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REVIEW PAPERS

The Role of DNA–DNA Hybridization and 16S rRNA Gene Sequencing in Solving Taxonomic Problems by the Example of the Order *Haloanaerobiales*

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Abstract—In this review, the validity of evolutionary conclusions inferred from the quantitative estimates of the similarity between bacterial genes is evaluated using the order *Halonanaerobiales* as an example. The haloanaerobic phenotype is briefly characterized, as are some specific features that allow the order *Haloanaerobiales* to serve as a reference taxon. Phylogenetic analysis provides a set of standard quantitative criteria for ranking bacterial taxa from species to families. Recommendations for the use of these standard criteria are given.

Key words: haloanaerobes, systematics, DNA–DNA hybridization, 16S rRNA gene analysis

INTRODUCTION

Beginning in the mid-1970s, the methods of molecular systematics have been used to establish whether taxa of a particular level are actually equivalent in different phylogenetic groups of living organisms [1]. For ranking taxa, a set of quantitative standards was developed based on comparison of genetic material.

This approach has been especially fruitful in determining the systematics of prokaryotes. The extent of genetic divergence was determined for many prokaryotic taxa of different levels by the three major methods developed for comparison of genetic material. These methods are the analysis of DNA nucleotide composition, DNA–DNA hybridization, and 16S rRNA gene sequencing. The results show that many traditional prokaryotic taxa are nonequivalent: in different physiological groups, taxa of the same level differed significantly in the quantitative parameters of their relationship. For this reason, the traditional phenotypic systematics of prokaryotes is currently undergoing revision. Researchers try to simplify and formalize the taxonomic operations by using quantitative estimates of the similarity between prokaryotic genome sequences.

Although comparison of 16S rRNA genes is widely used in bacterial phylogenetics and taxonomy, it remains unclear to what extent an individual gene sequence (monogenic phylogenetic constructs) reflects changes in the entire genome. The monogenic phylogenetic dendrograms are known to suffer from sampling error, which increases with decreasing size of the macromolecule studied [2]. Depending on the values of the sampling error, the dendrogram topology becomes uncertain over a large area, beginning in the roots. Thus, the dendrograms are based on the analysis of 5S rRNA (the shortest macromolecule studied in phylogenetics) and are almost entirely uncertain because of the extremely high sampling error. This is the reason 16S RNA analysis is used instead of 5S rRNA analysis to generate phylogenetic constructions: the sampling error and the uncertainty of the dendrograms are much lower with 16S RNA analysis.

One way of revealing the sampling error in a monogenic phylogenetic scheme is to compare several independent dendrograms. Universal phylogenetic dendrograms which were fairly congruent with the ribosomal dendrograms [5, 6] were constructed after comparison of the sequences for several structural genes [3, 4]. However, only the complete genome analysis with no sampling error can accurately determine the sampling error of the monogenic trees, particularly ribosomal trees.

One might expect that dendrograms based on the complete genome analysis will appear with the development of genomics, a new line of investigations dealing with the comparative analysis of complete genomes. Currently, complete genome sequencing has been performed for seventeen bacteria and six archaea, whereas several tens of prokaryotic genomes have been only partially sequenced. This is still insufficient for the construction of dendrograms as detailed as the monogenic ones, particularly for ribosomal dendrograms. In addition, these studies are only beginning, and the analysis of the complete genome is methodically difficult [7]. Nevertheless, the first results of genomics, primarily those concerning the general problems of prokaryote phylogeny and evolution, are already subjects of active discussion. Thus, genes with similar nucleotide sequences were found in genomes of two different

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prokaryotes, *Archaea* and *Bacteria*. This has been explained by two opposite hypotheses. One suggests the mosaicism of the prokaryotic genome due to horizontal gene transfer [8–11]. This suggests a revival of the network concept of prokaryote evolution, which was abandoned after the appearance of ribosomal phylogenetics [12, 13]. The other hypothesis suggests that similar genes belong to the portion of a common ancestral genotype inherited by all prokaryotes [14]. This hypothesis is supported by the fact that the rRNA trees correlate with trees based on complete genome analysis [15, 16]. Note that the reconstruction of a common ancestor shared by all living organisms is also a subject of discussion brought about by the use of genomics data [17, 18]. The first quantitative estimates of the similarities of the complete genomes of various organisms have been already reported, although they were obtained by the comparison of specific common genomics elements (signatures) rather than by the analysis of sequence homology [19]. The comparative analysis of whole genomes will be used in the future to resolve the major problems of evolution, phylogeny, and systematics of living organisms. However, the practical use of such an analysis in taxonomic studies has a long way to go.

