

Phylogenetic relationships of three amino-acid-utilizing anaerobes, *Selenomonas acidaminovorans*, '*Selenomonas acidaminophila*' and *Eubacterium acidaminophilum*, as inferred from partial 16S rDNA nucleotide sequences and proposal of *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov. and *Anaeromusa acidaminophila* gen. nov., comb. nov.

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16S rRNA gene sequences of three previously described amino-acid-fermenting anaerobes, *Selenomonas acidaminovorans*, '*Selenomonas acidaminophila*' and *Eubacterium acidaminophilum*, were determined. All three were found to cluster within the *Clostridium* and related genera of the subphylum of the Gram-positive bacteria. The thermophile, *S. acidaminovorans*, formed an individual line of descent and was equidistantly placed between *Dethiosulfovibrio peptidovorans* and *Anaerobaculum thermoterrenum* (similarity of 85%), both of which also form single lines of descent. '*S. acidaminophila*' was related to *Clostridium quercicolum*, a member of cluster IX, with a similarity of 90%, whereas *E. acidaminophilum* was closely related to *Clostridium litorale* (similarity of 96%) as a member of cluster XI. Based on the phylogenetic data presented in this report and the phenotypic descriptions of these bacteria published previously, it is recommended that *S. acidaminovorans* be transferred to a new genus, *Thermanaerovibrio* gen. nov., as *Thermanaerovibrio acidaminovorans* comb. nov. and '*Selenomonas acidaminophila*' be transferred to a new genus, *Anaeromusa* gen. nov., as *Anaeromusa acidaminophila* comb. nov. Though the transfer of *E. acidaminophilum* to a new taxon is justified, this is not recommended until the taxonomic status of all the members of cluster XI has been reviewed.

Keywords: amino acids, 16S rRNA analysis, *Selenomonas*, *Eubacterium*

INTRODUCTION

The trait of peptide and amino acid utilization as carbon, nitrogen and energy sources is distributed

The GenBank accession numbers for the 16S rRNA gene sequences of *Thermanaerovibrio acidaminovorans*, *Anaeromusa acidaminophila* and *Eubacterium acidaminophilum* are AF071414, AF071415 and AF071416, respectively.

amongst many anaerobic bacteria. This trait has been widely investigated in saccharolytic clostridia (McInerney, 1988; Paster *et al.*, 1993; Attwood *et al.*, 1998) but not in asaccharolytic clostridia (Nanninga *et al.*, 1987; Guangsheng *et al.*, 1992; Zindel *et al.*, 1988). Published data indicate that such bacteria may play an important role in the cycling of nitrogen, sulfur and carbon in diverse ecosystems such as the rumen



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Titre en Français : Relations phylogénétiques entre trois bactéries anaérobies dégradant les acides aminés, *Selenomonas acidaminovorans*, "*Selenomonas acidaminophila*" et *Eubacterium acidaminophilum*, à partir des séquences partielles de l'ADNr 16S, et proposition pour *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov. et *Anaeromusa acidaminophila* gen. nov., comb. nov.

Mots-clés matières : *Thermanaerovibrio* (*Selenomonas*) *acidaminovorans*, *Anaeromusa* (*Selenomonas*) *acidaminophila*, *Eubacterium acidaminophilum*, phylogénie, taxonomie, anaérobiose, acides aminés
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(Attwood *et al.*, 1998), human colon (Smith & Macfarlane, 1997) and waste digesters (Siebert & Toerien, 1969). We have recently initiated studies on understanding the role of obligate amino-acid-oxidizing and/or -fermenting anaerobic bacteria in dairy waste digesters. We were expecting to isolate new taxa from our studies and consequently, we searched the literature to identify and complete gaps in the taxonomy of amino-acid-degrading anaerobic bacteria. During this process, we identified only three such isolates, namely *Selenomonas acidaminovorans* (Guangsheng *et al.*, 1992), '*Selenomonas acidaminophila*' (Nanninga *et al.*, 1987) and *Eubacterium acidaminophilum* (Zindel *et al.*, 1988), which had all been validated using phenotypic rather than phylogenetic criteria. Our intention was to complete a phylogenetic analysis based on 16S rRNA gene sequences so that this data could then be used for comparison with our new isolates. The taxonomic position of the three isolates is reported.

