogen was produced from starch; the maximal hydrogen production yield was 6.5 mmol $H_2$ (g starch)$^{-1}$.

Cellular fatty acids of strain SW408$^T$ were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990). The fatty acid profile was determined and was comprised mainly of $C_{14:0}$ (36.5 %), $C_{16:0}$ (21.0 %), $C_{16:1}\omega7c/C_{15:0}$ iso 2-OH (12.7 %), $C_{12:0}$ (4.8 %) and $C_{18:1}\omega9c$ (4.3 %). A comparison of the fatty acid profile of strain SW408$^T$ with profiles of phylogenetically related strains is available in IJSEM Online (Supplementary Table S1).

Extraction of genomic DNA from strain SW408$^T$ was performed as described previously (Redburn & Patel, 1993) and the DNA G+C content was determined as 33.1 mol% by the thermal denaturation method (Marmur & Doty, 1962) using a DU800 spectrophotometer (Beckman) with *Escherichia coli* K-12 as reference.

To ascertain the phylogenetic position of strain SW408$^T$, the 16S rRNA gene was amplified by PCR and sequenced as described previously (Chen & Dong, 2004). The almost complete 16S rRNA gene sequence (1505 bp) was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. The best matching sequences were retrieved from the database, aligned and similarity analysis was performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic trees were con-

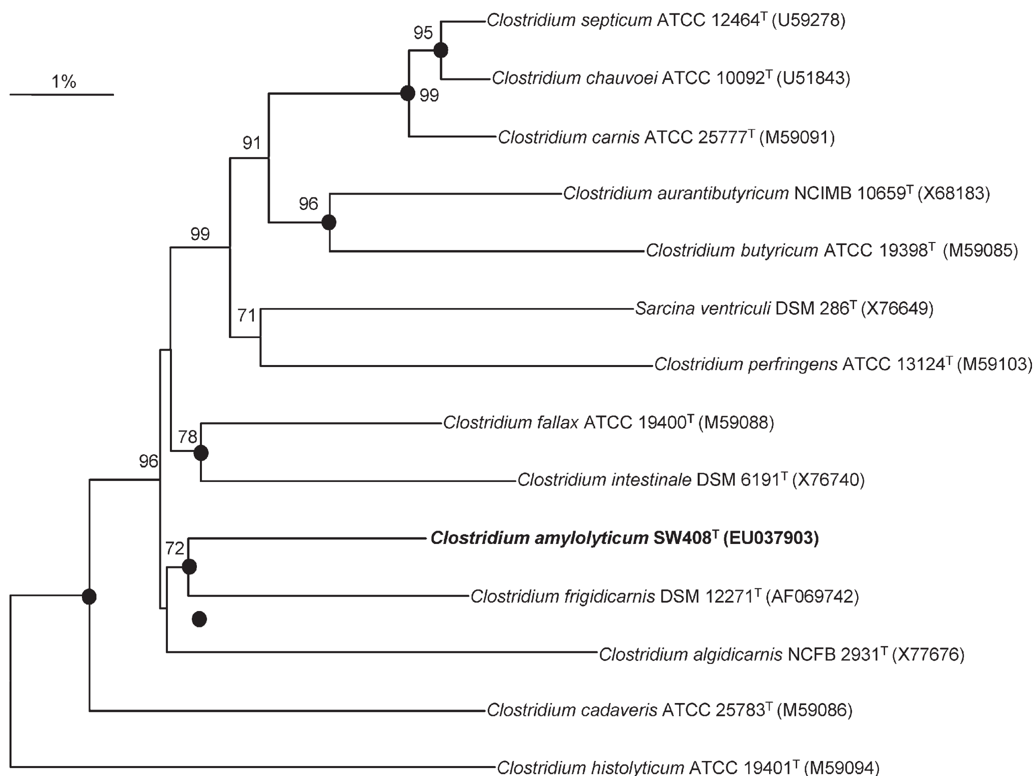


**Fig. 1.** Phylogenetic dendrogram of *Clostridium amylolyticum* sp. nov. SW408$^T$ and related species based on 16S rRNA gene sequence similarity. The tree was rooted with *Clostridium histolyticum* and constructed using the neighbour-joining method. Solid circles indicate that the corresponding nodes (groups) are also recovered in maximum-likelihood and maximum-parsimony methods. Levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets are shown at the nodes. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Bar, 1 % sequence divergence.

structed using the neighbour-joining, maximum-likelihood and maximum-parsimony methods implemented in the program MEGA2 (Kumar *et al.*, 2001) and the PHYLIP package (Felsenstein, 1993). The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. On the basis of a consensus16S rRNA gene sequence of 1411 bp, a phylogenetic tree rooted with *Clostridium histolyticum* ATCC 19401$^T$ was constructed (Fig. 1) and showed that strain SW408$^T$ was affiliated to *Clostridium* rRNA cluster I designated by Collins *et al.* (1994) encompassing the genus *Clostridium* and was most closely related to *C. frigidicarnis* DSM 12271$^T$ (94.9 % sequence similarity). This great sequence divergence indicated that strain SW408$^T$ could represent a novel species in this cluster.