Nowadays, DNA–DNA hybridization is the most widespread method used for approximate comparative analysis of the entire prokaryotic genome. However, the phylogenetic dendrograms derived from the data obtained by this method have a significant experimental error and a corresponding uncertainty. In addition, the DNA–DNA hybridization method is only adequate for the reconstruction of bacterial phylogenesis at a relatively low level (the top of the tree); at this level, the ribosomal dendrograms and those based on DNA–DNA hybridization can be compared.

The results of numerous studies using the method of DNA–DNA hybridization were summarized to set a quantitative standard for prokaryote relationships at the species level. All new prokaryotic species are currently described using the so-called quantitative genotypic species criterion. Only strains with a DNA homology of no less than 70% can be assigned to the same species [19]. However, the method of DNA–DNA hybridization is inconclusive when less-related organisms are studied. At the intrageneric level, this method is inadequate, although a DNA similarity of no less than 20% is accepted as an approximate criterion used to assign species to the same genus [20]. At higher taxonomic levels, the bacterial relationship cannot be assessed by DNA–DNA hybridization, because a similarity of less than 10–15% is close to the experimental error and the results are considered insignificant.

The ribosomal genes are more rigorously conserved than is the bulk of the genome, and, therefore, their comparative analysis is more informative than DNA–DNA hybridization. The quantitative estimates of the ribosomal gene similarity are used to characterize a broad region of the taxonomic spectrum from domains to those of the species that exhibit a 16S rRNA homology of about 97% [21]. However, many prokaryotic species are indistinguishable by this method, because they can have virtually identical 16S rRNA sequences but a low level of DNA similarity (as low as 25%) [21, 22]. Hence, the amount of information provided by 16S rRNA gene analysis decreases with an increase in the degree of relatedness, i.e., in the reverse direction as compared to the method of DNA–DNA hybridization.

The similarity estimates obtained by analyses of 16S rRNA and total DNA are related by the empiric formula [23]

$$\log S = 0.0350\,(\log\%\ \mathrm{DNA}) - 0.0698,$$

where S is the percent similarity of 16S rRNA gene sequences, and % DNA is the percent of hybridization of the entire genomes.

In the opposite regions of the taxonomic spectrum, where either of the methods becomes noninformative, the experimental data do not fit the above formula.

No conventional standards based on 16S rRNA analysis have yet been set for assessing the relationship between prokaryotes at the supraspecies level. The polyphasic analysis of the higher level taxa (beginning from the genus) has been recommended [24]. When the conclusions diverge significantly, the results of the phenotypic analysis are considered superior, although it is desirable to elucidate the reasons for the data conflict.

The well-studied reference taxa are helpful as examples for ranking new taxa, although a subjective attitude is inevitable when choosing the reference, since none of the bacterial taxa have been completely studied yet. The classification of the family *Enterobacteriaceae* is currently accepted as the most perfect and is often suggested as a reference classification to determine relationships between genera within other families [25].

I believe that the new taxonomic classification of the order *Haloanaerobiales* can also be accepted as a reference classification for the construction of dendrograms for prokaryotes [26].

A BRIEF PHENOTYPIC CHARACTERIZATION OF HALOANAEROBES

The first anaerobic organotrophic bacterial isolates growing at a high salinity (10–15% NaCl) were described as *Haloanaerobium praevalens* [27] and *Halobacteroides halobius* [28]. Subsequently, a series of new species and genera of haloanaerobes were identified and combined within the family *Haloanaerobiaceae* [29]. A list of presently known haloanaerobes, including nine genera and twenty species, is presented in Table 1.

Haloanaerobes usually inhabit cyanobacterial mats in hypersaline lagoons [49]. In addition, they occur on the surface and in the sediments of such saline ecosystems as the Dead Sea, in hypersaline lakes [50], in alka-

Table 1. Taxonomic structure of the order Haloanaerobiales

Order HALOANAEROBIALES [26]	
Family HALOANAEROBIACEAE [28]	Family HALOBACTERIACEAE [26]
Genus *Haloanaerobium* [27]	Genus *Halobacteroides* [28]
H. pravalens [27]	*Hb. halobius* [28]
H. (Haloincola) succharolyticum subsp. *saccharolyticum* [26, 30, 31]	*Hb. elegans* [40]
H. (Haloincola) saccharolyticum subsp. *senegalensis* [31]	Genus *Haloanaerobacter* [41]
H. alcaliphilum [32]	*Han. chitinovorans* [41]
H. salsuginis [33]	*Han. lacunaris* [26, 42]
H. (Halobacteroides) acetoethylicum [26, 34]	*Han. salinarius* [43]
H. congolense [35]	
H. kushneri [36]	Genus *Acetohalobium* [44]
H. lacusrosei [37]	*A. arabaticum* [44]
Genus *Halocella* [38]	Genus *Natroniella* [45]
Hc. cellulolytica [38]	*N. acetigena* [45]
Genus *Halothermothrix* [39]	Genus *Orenia* [26]
Ht. orenii [39]	*O. (Sporohalobacter) marismortui* [26, 46]
	O. sivashensis [47]
	Genus *Sporohalobacter* [48]
	S. lortetii [49]

litrophic lakes [45, 51] and in subterranean ecosystems, such as oil fields [33, 35].