METHODS

Source and culture of strains. *S. acidaminovorans* strain Su883^T (DSM 6589^T), '*S. acidaminophila*' strain Dkglu16^T (DSM 3853^T) and *E. acidaminophilum* strain al-2^T (DSM 3953^T) were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), and were grown on media and under conditions described previously (Guangsheng *et al.*, 1992; Nanninga *et al.*, 1987; Zindel *et al.*, 1988).

DNA extraction and amplification of 16S rRNA gene. DNA was extracted from the isolates as described previously (Redburn & Patel, 1993; Andrews & Patel, 1996). The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb corresponding to base positions 8–1542 based on *Escherichia coli* numbering of the 16S rDNA (Winker & Woese, 1991). A 50 µl reaction mix contained 1–20 ng genomic DNA, 1 µM each primer, 5 µl 10 × buffer, 200 µM dNTP, 3.5 mM MgCl₂ and 2.5 U *Taq* polymerase (Promega). PCR was carried out by an initial denaturation at 94 °C for 7 min, then 29 cycles of annealing at 55 °C for 2 min, extension at 72 °C for 4 min, denaturation at 94 °C for 1 min and finally an extension cycle of 55 °C for 2 min and 72 °C for 20 min. PCR products were purified with a QIAquick kit (Qiagen). DNA concentration of purified PCR product was estimated by comparison with the Low Mass Ladder (Gibco-BRL) on an agarose gel containing ethidium bromide.

Direct sequencing of PCR products. QIAquick-purified PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing kit containing AmpliTaq FS DNA polymerase and an ABI 373A sequencer. A 10 µl reaction mix contained 35 ng PCR product, 4 µl cycle sequencing reaction mix, 3.2 pmol primer (Andrews & Patel, 1996) and 2.5 µg BSA. Thermal cycling was carried out using a RapidCycler (Idaho Technology) at a temperature transition slope of 2, an initial denaturation of 94 °C for 15 s, then 25 cycles of denaturation at 94 °C for 0 s, annealing at 50 °C for 10 s and extension at 60 °C for 3 min.

Sequence alignments and phylogenetic inferences. The new sequence data that were generated were aligned and almost

full-length consensus 16S rRNA gene sequences were assembled and checked for accuracy manually using the alignment editor ae2 (Maidak *et al.*, 1996). These were compared with other sequences in the GenBank database (Benson *et al.*, 1993) using BLAST (Altschul *et al.*, 1997), and sequences from the Ribosomal Database Project, version 5.0 using SIMILARITY_RANK and SUGGEST_TREE (Maidak *et al.*, 1996). Reference sequences most related to our newly generated sequences were extracted from these databases and aligned. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1003 unambiguous nucleotides were computed using DNADIST (Jukes & Cantor option) and neighbour-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis (Van de Peer & De Wachter, 1993).

RESULTS AND DISCUSSION

Almost complete 16S rRNA gene sequences comprising 1426, 1530 and 1508 nucleotides, corresponding to *E. coli* positions 14–1540, 17–1539 and 16–1539 (Winker & Woese, 1991), were determined for *S. acidaminovorans*, '*S. acidaminophila*' and *E. acidaminophilum*, respectively. Several phylogenetic trees constructed from representative 16S rRNA sequences of members of the domain *Bacteria* revealed that the three strains were not related to each other and were clustered as members of the *Clostridium* and related genera of the subphylum of the Gram-positive bacteria (Fig. 1).

