



## Reevaluating the classification of *Halobacteroides* and *Haloanaerobacter* species based on sequence comparisons of the 16S ribosomal RNA gene

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### Abstract

The 16S rRNA gene (rDNA) sequence analysis of four halophilic anaerobes: *Halobacteroides halobius*, *H. lacunaris*, *Haloanaerobacter (Hb.) chitinovorans* and *H. acetoethylicus* confirmed that they were all members of the family Haloanaerobiaceae. *H. lacunaris* and *H. halobius* were found to be more closely related to each other and were distantly related to *Sporohalobacter lortetti* and the members of the genera *Haloanaerobium* and *Halothermothrix*. These data are in agreement with their assignment to the genus *Halobacteroides*. Further analysis indicated that *Hb. chitinovorans* was closely affiliated to members of the genus *Halobacteroides*, and therefore we propose to transfer it to the genus *Halobacteroides* as *H. chitinovorans* comb. nov. This transfer would invalidate the genus *Haloanaerobacter*, as *Hb. chitinovorans* is the only member of this genus. The 16S rDNA sequence analysis of *H. acetoethylicum* indicated that it was very closely related to members of the genus *Haloanaerobium*, viz. *Haloanaerobium (Ha.) praevalens*, *Ha. salsugo*, and *Ha. alcaliphilum*, and hence we propose to transfer it to the genus *Haloanaerobium* as *Ha. acetoethylicus* comb. nov.

**Keywords:** Haloanaerobiaceae; *Halobacteroides*; *Haloanaerobacter*; *Haloanaerobium*; 16S rDNA; Phylogeny; Taxonomy

### 1. Introduction

Halophilic anaerobic bacteria have been isolated from a variety of saline environments and include saline and hypersaline lakes, solar salterns, and oil field brines. *Sporohalobacter lortetii*, *Halobac-*

*teroides halobius* and *Haloanaerobium (Ha.) praevalens* were amongst the first that were isolated from such environments. Early studies on the 16S rRNA oligonucleotide catalogues of these halophilic anaerobic carbohydrate-fermenting bacteria, and confirmed more recently by 16S rRNA sequence analysis, indicated that they were more closely related to each other than to any other groups of bacteria, leading to the creation of a new family, Haloanaerobiaceae [11–13,20]. Another five new genera and nine species have been assigned to the family Haloanaerobiaceae recently, but with the exception

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of *Ha. salsugo* [1], *Ha. alcalophilum* [18] and *Haloferoxanthus orenii* [3] whose 16S rRNA gene (16S rDNA) sequences have been studied, the taxonomy has been based largely on 5S rRNA sequence analysis, DNA–DNA hybridization studies and/or differentiating phenotypic traits [2,8,15,16,21,22]. It is widely accepted that molecules such as 16S rRNA and 23S rRNA rather than 5S rRNA provide much better information for taxonomy and phylogenetic relationships at a generic level and that DNA–DNA hybridization tests are more appropriate for determining relationships below the genus level [17]. In order to confirm the taxonomic status of the members of *Haloanaerobacter* and *Halobacteroides*, viz. *Halobacteroides lacunaris*, *H. halobius*, *H. acetoethylicus* and *Haloanaerobacter (Hb.) chitinovorans*, we sequenced and analysed their 16S rDNA and report the results of these findings in this paper.

## 2. Materials and methods

*H. lacunaris* strain Z-7888<sup>T</sup> (strain Z-7888<sup>T</sup> = DSM 6640) and *Hb. chitinovorans* strain W5C8 (strain W5C8<sup>T</sup> = OCM 229) were obtained from DSM and Oregon Collection of Methanogens, respectively, cultured in a low Mg and Ca complex anaerobic media [2] and the DNA extracted and purified [9]. Liquid cultures of *H. halobius* strain MD-1<sup>T</sup> (strain MD-1<sup>T</sup> = ATCC 35273) and *H. acetoethylicus* strain EIGI<sup>T</sup> (strain EIGI<sup>T</sup> = DSM 3532) were purchased from ATCC and DSM, respectively, and the DNA extracted directly from these cultures using methods described previously [9]. Amplification, purification of the amplified product, and sequencing of the 16S rDNA was as previously reported [9,14]. Sequences were aligned manually, using the alignment editor ae2, to the sequences previously described for the members of the family Haloanaerobiaceae available from the RDP database, version 5.0 [10] and from recently published data [18]. Positions of sequence and alignment uncertainties were omitted from the analysis and pairwise evolutionary distances 906 nucleotides were computed using the method of Jukes and Cantor [6] and dendrograms constructed from the evolutionary distances using the Neighbor-joining method, both of which form part of the PHYLIP package [5]. Pro-

grams forming part of the Molecular Evolutionary Genetic Analysis (MEGA) Package, version 1 [7] were also used for phylogenetic analysis. All programs with the exception of MEGA were run on a Sun Sparc Workstation; MEGA was run on a 486 Compaq Contura 410CX notebook computer.