Most haloanaerobes are moderate halophiles, although some of them are extremely halophilic (*Hb. lacunaris*, *Natroniella acetigena*, and *Acetohalobium arabaticum*). Cells of haloanaerobes were established to contain high concentrations of Na^+ and K^+ [52].

Morphologically, haloanaerobes are typical bacteria with gram-negative type cell-wall ultrastructure. However, some species are spore-forming. All haloanaerobes described thus far are rod-shaped (sometimes these rods are long and thin); they can be nonmobile or mobile by means of peritrichous flagella. Cells of haloanaerobes belong to the major bacterial type: they propagate by binary fission, have no prosthecate or appendages, and form no aggregates.

Lipids of haloanaerobes contain aliphatic fatty acids and hydroxyacids, which is characteristic of gram-negative bacteria. Most strains contain the nonbranched fatty acids $nC_{14:0}$; $nC_{16:0}$; and $nC_{16:1}$ [31, 33, 34, 42, 44]. The thermophile *Halothermothrix* contains a branched $C_{15:0}$ acid which is intrinsic to thermophiles [39].

Haloanaerobes constitute a functionally diverse bacterial group including hydrolytic bacteria (such as the cellulolytic *Halocella*), saccharolytic organisms fermenting di- and monosaccharides (*Haloanaerobium*, *Halobacteroides*, *Sporohalobacter*, and *Halothermothrix*), and secondary anaerobes, such as the homoacetatic *Natroniella* and *Acetohalobium*. Bacteria of the genus *Acetohalobium* can grow either as lithoautotrophs consuming hydrogen and CO_2 or as methylotrophs or organotrophs consuming trimethylamine, betaine, lactate, formate, pyruvate, glutamate, aspartate, and histidine. All are anaerobic. Mostly acetate and sometimes ethanol were found among the products of sugar fermentation, in addition to H_2, CO_2, and C_2. Some strains also form butyrate, propionate, and formate [26, 53, 54].

Specific phenotypic features of haloanaerobes allow this group to serve as a convenient model for resolving taxonomic problems by molecular biological methods. These features are the following: (1) unlike many other physiological bacterial groups, the ecological, morphological, and physiological characteristics of haloanaerobes constitute a single complex typical of a monophyletic taxon; (2) due to their unique phenotypic properties, primarily morphological and ecological, haloanaerobes cannot be assigned to any of the known bacterial groups, which suggests that they form a high-level taxon; and (3) the functional diversity of the group suggests that the monophyletic taxon that they form has a complex structure.

PHYLOGENETIC PLACEMENT OF HALOANAEROBES

Due to the failure of the phenotypic analysis, the key role in the determination of the phylogenetic position of haloanaerobes among other microorganisms has been played by various methods of genotypic analysis.

The content of G+C pairs in the DNA of all haloanaerobes is low, as is the degree of variation in the

Table 2. Similarity levels of total DNA sequences (on the lower left) and 16S rRNA genes (on the upper right), %

No.	Haloanaerobe species	Strains	1	2	3	4	5	6	7	8	9
1	*Haloanaerobium praevalens*	DSM2228	100	99.9	99.0	98.5		98.6	97.9	96.6	98.8
2	*Haloanaerobium praevalens*	S-8		100	99.2	99.0		98.8	98.1	96.6	99.1
3	*Haloanaerobium acetoethylicum*	DSM3532	27		100	99.6		99.6	97.6	96.7	99.6
4	*Haloanaerobium saccharolyticum* subsp. *saccharolyticum*	Z-7787^T	15			100		99.2	97.1	96.5	99.3
5	*Haloanaerobium saccharolyticum*	Z-7487	11			90	100				
6	*Haloanaerobium saccharolyticum* subsp. *senegalense*[a]	DSM7379^T	9		11	71		100	97.6	96.6	99.2
7	*Haloanaerobium alcaliphilum*	DSM8275^T							100	95.5	97.6
8	*Haloanaerobium lacusrosei*[b]	H200^T	8		11	11		15		100	96.6
9	*Haloanaerobium kushneri*[c]	VS-751^T	18		37	28					100
10	*Haloanaerobium kushneri*	VS-732	16		40	25					95
11	*Haloanaerobium kushneri*	VS-511									98
12	*Haloanaerobium salsuginis*	VS-752^T									25
13	*Haloanaerobium congolense*[d]	SEBR4224^T	16		15	25		26	19		
14	*Halocella cellulolytica*	Z-10151^T				21					
15	*Halothermothrix orenii*	OCM544^T									
16	*Halobacteroides halobius*	DSM5150^T	4			5	8				
17	*Halobacteroides elegans*	Z-7287^T				7					
18	*Halobacteroides elegans*	Z-7187									
19	*Haloanaerobacter chitinovorans*[e]	W5C8^T	4								
20	*Haloanaerobacter chitinovorans*	W3C1	5								
21	*Haloanaerobacter salinarius*[f]	SG3903^T									
22	*Haloanaerobacter lacunaris*	Z-7888^T								3	
23	*Orenia marismortui*	DSM5156^T	10			10	10	1			
24	*Orenia sivashensis*	Z-7191^T	4								
25	*Natroniella acetigena*	Z-7937^T									
26	*Sporohalobacter lortetii*	DSM3070^T	9			4		1			
27	*Acetohalobium arabaticum*	Z-7288^T	7			7	7				
28	*Acetohalobium arabaticum*	Z-7492	10								