S. acidaminovorans (Guangsheng *et al.*, 1992) and '*S. acidaminophila*' (Nanninga *et al.*, 1987) are Gram-negative, non-spore-forming curved rods which are motile by means of a tuft of flagella and were assigned to the genus *Selenomonas* based on these characteristics. However, phylogenetic analysis revealed that the sequence similarity of these two isolates was low (80%) and they were therefore not specifically related to each other. The analysis also showed that both isolates were distantly placed from members of the genus *Selenomonas* with a similarity of only 80%. Therefore, the two isolates cannot be ascribed as members of the genus *Selenomonas*.

S. acidaminovorans was equidistantly placed between *Dethiosulfovibrio peptidovorans* and *Anaerobaculum thermoerrenum* (similarity of 85%). All three strains shared in common the property of strict anaerobiosis for growth and had curved cell morphology; each member formed an independent line of descent in the vicinity of cluster V (Rees *et al.*, 1997; Magot *et al.*, 1997; Collins *et al.*, 1994). The phylogenetic separation alone is sufficient grounds for proposing that *S. acidaminovorans* be transferred to a new genus but, in addition, the considerable differences in the G+C content and phenotypic characteristics amongst *S. acidaminovorans*, *D. peptidovorans* and *A. thermoerrenum* further strengthen the argument for the creation of a new genus (Table 1). *S. acidaminovorans* is a thermophilic strain isolated from a sludge sample.

