Aminiphilus circumscriptus gen. nov., sp. nov., an anaerobic amino-acid-degrading bacterium from an upflow anaerobic sludge reactor

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Strain ILE-2^T was isolated from an upflow anaerobic sludge bed reactor treating brewery wastewater. The motile, non-sporulating, slightly curved cells (2–4×0.1 µm) stained Gram-negative and grew optimally at 42 °C and pH 7.1 with 0.5 % NaCl. The strain required yeast extract for growth and fermented Casamino acids, peptone, isoleucine, arginine, lysine, alanine, valine, glutamate, histidine, glutamine, methionine, malate, fumarate, glycerol and pyruvate to acetate, propionate and minor amounts of branched-chain fatty acids. Carbohydrates, formate, acetate, propionate, butyrate, isovalerate, methanol, ethanol, 1-propanol, butanol, lactate, succinate, starch, casein, gelatin, xylan and a number of other amino acids were not utilized. The DNA G+C content of strain ILE-2^T was 52.7 mol%. 16S rRNA gene sequence analysis revealed that ILE-2^T was distantly related to members of the genera *Aminobacterium* (83 % similarity) and *Aminomonas* (85 % similarity) in the family *Syntrophomonadaceae*, order *Clostridiales*, phylum *Firmicutes*. On the basis of the results of our polyphasic analysis, strain ILE-2^T represents a novel species and genus within the family *Syntrophomonadaceae*, for which the name *Aminiphilus circumscriptus* gen. nov., sp. nov. is proposed. The type strain of *Aminiphilus circumscriptus* is ILE-2^T (=DSM 16581^T =JCM 14039^T).

Amino-acid degradation is carried out by a variety of anaerobic saccharolytic and non-saccharolytic bacteria (Smith & Macfarlane, 1997; Plugge *et al.*, 2000) that include members of the genera *Campylobacter* (Laanbroek *et al.*, 1978), *Clostridium* (Paster *et al.*, 1993; Örlýgsson *et al.*, 1996), *Peptostreptococcus* (Chen & Russell, 1989), *Acidaminococcus* (Rogosa, 1969), *Synergistes* (McSweeney *et al.*, 1993), *Anaeromusa* (Nanninga *et al.*, 1987; Baena *et al.*, 1999a), *Aminomonas* (Baena *et al.*, 1999b) and *Dethiosulfovibrio* (Magot *et al.*, 1997; Surkov *et al.*, 2001). In other cases, amino-acid degradation occurs in syntrophic association with hydrogen-scavenging organisms such as methanogens (Stams, 1994; Schink, 1997). Mesophilic and thermophilic amino-acid degraders such as *Acidaminobacter hydrogenoformans* (Stams & Hansen,

1984), *Eubacterium acidaminophilum* (Zindel *et al.*, 1988), *Aminobacterium colombiense* (Baena *et al.*, 1998), *Aminobacterium mobile* (Baena *et al.*, 2000), *Thermanaerovibrio acidaminovorans* (Cheng *et al.*, 1992; Baena *et al.*, 1999a), *Caloramator proteoclasticus* (Tarlera & Stams, 1999), *Caloramator coolhaasii* (Plugge *et al.*, 2000) and *Gelria glutamica* (Plugge *et al.*, 2002) use syntrophic relationships to convert amino acids. We have isolated a number of different amino-acid-degrading bacteria from an upflow anaerobic sludge bed (UASB) reactor treating brewery wastewater. One of these isolates, a novel mesophilic, non-saccharolytic amino-acid-degrading bacterium designated strain ILE-2^T, is described here. Studies on these novel strains of protein- and amino-acid-degrading bacteria could help us better understand their role in protein-rich anaerobic wastewater treatment.

Flocculent sludge (pH 7.0) with a chemical oxygen demand (COD) of 2600 mg l⁻¹ was collected from a UASB reactor treating brewery wastewater (Industria Cervecería Bavaria S.A., Bogotá, Colombia) maintained at 30 °C. Unless indicated otherwise, cultures were incubated at 37 °C using

Abbreviation: UASB, upflow anaerobic sludge bed.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ILE-2^T is AY642589.

