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TAXONOMY OF THE FAMILY HALOBACTERIACEAE AND THE DESCRIPTION OF TWO NEW GENERA *HALORUBROBACTERIUM* AND *NATRIALBA*

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Complete sequences of 16S rRNA encoding genes from extreme halophiles *Halobacterium saccharovorum*, *Halobacterium lacusprofundi*, and *Halobacterium distributum* were determined. The polar lipids, particularly the glycolipids, of these and 20 other isolates were also analyzed. Based on both genetic and chemotaxonomic data, the following two novel genera are proposed: *Halorubrobacterium* (comprising *Halorubrobacterium saccharovorum*, *Halorubrobacterium sodomense*, *Halorubrobacterium lacusprofundi*, *Halorubrobacterium distributum*, and *Halorubrobacterium coriensis*) and *Natrialba* (comprising *Natrialba asiatica*). Evidence for a third new genus is also presented.

Halobacteria are a group of microorganisms forming part of the domain Archaea, and require high salt concentrations for growth (*11*). For many years the taxonomy of halobacteria has been confused, partly the result of a classification system not based on rRNA sequence data, and partly due to many of the deposited strains retaining their original classification in the genus *Halobacterium* even after the establishment of the genera *Haloarcula* and *Haloferax* (*36*). Adding to this confusion has been the use of misidentified or mislabeled cultures in taxonomic studies, giving rise to conflicting data, e.g., *Hb. saccharovorum* (*32*,*34*) and *Hb. trapanicum* (*34*), and delaying a clear definition of genera. The ramifications of this situation are directly affecting progress in the area and there is an urgent need for reform (*32*). This is becoming more pressing as new halobacteria are discovered (*22*,*24*,*27*,*28*).

Currently, the family Halobacteriaceae contains six genera of extremely halophilic archaea: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, and the two alkaliphilic genera, *Natronobacterium* and *Natronococcus* (*11*). These divisions are

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based largely on chemotaxonomic criteria, particularly polar lipid composition. The presence of phosphatidylglycerosulfate (PGS) and types of glycolipids are used to assign new isolates of halobacteria to existing genera (*11*,*36*).

Within the genus *Halobacterium* there are three species, *Hb. saccharovorum*, *Hb. sodomense* and *Hb. lacusprofundi*, that are taxonomically quite distinct from the classical *Hb. salinarium* complex, but to date there has been no formal revision of their classification. The results of 16S rRNA/DNA hybridization (*8*,*29*), 16S rRNA sequencing (*5*), and polar lipid (particularly glycolipid) analyses (*12*,*32*,*33*) have all demonstrated that the *Hb. saccharovorum*/*Hb. sodomense*/*Hb. lacusprofundi* group deserves novel generic status within the family Halobacteriaceae.

In this study, the complete sequences of 16S rRNA encoding genes from *Hb. lacusprofundi* JCM 8891, *Hb. saccharovorum* JCM 8865, and *Hb. distributum* JCM 9100, as well as a culture of [*Hb. saccharovorum*] obtained from F. Rodriguez-Valera (University of Alicante, Spain) were determined. The polar lipids of these and other isolates were also analyzed. Based on both genetic and chemotaxonomic criteria, two new genera are proposed: *Halorubrobacterium* (comprising *Hr. sodomense*, *Hr. saccharovorum*, *Hr. lacusprofundi*, *Hr. distributum*, and isolate Ch2 (*24*)) and *Natrialba* (comprising the isolates B1T and 172P1).

MATERIALS AND METHODS

Bacteria and media. The halobacteria used in this study are given in Table 1. *Hb. saccharovorum* JCM 8865 (derived from IFO 14717 which came from DSM 1137) and *Hb. distributum* JCM 9100 (derived from VKM B-1733) were grown in JCM medium 168 (the same as DSM medium 372), *Hb. sodomense* IFO 14740 (derived from ATCC 33755) in IFO medium 258 (the same as ATCC medium 1218), and *Hb. lacusprofundi* JCM 8891 (derived from ATCC 49239) was grown in JCM medium 170 (the same as ATCC medium 1682). [*Hb. saccharovorum*] obtained from Rodriguez-Valera and other neutrophilic extreme halophiles were grown in JCM 168 medium. Cells were grown at 37°C in a shaker-incubator (120 rpm) until they reached the stationary phase.

Lipid composition. Cells grown in 20 ml of media were harvested from stationary phase cultures, and total lipids extracted and analyzed by thin-layer chromatography (TLC) as described previously (*36*).