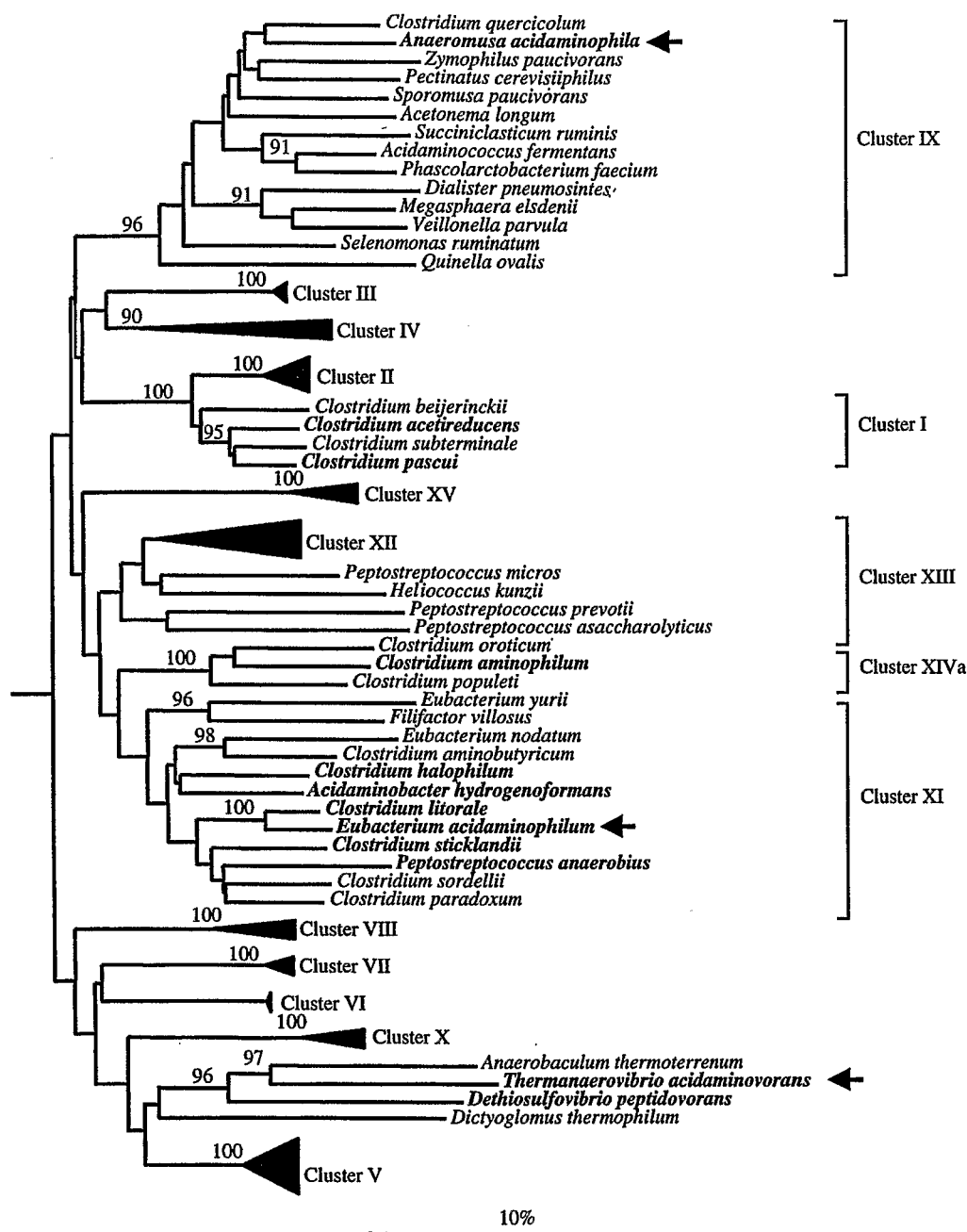


Fig. 1. Unrooted dendrogram based on 16S rRNA sequence data indicating the phylogenetic positions of *Thermanaerovibrio acidaminovorans* strain DSM 6589^T (*Selenomonas acidaminovorans*), *Anaeromusa acidaminophila* strain DSM 3853^T (*Selenomonas acidaminophila*) and *Eubacterium acidaminophilum* (strain DSM 3953^T) within the radiation of representatives of the low-G+C-containing Gram-positive bacteria. The proposed new taxa studied in this paper are indicated by arrows and names of various amino-acid-degrading bacteria are indicated in bold type. All the sequences used in the analysis, with the exception of *Anaerobaculum thermoterrenum*, *Dethiosulfovibrio peptidovorans* and *Acidaminobacter hydrogenoformans* (GenBank nos U50711, U52817 and AF016691, respectively) and *Clostridium acetireducens* and *Clostridium pascui* (EMBL nos X79862 and X96736, respectively), were obtained from the Ribosomal Database Project, version 5.0 (Maidak *et al.*, 1996). Bootstrap values, expressed as a percentage of 100 replications are shown at the branching points. Only values above 90% were considered significant and therefore reported. Scale bar, 10 nucleotide substitutions per 100 nucleotides.

t has a DNA G+C content of 56.5 mol% and ferments carbohydrates (e.g. glucose, fructose) and a number of amino acids (e.g. glutamate, histidine). It also oxidizes amino acids in syntrophic association

with a hydrogen scavenger (*Methanobacterium thermoautotrophicum*). *A. thermoterrenum* is a thermophilic strain isolated from a petroleum reservoir and has a DNA G+C content of 44 mol%. It grows on a range

Table 1. Distinguishing features of the amino-acid-degrading bacteria *Thermanaerovibrio acidaminovorans*, *Anaeromusa acidaminophila*, *Clostridium acidaminophila* and their closest phylogenetic relatives

Strains: 1, *Selenomonas acidaminovorans* [proposal to reassign as *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov. (cluster V)]; 2, *Anaerobaculum thermoterrenum*; 3, *Dethiosulfovibrio peptidovorans*; 4, '*Selenomonas acidaminophila*' [proposal to reassign as *Anaeromusa acidaminophila* gen. nov., comb. nov. (cluster IX)]; 5, *Clostridium quercicolum*; 6, *Eubacterium acidaminophilum* (to be considered for reassignment as part of the taxonomic revision of members of cluster XI); 7, *Clostridium litorale*. Abbreviations: AA, amino acid; P, peptides; A, acetate; Pr, propionate; I-B, isobutyrate; I-V, isovalerate; 2-MB, 2-methylbutyrate; Et, ethanol; Am, ammonia; Su, succinate; NR, not reported.