A phase-contrast photomicrograph of cells of strain ILE-2^T is available as supplementary material with the online version of this paper.

anaerobic techniques (Baena *et al.*, 2000). Most-probable number (MPN) estimations were performed by inoculating 10-fold serial dilutions of the UASB reactor samples in basal medium supplemented with Casamino acids (1 %) (Baena *et al.*, 2000) and incubating them at 37 °C. The highest dilutions at which MPN tubes were positive for growth were used to initiate enrichment cultures. Serial dilutions of the enrichment cultures were subcultured and then the roll-tube technique (using media supplemented with 2 % noble agar, 1 % Casamino acids and 0.05 % yeast extract) was used to isolate pure cultures. Several well-isolated small, round colonies with smooth edges that developed in the roll-tube cultures after 2 days incubation at 37 °C were picked and subcultured in the same medium lacking agar and the purification was repeated at least twice before the isolate was deemed to be pure. An isolate designated strain ILE-2^T was selected for further characterization. As strain ILE-2^T was a strict anaerobe, all media were prepared anaerobically (Baena *et al.*, 2000). Strain ILE-2^T did not grow on carbohydrates, and hence a lack of growth in basal medium containing 20 mM glucose was routinely used as a means of assessing culture purity.

Light microscopy revealed that the cells of strain ILE-2^T were motile, non-sporulating, slightly curved cells (2–4 × 0.1 µm) that stained Gram-negative (see Supplementary Fig. S1 in IJSEM Online). Electron microscopy of thin sections, performed using the procedure of Fardeau *et al.* (1997), revealed that strain ILE-2^T possessed a single, thick, layered cell wall typical of Gram-positive bacteria. Electron microscopy of cells stained with 1 % uranyl acetate revealed the presence of peritrichous flagella.

Unless otherwise indicated, all subsequent experiments were performed in duplicate and the strain was subcultured at least once under the same experimental conditions before use in any characterization experiment. Strain ILE-2^T grew optimally at pH 7.1 (range pH 5.5–8.5) in basal medium that contained 1 % Casamino acids and 0.2 % yeast extract and had been adjusted to pH 6.0–9.0 with anaerobic stock solutions of NaHCO₃ (10 %) or Na₂CO₃ (8 %). The optimum temperature for growth was determined in the same medium at pH 7.1 and was found to be 42 °C; the temperature range for growth was 25–55 °C. Strain ILE-2^T grew best with 0.5 % NaCl; the NaCl range for growth was 0–2.5 %.

Unless indicated otherwise, substrate-utilization tests were performed in basal medium containing 0.2 % yeast extract. For control experiments, the medium was the same except that no substrates were included, and all values were corrected for the small amounts of growth and end products that were produced. Results were recorded after 2 weeks incubation at 37 °C. Strain ILE-2^T grew in the presence of 20 mM isoleucine, arginine, lysine, alanine, valine, glutamate, histidine, glutamine and methionine but not with tryptophan, glycine, aspartate, threonine, asparagine, cysteine, phenylalanine, leucine, tyrosine or serine. The strain fermented yeast extract, Casamino acids,

peptone, malate, fumarate, glycerol and pyruvate but not lactate, succinate, formate, acetate, propionate, butyrate, isobutyrate, isovalerate, gelatin, casein, xylan or starch at a final concentration of 1 %. Carbohydrates (fructose, D-cellobiose, L-xylose, D-xylose, D-glucose, D-rhamnose, D-mannose, D-mannitol, D-sorbitol, maltose, melibiose and D-ribose) and alcohols (glycerol, ethanol, methanol, 1-propanol and butanol) were each tested at a final concentration of 20 mM and were not utilized. Analysis of the fermentation end products from growth on peptone and Casamino acids, using the method of Fardeau *et al.* (2000), showed the presence of acetate, propionate and minor amounts of branched-chain fatty acids.