## 3. Results and discussion

The 16S rDNA genes of *H. halobius*, *H. lacunaris*, *H. acetoethylicus* and *H. chitinovorans*, from positions 34–1542, 30–1542, 8–1524, and 30–1542 (according to *Escherichia coli* numbering [19]) representing 1505, 1491, 1526 and 1284 nucleotides, were determined, and deposited in GenBank under accession numbers U32595, U32593, U32594 and U32596, respectively. The G + C content of the 16S rDNA genes ranged between 51.3% mol G + C and 52.5% mol G + C.

Comparative 16S rDNA gene sequence analysis of the four anaerobic halophiles with representative members of the 13 phyla of the domain Bacteria [19] indicated that they were related in general to the Gram-positive phylum whose members contained DNA with a G + C content of less than 50% and, more specifically related to the members of the family Haloanaerobiaceae (Table 1). Their membership to the family Haloanaerobiaceae was confirmed by identifying the presence of the unique 22 nucleotide signature sequence GGAUACUAGGUGU-UGGRGGUUC (positions 821–842, *E. coli*) numbering according to Winker and Woese [19] as has been described previously [3].

The 16S rDNA sequence analysis of *H. acetoethylicus* with members of family Haloanaerobiaceae indicated that it was a very close phylogenetic relative of *Haloanaerobium* species, namely *H. praevalens*, *H. salsugo* and *H. alcaliphilum* (evolutionary distances of 1.3, 3.5 and 2.6, respectively) and was only distantly related to *H. lacunaris*, *Hb. chitinovorans* and *H. halobius* (evolutionary distances of 19.9, 18.5 and 18.9, respectively). We therefore propose that *H. acetoethylicus* be transferred to the genus *Haloanaerobium* as *Ha. acetoethylicus* comb. nov. Though *H. lacunaris* and *Hb. chitinovorans* are members of different genera and possess markedly different phenotypic traits [8,21],

Table 1

Evolutionary distances of members of the family Haloanaerobiaceae and various representatives of the Gram-positive phylum based on the method of Jukes and Cantor [6]

1 <i>Escherichia coli</i>																		
2 <i>Haloanaerobacter chitinovorans</i>	30.2																	
3 <i>Halobacteroides lacunaris</i>	29.7	4.4																
4 <i>Halobacteroides halobius</i>	28.9	11.9	13.8															
5 <i>Sporohalobacter lortetii</i>	29.5	11.6	13.4	13.1														
6 <i>Halobacteroides acetoethylicus</i>	29.0	18.8	20.1	19.0	17.9													
7 <i>Haloanaerobium praevalens</i>	29.2	18.7	20.1	17.9	18.1	1.3												
8 <i>Haloanaerobium salsugo</i>	28.5	20.3	21.3	21.2	18.4	3.5	4.6											
9 <i>Haloanaerobium alcaliphilum</i>	30.7	20.0	21.4	20.3	19.4	2.6	2.0	5.9										
10 <i>Halothermothrix orenii</i>	27.6	18.3	19.4	19.4	15.7	13.1	14.0	12.3	15.5									
11 <i>Sporomus paucivorans</i>	26.6	23.4	24.4	23.2	22.3	23.4	23.5	22.9	25.2	22.0								
12 <i>Megasphaera elsdenii</i>	25.1	24.1	26.1	23.2	22.5	23.4	23.5	24.0	25.5	22.2	16.2							
13 <i>Heliobacterium chlorum</i>	25.2	22.9	24.4	24.0	22.2	23.8	24.1	22.5	25.7	20.7	18.3	19.8						
14 <i>Acidaminococcus fermentans</i>	27.1	23.2	24.8	22.0	21.2	22.0	21.7	21.3	23.7	19.3	12.1	15.1	18.7					
15 <i>Moorella thermoacetica</i>	26.1	21.6	22.8	21.0	19.6	22.2	22.9	21.4	24.1	20.0	21.7	19.4	17.2	19.6				
16 <i>Thermoanaerobacterium saccharolyticum</i>	25.4	19.4	20.6	18.6	18.4	19.6	19.8	19.8	21.6	19.3	17.9	17.2	16.7	18.0	13.6			
17 <i>Syntrophospora bryantii</i>	23.1	19.6	20.7	20.3	19.6	22.2	22.6	21.7	24.4	19.4	19.0	18.8	16.1	18.3	16.9	13.8		
18 <i>Syntrophomonas wolfei</i>	23.5	20.3	21.9	21.4	22.0	22.3	22.5	21.7	24.3	21.3	20.6	19.0	16.6	19.1	17.6	14.2	4.6	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