Character	1	2	3	4	5	6	7
Habitat	Methanogenic digester	Petroleum reservoir	Oil-producing well	Anaerobic digester	Tissue of oak tree	Black anaerobic sediment	Marine sediments
Rod morphology	Curved	Curved	Curved	Curved	Straight	Straight	Straight
Spores	—	—	—	—	+	—	+
Flagella	6-8, lateral	No	1-5, lateral	Up to 16, lateral	Peritrichous	1, polar to subpolar	Yes; details not reported
NaCl required for optimum growth	No	Yes (1%)	Yes (3%)	No	No	NR	Yes (1%)
Growth temperature (°C)*	55 (40-58)	55	42 (20-45)	38 (25-46)	25-30	32-36 (15-40)	28 (13-39)
Growth pH*	6.5-8.1	7.0-7.6 (5.5-8.6)	7.0 (5.5-8.8)	6.8-7.2 (5-8.5)	NR	7.1-7.4 (6.5-8.5)	7.3 (6.5-8.4)
G+C (mol%)	56.5	44	56	48	52-54	44	26
Carbohydrates utilized	+	+	—	—	+	—	—
P/AA utilized	AA	AA, P	AA, P	AA	NR	AA, P	AA
Sulfur compounds used as electron acceptors	—	Thiosulfate, S ⁰ , cystine	Thiosulfate, S ⁰	—	—	—	—
Fermentation products	A, Pr	A	A, I-V, I-B, 2-MB, CO ₂ , H ₂	A, Pr, Su	NR	A, Et, CO ₂ , H ₂ , Am	A, B
Reference	Guangsheng <i>et al.</i> (1992)	Rees <i>et al.</i> (1997)	Magot <i>et al.</i> (1997)	Nanninga <i>et al.</i> (1987)	Stankewich <i>et al.</i> (1971)	Zindel <i>et al.</i> (1988)	Fendrich <i>et al.</i> (1990)

* Optimum is given; ranges are shown in parentheses.

of carbohydrates, organic compounds, protein extracts and Casamino acids. On the other hand, *D. peptidovorans*, a mesophilic isolate from an oilfield, has a DNA G+C content of 56 mol%, is an obligate peptide and amino acid degrader that is unable to utilize carbohydrates or oxidize amino acids in the presence of a hydrogen scavenger (Magot *et al.*, 1997). Based on this evidence, we propose to create a new genus, *Thermanaerovibrio* gen. nov. and transfer *S. acidaminovorans* to the genus as *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov.

'*S. acidaminophila*' (Nanninga *et al.*, 1987), which has not yet been formally taxonomically validated, was related to *Clostridium quercicolum*, a member of cluster IX, with a similarity of 90%. This result alone signifies that '*S. acidaminophila*' should be accorded genus status. The argument is further strengthened by the numerous phenotypic and genotypic differences between '*S. acidaminophila*' and *C. quercicolum* (Table 1). '*S. acidaminophila*', an isolate of an anaerobic digester, is a mesophilic non-spore-former, has a DNA G+C content of 48 mol% and ferments a very limited range of substrates including glutamate, aspartate, lactate and pyruvate, but not carbohydrates. *C. quercicolum* is also a mesophile but was isolated from oak tree tissue (Stankewich *et al.*, 1971). It forms spores, ferments carbohydrates and has a DNA G+C content of 52-54 mol%. Based on phylogenetic evidence and the distinct phenotypic characteristics, we propose that a new genus, *Anaeromusa* gen. nov., be

created and that '*S. acidaminophila*' be transferred to this genus as *Anaeromusa acidaminophila* gen. nov., comb. nov. The cluster contains a heterogeneous collection of spore-forming and non-spore-forming bacteria, many of which are Gram-negative, but the cluster is a cohesive group at suprageneric level supported by a bootstrap value of 99% (Collins *et al.*, 1994).