Note: Data on DNA–DNA hybridization are from A.M. Lysenko and from the following papers: a, [30]; b, [36]; c, [35]; d, [34]; e, [40]; and f, [42].

10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
		95.4	97.9	88.4	84.6	81.7	81.1		81.2		81.3	81.4	83.3	80.9	82.8	82.3	79.7
		95.5	97.9	88.5	84.8	81.7	81.0		81.2		81.0	81.6	83.3	81.1	82.9	82.0	80.4
		95.7	98.2	89.0	85.6	81.7	81.0		81.5		81.0	81.5	83.0	80.6	83.0	82.2	80.6
		95.4	98.0	88.5	84.8	81.7	80.9		81.0		80.7	81.1	82.7	80.3	83.0	81.8	79.8
		95.3	98.0	88.4	85.0	81.3	81.1		81.1		81.0	81.4	82.3	80.2	82.6	82.2	79.5
		93.9	96.5	87.3	83.9	80.1	80.5		80.6		80.8	81.2	82.1	79.4	82.1	81.8	79.4
		95.2	96.1	88.5	85.0	80.9	80.8		80.8		80.6	80.7	82.2	80.5	82.7	81.6	79.8
		96.0	98.2	88.7	85.3	81.3	81.5		81.0		81.4	81.7	82.9	80.6	82.7	82.9	80.2
100																	
89	100																
22		100	95.5	89.2	85.7	80.2	80.2		80.1		80.3	80.6	82.0	80.2	82.9	81.5	79.5
			100	89.0	85.0	81.9	81.5		81.8		80.2	82.0	83.6	80.7	82.8	82.6	80.6
				100	88.4	82.5	82.6		82.6		81.5	82.3	84.4	80.6	83.8	81.7	80.2
					100	82.4	80.4		82.5		81.5	81.5	83.9	80.8	84.0	80.3	81.6
						100	87.5		88.2		87.0	87.8	89.4	87.3	88.6	86.0	85.8
				9		42	100		95.7		94.8	94.0	87.1	85.7	87.4	86.7	84.9
							92	100									
									100		98.3	94.4	88.3	86.6	87.3	88.4	84.9
									92	100							
									67		100	93.2	87.5	85.9	87.6	87.1	84.3
						45	44					100	87.2	83.3	87.3	87.4	86.1
			1			13							100	91.4	90.9	86.1	84.0
													44	100	88.7	84.0	82.4
															100	86.3	85.2
						9							13			100	91.8
						7							15		27	15	100
						5										16	91

nucleotide composition: from 27–34 mol % G+C in mesophilic strains to 39.6 mol % G+C in the thermophile *Halothermotrix*. This lends support to the notion that haloanaerobes constitute a single group, whereas the phylogenetic placement and the degree of genetic variation in this group remain unclear.

The idea that haloanaerobes have a common ancestry was confirmed by comparative analysis of 5S [30] and 16S rRNA [46] sequences, although the precise phylogenetic placement of these bacteria still remain unclear. The resolution of this issue came from a comparative analysis of the complete nucleotide sequences of 16S rRNA genes. To date, all haloanaerobe species have been studied by this method.