Sequencing of 16S rRNA genes. Genomic DNA was extracted from halobacterial cells, and 16S rRNA genes were amplified by PCR and cloned into plasmid pUC119 and sequenced as described previously (*13*,*14*). DNA sequences were determined from ssDNA (i.e., M13 phage) templates using a chain-termination method on an ABI 373A automatic sequencing machine. To eliminate the possibility of errors introduced during gene amplification by PCR with Taq DNA polymerase, and to detect multiple heterogeneous genes, if any, of 16S rRNA as in the case of *Ha. marismortui* (*23*), several (eight to ten) different clones from each bacterial strain were analyzed. The accession numbers of sequences deposited with

Table 1. Bacteria used in this study and DNA database accession numbers of 16S rRNA sequences.

Species/Isolate name	Strain number or laboratory of origin	Accession number
Haloarcula vallismortis	ATCC 29715^T	U17593
Haloarcula hispanica	ATCC 33960^T	
Haloarcula japonica	JCM 7785^T	
Haloarcula marismortui	ATCC 43049^T	A, X61688; B, 61689
"Haloarcula sinaiiensis"	ATCC 33800	Major, D14129, minor, D14130
Haloarcula sp. GN-1		
Halobacterium salinarium	CCM 2148	
Halobacterium halobium	Strain R1, (CECT 396)	M38280, M11583
Halobacterium cutirubrum	(NRC 34001)	K02971
Halobacterium distributum	JCM 9100^T (←VKM B-1733^T)	D63572
Halobacterium sp. Y12	F. Rodriguez-Valera[a]	D14127
Halobacterium sodomense	IFO 14740^T (←ATCC 33755^T)	D13379
Halobacterium saccharovorum	JCM 8865^T (←IFO 14717^T ←DSM 1137^T), (ATCC 29252^T)	U17364
[*Halobacterium saccharovorum*]	F. Rodriguez-Valera[a]	
Halobacterium lacusprofundi	JCM 8891^T (←ATCC 49239^T), (DSM 5036)	U17365
Halobacterium trapanicum	NCIMB 767^T	D14125
Halococcus morrhuae	ATCC 17082^T	X00662
Halococcus morrhuae	NRC 16008	D11106
Haloferax volcanii	(NCIMB 2012^T)	K00421
Haloferax mediterranei	ATCC 33500^T, (CCM 3361^T)	D11107
Haloferax gibbonsii	ATCC 33959^T	D13378
Haloferax denitrificans	ATCC 35960^T, (DSM 4425)	D14128
Natronococcus occultus	NCIMB 2192^T	Z28378
Natronobacterium magadii	NCIMB 2190^T	X72495
172P1	JCM 9576^T	D14123
B1T	K. Tojo,[b] JCM 9577	D14124
L-11 (GSL-11)	F. J. Post[c]	D14126
Ch2	JCM 9275^T (←ACM 3911^T)	L00922

[a] Dr. F. Rodriguez-Valera, University of Alicante, Spain.

[b] Dr. K. Tojo, Nihon University, Japan.

[c] Dr. F. J. Post, Dept. of Biology, Utah State University, U.S.A. (Ref. *28*)).

ACM, Australian Collection of Microorganisms, University of Queensland, Brisbane, Australia; ATCC, American Type Culture Collection, Rockville, U.S.A.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan; NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland; NRC, National Research Council, Ottawa, Canada; VKM, All-Russian Collection of Microorganisms, Moscow, Russia. ^{T}, type strain.

DNA databases (DDBJ, GenBank) are listed in Table 1, together with those deposited previously.

Determination of guanine+cytosine contents and DNA-DNA reassociation values. DNA was extracted and purified as described previously (*9*). The guanine-plus-cytosine content of the DNA was determined from the midpoint value (T_m) of the thermal denaturation profile. DNA-DNA homology was determined by the competition hybridization method as described previously (*9*).

Physiological characterization. Physiological tests of isolates B1T and 172P1 were performed according to a standard bacteriological handbook (*7*) and to relevant references (*5,36*).