E. acidaminophilum (Zindel *et al.*, 1988) is a member of cluster XI (similarity of 87-96%) and is most closely related to *Clostridium litorale* (similarity of 96%). There are sufficient phenotypic and genotypic differences between *C. litorale* and *E. acidaminophilum* to support the notion that they should be designated separate species (Table 1). *E. acidaminophilum*, a non-sporulating strain isolated from anaerobic mud, has a DNA G+C content of 44 mol%, oxidizes amino acids if a hydrogen scavenger or chemical electron acceptors are present and ferments serine and glycine. On the other hand, *C. litorale* (Fendrich *et al.*, 1990) is a halotolerant spore-forming strain, isolated from anoxic marine sediments. It oxidizes amino acids by the Stickland reaction and has a DNA G+C content of 26 mol%. Over the past five years, there has been a slow and gradual revision of the taxonomy of the clostridial and non-clostridial members of the low-G+C-containing Gram-positive bacteria phylum. Collins *et al.* (1994) indicated that cluster XI is a taxonomically heterogeneous group consisting of several sublines of descent. They suggested that all

members should be incorporated into a single supra-generic family consisting of several genera. *C. litorale* forms an independent line of descent (similarity less than 92% with other members of the cluster) and hence, according to this suggestion, a new genus should be created to accommodate *C. litorale* and subsequently *E. acidaminophilum*. However, given that this cluster contains numerous other amino acid utilizers (Fig. 1) and other strains have still to be analysed both for phylogeny and the ability to degrade amino acids, it would be prudent to wait until a more comprehensive overhaul of this group has been discussed formally before *E. acidaminophilum* is reassigned as a new taxon.

Description of *Thermanaerovibrio* gen. nov.

Thermanaerovibrio (Therm.an.ae.ro.vib'ri.o. Gr. adj. *thermos* hot; Gr. pref. *an* not; Gr. n. *aer* air; M.L. masc. n. *vibrio* that vibrates; M.L. masc. n. *Thermanaerovibrio* a thermophilic vibrating anaerobe).

Strictly anaerobic curved cells with rounded ends. Motile and Gram-negative. Non-spore-former. Uses amino acids and carbohydrates. The type species is *Thermanaerovibrio acidaminovorans* comb. nov.

Description of *Thermanaerovibrio acidaminovorans* (Guangsheng, Plugge, Roelofsen, Houwen and Stams 1992) comb. nov.

Thermanaerovibrio acidaminovorans (a.ci.dam.in.o'vor.ans. M.L. neut. n. *acidum* acid; M.L. neut. n. *aminum* amine; L. part. adj. *vorans* devouring; M.L. part. adj. *acidaminovorans* amino-acid-digesting).

The description of *Thermanaerovibrio acidaminovorans* comb. nov. is identical to that proposed for *Selenomonas acidaminovorans* (Guangsheng, Plugge, Roelofsen, Houwen and Stams 1992). The type strain is DSM 6589^T.

Description of *Anaeromusa* gen. nov.

Anaeromusa (An.ae.ro'mu.sa. L. v. Gr. pref. *an* not; Gr. n. *aer* air; M.L. n. *musa* a banana; M.L. fem. n. *Anaeromusa* an anaerobic banana).

Strictly anaerobic Gram-negative curved rods. Non-spore-former. Ferments and oxidizes amino acids. The type species is *Anaeromusa acidaminophila* comb. nov.

Description of *Anaeromusa acidaminophila* (Nanninga, Drent and Gottschal 1987) comb. nov.

Anaeromusa acidaminophila (a.ci.dam.in.o'phi.la. M. L. neut. n. *acidum* acid; M.L. neut. n. *aminum* amine; Gr. adj. *philus* loving; M.L. part. adj. *acidaminophila* amino-acid-loving).

The description of *Anaeromusa acidaminophila* comb. nov. is identical to that proposed for '*Selenomonas acidaminophila*' (Nanninga, Drent and Gottschal 1987). The type strain is DSM 3853^T.

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