Phylogenetic analysis [26, 55] using various algorithms for constructing phylogenetic trees has confirmed the common ancestry of haloanaerobes and combined them in a monophyletic cluster branching off from the basis of the line of descent leading to gram-positive bacteria. Haloanaerobes did not cluster with any of the subdivisions of this line, including those with such gram-negative members as *Heliobacterium* and *Sporomusa*. As judged from 16S rRNA gene sequences, haloanaerobes were 75–80% similar to other representatives of the gram-positive bacterial line. An additional analysis of 16S rRNA signature positions and secondary structure confirmed that haloanaerobes differ considerably from each of the subdivisions in the gram-positive bacterial line. It can be inferred that haloanaerobes constitute a distinct phylogenetic branch of the gram-positive bacterial line. The phylogenetic placement of haloanaerobes confirms that a trait such as the ability to form endospores is taxonomically important because spore-forming bacteria have so far only been found beyond the gram-positive bacterial line of descent [56]. Thus, the list of bacteria contributing to this line was supplemented by haloanaerobes and became so extended that now it should be called the line of gram-positive bacteria and their gram-negative relatives.

Since haloanaerobes were assigned to a separate phylogenetic branch, they have been promoted to the rank of a separate order *Haloanaerobiales* [26] (instead of the family *Haloanaerobiaceae* Oren 1984); this suggestion has been validated. Thus, the idea that haloanaerobes should form a high-level monophyletic taxon was correct.

In the newly established order *Haloanaerobiales*, there is much agreement between the data of phenotypic and genotypic analyses, which is seldom the situation in bacterial systematics. The order *Haloanaerobiales* can, therefore, be used as a reference taxon to set quantitative genotypic standards for taxon ranking.

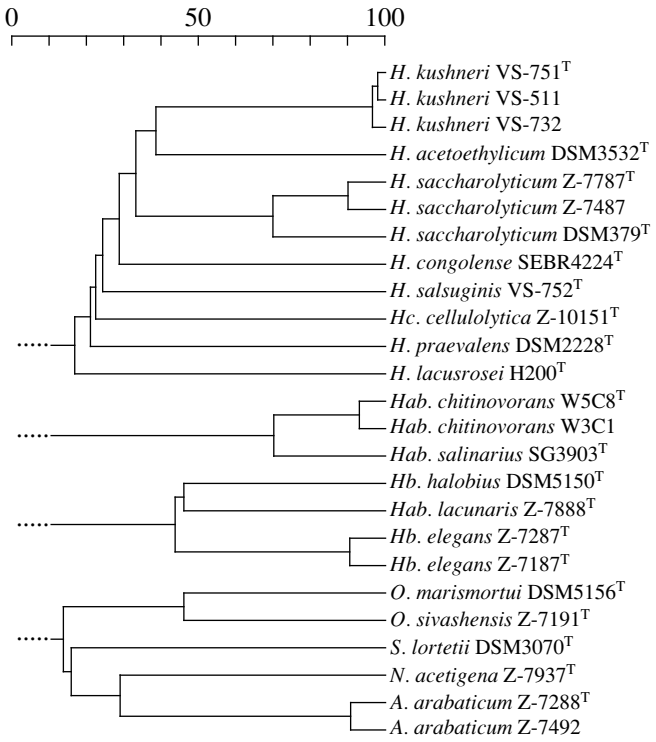

Fig. 1. Approximative dendrogram of the relationships among the members of the order *Haloanaerobiales* based on the data of DNA–DNA hybridization. The approximative similarity matrix was used; the lacking values were calculated from the mean values of a cluster. The trees were constructed from the approximative matrix using cluster analysis (UPGMA) implemented in the PHYLIP software package [63]. The scale shows % of DNA–DNA hybridization.

TAXONOMIC STRUCTURE OF THE ORDER *HALOANAEROBIALES*

Two methods have been extensively used to construct phylogenetic dendrograms for the order *Haloanaerobiales*: either nucleotide sequences of the entire genome (DNA–DNA hybridization) or complete 16S rRNA genes sequences were compared. The results obtained by both methods are presented in Table 2. As in other taxonomic bacterial groups, the phylogenetic dendrograms based on DNA–DNA hybridization and on 16S rRNA analysis show no crucial differences (Figs. 1 and 2), although, as expected, they differed in the information content within certain regions. Both types of dendrograms confirmed that haloanaerobe phylogenetic diversity corresponds to their functional diversity. The relationships revealed by the phylogenetic analysis coincide with the taxonomic structure of the order *Haloanaerobiales* determined by phenotypic analysis.