Thiosulfate (20 mM), sulfate (20 mM), elemental sulfur (0.1 %), sulfite (2 mM), nitrate, nitrite (both 20 mM) and oxygen could not be used as electron acceptors in basal medium containing 1 % Casamino acids and 0.2 % yeast extract.

Amino acid degradation via the Stickland reaction was not observed in a basal medium containing 20 mM alanine and 20 mM isoleucine as the electron donors and 20 mM methionine, 20 mM glycine or 20 mM cysteine as the electron acceptor.

The G+C content (mol%) of the DNA of strain ILE-2^T was 52.7 %, as determined by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) using the method of Mesbah *et al.* (1989).

Methods for purification of the DNA and PCR-amplification and sequencing of the 16S rRNA gene were as described previously (Woo *et al.*, 1997; Redburn & Patel, 1993). The raw sequence data obtained from the sequencing were imported into the sequence editor BioEdit, version 5.0.9 (Hall, 1999), the base calling was examined and a contiguous consensus sequence was generated. The closest relatives of the consensus sequence were identified using the GenBank database and BLASTN (Altschul *et al.*, 2001; Benson *et al.*, 1999). The consensus sequence was aligned using the Ribosomal Database Project Sequence Aligner program (Maidak *et al.*, 2001) and was subsequently manually adjusted to conform to the 16S rRNA secondary-structure model (Winker & Woese, 1991). The sequences used in the phylogenetic analysis were extracted from GenBank and the Ribosomal Database Project, positions of sequence and alignment ambiguity were omitted, pairwise evolutionary distances were calculated using the method of Jukes & Cantor (1969) and dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987) as implemented in TREECON (Van de Peer & De Wachter, 1994). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985). Sequence alignment and subsequent comparisons with sequences of representative members of the domain *Bacteria* consistently placed strain ILE-2^T within the family *Syntrophomonadaceae*, phylum *Firmicutes*, the closest phylogenetic relative being the sole member of the genus *Aminomonas*, *Aminomonas paucivorans* (85 % similarity) (see Fig. 1).

Fig. 1. Phylogenetic analysis of strain ILE-2^T within the radiation of the family Syntrophomonadaceae. The dendrogram was generated from 1416 unambiguous base pairs that had been extracted from the pairwise alignment of 28 16S rRNA gene sequences. Accession numbers are indicated in parentheses. Clusters of sequences relating to the same genus are indicated by filled triangles as follows: *Thermanaerovibrio acidaminovorans* DSM 6589^T (GenBank accession no. AF071414) and *Thermanaerovibrio velox* Z-9701^T (AF161069); *Aminobacterium colombiense* DSM 12261^T (AF069287) and *Aminobacterium mobile* DSM 12262^T (AF073521); *Anaerobaculum thermoterrenum* RWcit^T (U50711) and *Anaerobaculum mobile* ATCC BAA-54^T (AJ243189); *Anaerobranca horikoshii* ATCC 700319^T (U21809) and *Anaerobranca gottschalkii* ATCC BAA-51^T (AF203703); *Dethiosulfovibrio marinus* DSM 12538^T (AF234542), *Dethiosulfovibrio peptidovorans* G4207^T (U52817) and *Dethiosulfovibrio acidaminovorans* DSM 12590^T (AY005466); *Thermoerobacter subterraneus* C21^T (AF343566), *Thermoerobacter marianensis* 7p75a^T (AB011495) and *Thermoerobacter nagasakiensis* JCM 11223^T (AB061441); *Caldicellulosiruptor lactoaceticus* DSM 9545^T (X82842), *Caldicellulosiruptor owensensis* ATCC 700167^T (U80596) and *Caldicellulosiruptor kristjanssonii* ATCC 700853^T (AJ004811). The cluster represented by members of the family Acidaminococcaceae, which were used as the outgroup sequences, includes *Acidaminococcus fermentans* DSM 20731^T (GenBank accession no. X78017), *Papillibacter cinnamivorans* DSM 12816^T (AF167711), *Phascolarctobacterium faecium* DSM 14760^T (X72865), *Selenomonas sputigena* ATCC 35185 (AF373023), *Sporomusa malonica* DSM 5090^T (AJ279799) and *Veillonella atypica* DSM 20739^T (X84007). Bar, 2 substitutions per 100 nt.