Distinct phenotypic features listed in Table 1 enabled strain SW408$^T$ to be distinguished from phylogenetically related strains. Strain SW408$^T$ could be differentiated from *C. frigidicarnis*, *C. fallax*, *C. intestinale* and *C. algidicarnis* by the formation of ethanol, acetate, hydrogen and carbon dioxide as the fermentation end products from glucose; none of the four species produced this combination of compounds (Broda *et al.*, 1999; Cato *et al.*, 1986; Lee *et al.*, 1989; Lawson *et al.*, 1994). Secondly, strain SW408$^T$ hydrolysed starch, whereas the other four species did not. In addition, different profiles of fermentable sugars among the species were also determined (Table 1).

Based on the combination of a distant phylogenetic relationship with related taxa, divergent DNA G+C contents and distinct physiological and biochemical traits, it is evident that strain SW408$^T$ represents a novel distinct species within *Clostridium* rRNA cluster I for which the name *Clostridium amylolyticum* sp. nov. is proposed.

## Description of *Clostridium amylolyticum* sp. nov.

*Clostridium amylolyticum* (am.y.lo.ly′ti.cum. Gr. n. *amulon* starch; Gr. adj. *lytikos* able to loosen, able to dissolve; N.L. neut. adj. *amylolyticum* starch-dissolving).

Gram-reaction-positive, motile rods. Peritrichous flagella. Cells are 0.5–0.7 × 2.0–7.5 μm. Obligately anaerobic. Oxidase and catalase are not produced. Optimum growth occurs at 37 °C (no growth at or below 22 °C or at or above 47 °C). The pH range for growth is 4.0–9.0 (no growth at or below pH 3.6 or at or above pH 9.3), with optimum growth at pH 7.0. Chemo-organotrophic. Peptone may serve as a nitrogen source. Produces ethanol, acetate, hydrogen and carbon dioxide from glucose fermentation. Hydrolyses aesculin and starch, but not gelatin. Milk reaction is negative. Utilizes glucose, ribose, galactose, fructose, mannose, mannitol, sucrose, cellobiose, lactose, maltose and trehalose, but not glycerol, xylose, arabinose, sorbitol, rhamnose, xylan or cellulose. Complex substrates such as yeast extract and peptone are fermented. Nitrate is not reduced. $H_2S$, but not indole, is produced from peptone/yeast extract/glucose medium. The predominant cellular fatty acids are $C_{14:0}$ (36.5 %), $C_{16:0}$ (21.0 %), $C_{16:1}\omega7c$/$C_{15:0}$ iso 2-OH (12.7 %), $C_{12:0}$ (4.8 %) and $C_{18:1}\omega9c$ (4.3 %).

The type strain, SW408$^T$ (=AS 1.5069$^T$=JCM 14823$^T$), was isolated from granules of a laboratory-scale $H_2$-producing

**Table 1.** Differential characteristics of *Clostridium amylolyticum* sp. nov. SW408$^T$ and its phylogenetic relatives

Strains: 1, SW408$^T$; 2, *C. frigidicarnis* DSM 12271$^T$ (data from Broda *et al.*, 1999); 3, *C. fallax* ATCC 19400$^T$ (data from Cato *et al.*, 1986); 4, *C. intestinale* DSM 6191$^T$ (data from Lee *et al.*, 1989); 5, *C. algidicarnis* NCFB 2931$^T$ (data from Lawson *et al.*, 1994) +, Positive; −, negative; NR, not reported; W, weakly fermented; − (W), generally negative, although weak positive result obtained occasionally; C (milk), curdled.

| Phenotypic characteristic | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Fermentation products* | A,2 | A,B,ib,iv,l,o,2,4 | A,B,L,s,2,4 | A,B,l,f,s | A,B |
| Hydrolysis of: | | | | | |
| Aesculin | + | − | + | + | − |
| Starch | + | − | − | − | − |
| Milk reaction | − | NR | C | NR | − |
| DNA G+C content (mol%) | 33.1 | 27.3 | 26 | 27 | NR |
| Utilization of: | | | | | |
| Cellobiose | + | NR | − (W) | + | − |
| Lactose | + | − | − (W) | + | − |
| Maltose | + | + | + | − | − |
| Mannitol | + | + | − | + | − |
| Ribose | + | NR | W | − | + |
| Sorbitol | − | + | − | + | − |
| Sucrose | + | NR | − | + | − |
| Trehalose | + | + | − | + | − |
| Xylose | − | − | − | − | + |

*Products: a, acetate; b, butyrate; l, lactate; ib, isobutyrate; iv, isovalerate; f, formate; s, succinate; o, oxaloacetate; 2, ethanol; 4, butanol. Upper- and lower-case letters indicate major and minor fermentation products, respectively. All products are from fermentation of glucose.

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UASB reactor at ambient temperature. The DNA G+C content of the type strain is 33.1 mol% ($T_m$ method).

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