As evidenced by the ribosomal phylogenetic dendrogram, two distinctly different clusters occur within the order *Haloanaerobiales* (Fig. 2). The first is comprised of the genera *Haloanaerobium*, *Halothermothrix*, and *Halocella*; and the second is comprised of the

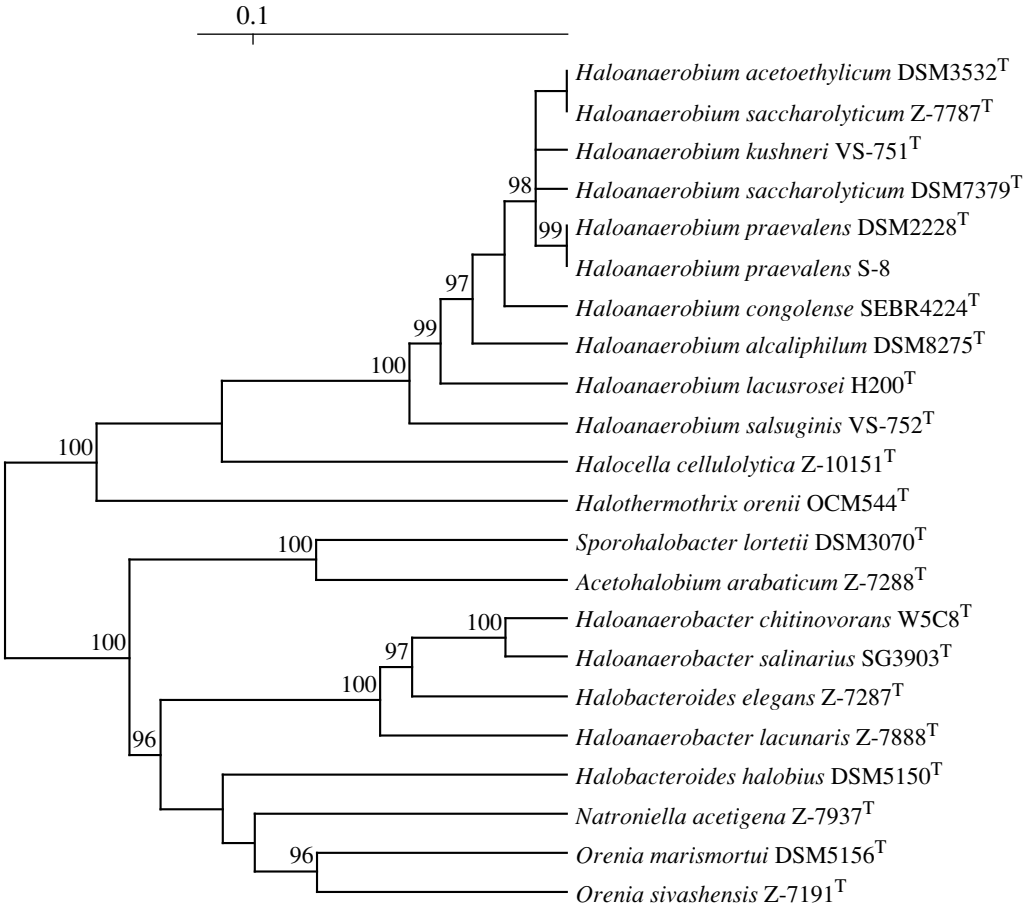

Fig. 2. Dendrogram of the relationships between the members of the order *Haloanaerobiales* based on 16S rRNA gene analysis and constructed using cluster analysis (UPGMA) in the TREECON software package [64]. The scale bar corresponds to 10 nucleotide substitutions per 100 nucleotides. Numbers indicate statistical significance of the branching order determined using bootstrap analysis of 100 alternative trees; values less than 95% are not shown.

genera *Halobacteroides*, *Haloanaerobacter*, *Acetohalobium*, *Sporohalobacter*, *Orenia*, and *Natroniella*. This phylogenetic clustering corresponds to the taxonomic subdivision of the order *Haloanaerobiales* into two families, *Haloanaerobiaceae* and *Halobacteroidaceae*, which differ phenotypically in the capacity to form spores [26, 55]. Note that the phylogenetic clustering within the order *Haloanaerobiales* was statistically significant to the extent characteristic of taxonomic subdivision into families and genera; this is evidenced by the high values (no less than 96%) yielded by bootstrap analysis for the corresponding points of branching (Fig. 2).

The dendrograms based on DNA–DNA hybridization fail to reveal the subdivision of haloanaerobes into the two major clusters (Fig. 1). This is likely due to the fact that this subdivision fell into the uncertainty region characteristic of such dendrograms (the level of DNA similarity was less than 10–15%).

The generic structure of both families within the order *Haloanaerobiales* can be determined only by ribosomal analysis since the level of DNA hybridization between the members of different genera was less than 20%. The history of the changes in taxonomy of haloanaerobes at the generic level illustrates this.

Spore-forming haloanaerobes, now assigned to different genera, *Sporohalobacter* and *Orenia*, were originally combined in the genus *Sporohalobacter* as two different species, *S. lortetii* and *S. marismortui* [46]. The level of DNA hybridization between these species (less than 15%) could not be interpreted unambiguously. Only after 16S rRNA comparison did the phylogenetic relationships of these organisms become clear. According to the ribosomal dendrograms, the species *S. lortetii* and *S. marismortui* belong to different clusters. For this reason, the latter species was reclassified as a representative of the new genus, *Orenia* [26].