The sequences used in distance calculations were obtained from the Ribosomal Database Project, version 5.0 [10] with the exception of the sequence of *H. alcaliphilum*, which was extracted from EMBL (accession number X81850). A total of 906 unambiguous nucleotides were used in the analysis. *Clostridium thermoaceticum* and *Clostridium thermosaccharolyticum* have been recently renamed *Moorella thermoacetica* and *Thermoanaerobacterium saccharolyticum* [4].

their 16S rDNA sequence analysis indicates that they are very close phylogenetic relatives of each other (an evolutionary distance of 4.5). The 16S rDNA sequence analysis also established that *H. lacunaris* and *Hb. chitinovorans* are phylogenetic relatives of *H. halobius* (evolutionary distances of 13.8 and 11.9, respectively). Based on these results, we propose that *Hb. chitinovorans* be transferred to the genus *Halobacteroides* as *H. chitinovorans* comb. nov. This transfer would invalidate the genus *Haloanaerobacter* as *Hb. chitinovorans* is the only member in this genus.

Table 2

Signature sequences and nucleotide bases defining cluster I consisting of *Halobacteroides lacunaris*, *H. chitinovorans*, *H. halobius*, and *Sporohalobacter lortetii* from cluster II consisting of *Haloanaerobium* species and *Halothermothrix orenii*

Escherichia coli position	Signature sequence and nucleotide(s) for:	
	<i>Halobacteroides</i> / <i>Sporohalobacter</i> (cluster I)	<i>Haloanaerobium</i> / <i>Halothermothrix</i> (cluster II)
95–99	AGUAA	UAGAU
134	A	G
135	C	U
148:174	G:A or U:U	G:C
154:167	U:A or U:G	C:G
199:218	A:U	G:U
291:309	C:G	U:A
293:304	A:U	C:G
294:303	C:G	U:A
353	U	A
453	A	C
459:473	G:C	A:U or U:A
462:470	G:C	A:G
479	U	C
504:541	C:G	U:A
590:649	C:G	U:A
591:648	U:A	A:U
772:807	A:U	G:C
811	U	C
843	A	G
896:903	C:G	U:A
943:1340	C:G	U:A
987:1218	G:C	A:U
1047:1210	G:C	G:U
1201	A	C

The 16S rRNA and 16S rDNA sequences of the members of family Haloanaerobiaceae used in the comparisons were obtained from the Ribosomal Database Project, version 5.0 [10] and that of *H. alcaliphilum* was extracted from EMBL (accession number X81850).

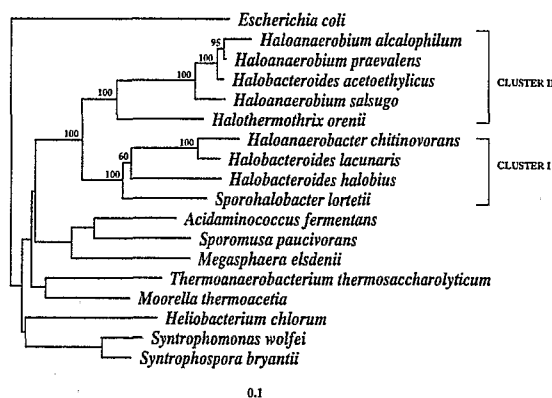


Fig. 1. Phylogenetic relationship of *Haloanaerobacter* and *Halobacteroides* species to members of the family Haloanaerobiaceae and some other members of the low G+C DNA containing Gram-positive phylum. Bootstrap values were obtained by examining 1000 trees and are indicated for members of the Haloanaerobiaceae family only. Bar indicates 10 nucleotide changes per 100 nucleotides.

*H. chitinovorans*, *H. lacunaris* and *H. halobius* form a loose cluster (cluster I) with *Sporohalobacter lortetii* and are separated from it by evolutionary distances of 11.6, 13.4, and 13.1, respectively. All the four strains, in addition, share common defining nucleotides, nucleotide pairs and secondary structures at the exclusion of the members of cluster II which includes the genera *Haloanaerobium* and *Halothermothrix* (Table 2 and Fig. 1). It can be argued on the basis of these results that *H. lacunaris*, *H. chitinovorans*, *H. halobius* and *S. lortetii* should be included as members of the same genus. However, bootstrap analysis does not confidently support this (Fig. 1) and new isolates should be studied before such conclusions can be drawn and further revision considered.

Description of *H. chitinovorans* comb. nov. The description of the species has not been amended and is the same as described in the original report [8]. The transfer of the species to the genus *Halobacteroides* does not change the description of the genus.

Description of *Ha. acetoethylicus* comb. nov. The description of the species has not been amended and is the same as described in the original report [15]. The transfer of the species to the genus *Haloanaerobium* does not change the description of the genus. The description of the genus has been amended

recently from its original description [20] and includes new traits [1,18].

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