In addition to the large phylogenetic distance separating strain ILE-2^T and *Aminomonas paucivorans*, significant phenotypic and genotypic differences were also discernible. *Aminomonas paucivorans* utilizes a very restricted range of substrates for growth (arginine, threonine, glutamate, histidine and glycine), whereas strain ILE-2^T utilizes a wider spectrum of substrates (yeast extract, Casamino acids, peptone, isoleucine, arginine, lysine, alanine, valine, glutamate, histidine, glutamine, methionine, malate, fumarate, glycerol and pyruvate). Moreover, the G + C content of its DNA (52.7 mol%) is far higher than that of *Aminomonas paucivorans* (43 mol%).

Strain ILE-2^T, isolated from anaerobic sludge from a brewery wastewater-treatment plant, is a mesophilic and strictly anaerobic amino-acid-degrading bacterium. The strain ferments peptides in the form of peptone and yeast extract and amino acids in the form of Casamino acids and single amino acids, but not carbohydrates. These characteristics are shared with the non-saccharolytic

peptidolytic and aminolytic species *Acidaminobacter hydrogeniformans* (Stams & Hansen, 1984), *Dethiosulfovibrio peptidovorans* (Magot *et al.*, 1997), *Aminobacterium colombiense* (Baena *et al.*, 1998), *Aminomonas paucivorans* (Baena *et al.*, 1999b), *Aminobacterium mobile* (Baena *et al.*, 2000) and *Dethiosulfovibrio russensis* (Surkov *et al.*, 2001). However, the phylogenetic distance that separates strain ILE-2^T from the group of amino-acid- and peptide-degrading bacteria is greater than 15% 16S rRNA gene sequence dissimilarity.

The phenotypic and phylogenetic characteristics presented in our studies sets strain ILE-2^T apart from all other related amino-acid- and peptide-degrading bacteria. Consequently, strain ILE-2^T represents a novel species and genus within the family Syntrophomonadaceae, for which the name *Aminiphilus circumscriptus* gen. nov., sp. nov. is proposed. Interestingly, the genera *Syntrophomonas*, *Syntrophospora*, *Aminobacterium*, *Aminomonas*, *Dethiosulfovibrio* and *Thermanaerovibrio*, which comprise this family, are all

characterized by the common traits of amino acid and peptide utilization.

Description of *Aminiphilus* gen. nov.

Aminiphilus (A.mi.ni.phi'lus. N.L. n. *aminum* amine; Gr. adj. *philos* loving; N.L. masc. n. *Aminiphilus* amine lover).

Strictly anaerobic, non-spore-forming, mesophilic, curved cells. Peptide compounds, amino acids, malate, fumarate, glycerol and pyruvate are fermented. Oxygen, sulfite, thiosulfate, sulfate, nitrite, nitrate and elemental sulfur do not serve as electron acceptors for growth on Casamino acids. The DNA G + C content of the type strain of the type species is 52.7 mol% (by HPLC). The type species is *Aminiphilus circumscriptus*.

Description of *Aminiphilus circumscriptus* sp. nov.

Aminiphilus circumscriptus (cir.cum.scrip'tus. L. masc. part. adj. *circumscriptus* rounded, periodic, restricted, limited).

Displays the following properties in addition to those given in the genus description. Cells are motile and slightly curved ($2-4 \times 0.1 \mu\text{m}$) and stain Gram-negative. Grows optimally at 42 °C, at pH 7.1 and with 0.5% NaCl. Casamino acids, peptone, isoleucine, arginine, lysine, alanine, valine, glutamate, histidine, glutamine, methionine, malate, fumarate, glycerol and pyruvate are degraded to acetate, propionate and minor amounts of branched-chain fatty acids.

The type strain, ILE-2^T (=DSM 16581^T =JCM 14039^T), was isolated from a UASB reactor treating brewery wastewater.

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