However, the two methods used for comparison of nucleotide sequences, as well as the phenotypic analysis, sometimes yield conflicting results. Thus, the relationships between the type genus of the family *Halobacteroides* family and the genus *Haloanaerobacter* have been debated. Two species, *Hb. halobius* (the type species) [28] and *Hb. lacunaris* [42] were originally assigned to the genus *Halobacteroides*, whereas the

single species *Hab. chitinovorans* constituted the genus *Haloanaerobacter* [41]. On a dendrogram based on DNA–DNA hybridization (Fig. 1), the *Hab. chitinovorans* strains form a separate cluster: the similarity level between their genomes and those of other haloanaerobe species is extremely low (about 8%) and falls into the uncertainty region, which can be interpreted as support for their generic status. In contrast, *Hb. halobius* and *Hb. lacunaris* were assigned to the same genus because of the relatively high level of interspecific DNA hybridization (45%). However, 16S rRNA gene sequencing showed that *Hab. chitinovorans* is much more closely related to *Hb. lacunaris* than the latter species is related to *Hb. halobius* (the similarity levels of 94.8 and 87.8%, respectively).

Two alternative ways to change the classification were proposed: (a) to assign all of these species to the genus *Halobacteroides* and to abolish the genus *Haloanaerobacter* [57], or (b) to move *Hb. lacunaris* into the genus *Haloanaerobacter* with emendation of the descriptions of both genera [26]. The latter proposal has been accepted. Recently, a new species of this genus, *Hab. salinarius*, has been described, which is similar to the species *Hab. chitinovorans* both phylogenetically and phenotypically [43]. Description of a new haloanaerobe species, *Hb. elegans*, has caused more complications [40]. As inferred from the similarities between 16S rRNA gene sequences, this species was considerably more closely related to the species of the genus *Haloanaerobacter* (95.7%) than to *Hb. halobius* (87.5%); i.e., by this criterion, it belonged to the genus *Haloanaerobacter.* However, the DNA of *Hb. elegans* was rather homologous (44%) to the DNA of both *Hab. lacunaris* and *Hb. halobius*, and, in addition, *Hb. elegans* is phenotypically so similar to *Hb. halobius* that it was originally described as a representative of the latter species [49]. Thus, the study of the species *Hb. elegans* provided further support for the proposal to combine all of these species into a single genus, *Halobacteroides.* However, one has to recognize that this proposal is inconsistent with the results of phylogenetic analysis.

At the intrageneric (interspecies) level, the data from DNA hybridization and 16S rRNA gene sequencing show much better correlation. This is well demonstrated by the example of the most voluminous taxon of haloanaerobes, the type genus of the family *Haloanaerobiaceae*, *Haloanaerobium*, which encompasses eight species whose relationships determined by both methods largely coincide (Figs. 1 and 2). The same is true of the relationships between the type species of the genus *Orenia* (*O. marismortui*) and the new species of this genus *O. sivashensis.*

The two methods of nucleotide sequence analysis applied concurrently made it possible, in some cases, to alter the taxonomic structure of haloanaerobes determined on the basis of phenotypic analysis. Thus, the similarity between the species *Hb. acetoethylicus* of the genus *Halobacteroides* [34] and the type species *H. pravaelens* of the genus *Haloanaerobium* proved to be too high (27%) for the intergeneric level. Comparative analysis of 16S rRNA confirmed that *Hb. acetoethylicus* belongs to the cluster formed by the *Haloanaerobium* species. As a result, *Hb. acetoethylicus* was reclassified as *H. acetoethylicum* [26].

A group of haloanaerobe strains was originally assigned to a separate new genus, *Haloincola*, composed of a single species, *Hi. saccharolytica* [30]. DNA–DNA hybridization showed that the *Haloincola* strains belong to the species cluster of the genus *Haloanaerobium.* Since the level of DNA similarity did not, however, exceed 15%, this was accepted as support for the generic status of *Haloincola.* Analysis of 16S rRNA sequences confirmed the relatedness between *Haloincola* strains and the species contributing to the genus *Haloanaerobium.* However, the level of similarity revealed (99.2%) was too high to support the generic status of *Haloincola.* Therefore, the genus *Haloincola* was abolished, and its members were assigned to the genus *Haloanaerobium* as individual species [26].

At the intraspecific level, only the data on the similarity of total genomes proved informative. For example, the 16S rRNA sequences of two subspecies of *H. saccharolyticum* were virtually identical; the differences found were most caused by experimental error. Hence, if the results of DNA–DNA hybridization support the intraspecific similarity of the strains studied, it is reasonable to conclude that these strains belong to a single species. Appropriate examples are strains Z-7288 and Z-7492 of *A. arabaticum*, Z-7187 and Z-7287 of *Hb. elegans*, Z-7787 and Z-7487 of *H. saccharolyticum*, W3C1 and W5C8 of *Hab. chitinovorans*, and VS-751, VS-732, and VS-511 of *H. kushneri.*

The results of the investigations of the taxonomic structure of bacteria described to date as the members of the order *Haloanaerobiales* make it possible to specify quantitative genotypic parameters allowing taxon ranking within this bacterial group (Table 3).

It can be seen that, at the boundaries of different-level taxa, the ranges of the parameters given in Table 3 overlap, which is due to the taxonomic requirement that the evidence of the phenotypic analysis should be superior to that of the genotypic analysis [24]. As a result, taxa of different levels may sometimes have the same level of genotypic divergence. Within the family *Halobacteriaceae*, this is illustrated by the examples of two pairs, *A. arabaticum–S. lortetii* and *O. marismortui–O. sivashensis*, which display approximately the same level of 16S rDNA similarity (91.8 and 91.4%, respectively) but different levels of total DNA similarity (15 and 44%, respectively). Note that considerable phenotypic differences between *A. arabaticum* and *S. lortetii* suggest their affiliation to different genera [43], whereas *O. marismortui* and *O. sivashensis* have enough phenotypic features in common to be combined in a single genus [47].

Table 3. Quantitative data characterizing the relationships of various-level taxa within the order Haloanaerobiales

Taxonomic level	Sequence similarity, %	
	total DNA	16S rRNA genes
Between families	Less than 10	79.5–84.4
Between genera of the same family	5–27	83.3–91.8
Between species of the same genus	8–67	91.4–99.8
Between strains of the same species	71–98	99.2–99.9

The same taxonomic situation is inevitable when other prokaryotic groups are studied, and it is not only characteristic of these two pairs of haloanaerobes. In extreme cases, the data of phenotypic and genotypic analyses may differ considerably, hindering the taxonomic interpretation of phylogenetic dendrograms. On the one hand, some genotypically related organisms cannot be combined in a separate taxon because of the basic differences in their phenotype, as is the case with the genera *Synthrophomonas* and *Synthrophospora* [58]. On the other hand, the numerous cases where considerable genotypic differences were revealed by DNA–DNA hybridization while the phenotypes were similar or even identical, created the need to control the use of the term *genospecies* [59].

Similarly, at higher taxonomic levels, for example, in the genera *Bacillus* [60, 61] and *Clostridium* [62], ribosome gene analysis necessitated their reclassification, which resulted in the description of new genera. The same problem arose with the haloanaerobe genera *Halobacteroides* and *Haloanaerobacter* (see above). Such situations are evidently due to some regularities of the evolutionary process and should not be unexpected for bacterial taxonomists.

We believe that the taxonomic structure of the order *Haloanaerobiales* is sufficiently complex and well enough studied both phenotypically and genotypically for this order to be recommended as a reference taxon in bacterial systematics for the interpretation of interpret the data obtained by DNA–DNA hybridization and 16S rRNA gene sequencing.

CONCLUSION

Several recommendations can be made concerning the use of quantitative data for resolving the taxonomic problems of bacterial gene similarity.

DNA–DNA hybridization is inconclusive at high taxonomic levels such as families, genera, and, possibly at the species level (those with the similarity level of no less than 90% as determined by 16S rRNA gene sequencing). Nowadays, high level taxa are mostly formed on the basis of the phylogenetic analysis, whereas phenotypic evidence is additional.

At lower taxonomic levels, such as related genera and most species, i.e., at a 16S rRNA gene similarity of 90 to 97%, a combined application of both methods is advantageous. For closely related genera and remote species, the results obtained by 16S rRNA gene sequencing are basic, whereas those of DNA–DNA hybridization provide only additional quantitative characteristics. However, the data obtained by phenotypic analysis are of special importance at this taxonomic level; only phenotypic analysis can establish whether the taxa studied are close genera or remote species.

For the closest species, with more than 97% similarity of 16S rRNA genes [21], DNA–DNA hybridization studies and phenotypic analysis are required. Thus, strain SG3903, genotypically related to the species *Hab. chitinovorans* (67% of DNA similarity and 98.3% of 16S rRNA similarity), was classified as the individual species *Hab. salinarius*, primarily on the basis of its phenotypic features [43].

At the intraspecific level, i.e., at more than 60–70% of total DNA similarity, 16S rRNA gene analysis makes no sense, since these sequences would most likely prove to be identical within the limits of experimental error.

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