Phylogenetic reconstruction. Complete 16S rRNA sequences were aligned with the aid of the aligned sequence database of the RDP (Ribosome Database Project; Ref. *19*)). Positions with deletions, ambiguous bases, or uncertain alignment were removed and the remaining positions used to construct phylogenetic trees of the halobacteria using the programs PHYLIP (*3*), PAUP (*31*), and fastDNAml (*25*). Distance matrix (dnadist/neighbor joining), maximum likelihood (fastDNAml), and parsimony (dnapars, PAUP) algorithms were used for tree reconstructions. Bootstrap sampling was performed to estimate confidence levels of the nodes. Methanogen outgroup species were selected on the basis of the phylogenetic tree of the Archaea available from the RDP (*19*).

RESULTS

Polar lipid analyses

Membrane lipid analyses supported the classification of many of the isolates examined (Fig. 1, Table 2). Most of the recognized genera had distinctive lipids, making placement of isolates fairly straightforward. A potential difficulty could arise between the genera *Halococcus* and *Haloferax*, which both contain S-DGD-1, but in practice there would be little problem in distinguishing members of the two groups on other grounds; e.g., morphology, resistance of cells to low salt concentrations, C_{20}, C_{25} diether lipids, and difficulties in the lipid extraction from *Halococcus* cells (sonication or grinding with quartz sands is a necessary prerequisite).

On the other hand, there were a number of isolates that could not be placed in the existing genera or for which the lipid analyses identified an incorrect classification:

i) *Hb. lacusprofundi*, *Hb. saccharovorum* JCM 8865, *Hb. distributum* JCM 9100, and *Hb. sodomense* are all currently placed in the genus *Halobacterium*, yet all four possess the distinctive lipid S-DGD-3 (*15,37*), which is not found in *Halobacterium salinarium* strains (Fig. 1A). Strain Ch2 also possessed the same glycolipid (Fig. 1B).

ii) The strain of [*Hb. saccharovorum*] obtained from F. Rodriguez-Valera did not contain S-DGD-3, but instead has lipids characteristic of *Hb. salinarium* strains (i.e., S-TGD and S-TeGD). An authentic isolate of *Hb. saccharovorum* (JCM 8865) did contain S-DGD-3 as mentioned above.

iii) *Hb. trapanicum* NCIMB 767 had a lipid profile identical with that of isolate L-11 (Fig. 1C, although structures of the glycolipids have not been elucidated), which is different from those of all other isolates, and unlike that of *Hb. lacusprofundi*, *Hb. saccharovorum*, *Hb. sodomense*, and Ch2.

iv) The strains 172P1 and B1T contained a novel glycolipid, the structure of which has been determined as S_2-DGD-1 recently (*22*).

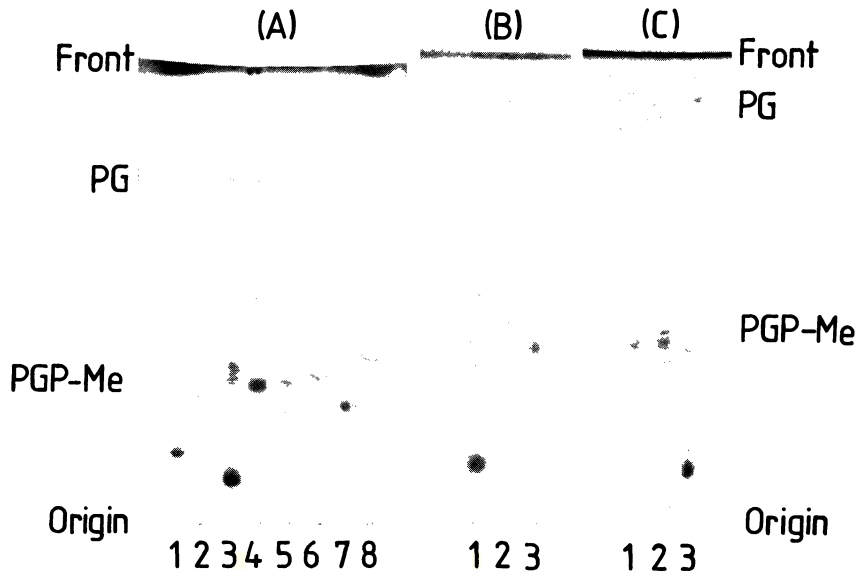

Fig. 1. TLC plates of polar lipids from extreme halophiles.

(A) 1, *Halobacterium* sp. Y12; 2, *Hb. lacusprofundi*; 3, strain 172P1; 4, *Haloferax mediterranei*; 5, *Hb. saccharovorum*; 6, *Hb. trapanicum*; 7, *Hb. sodomense*; 8, *Haloarcula hispanica*. (B) 1, strain 172P1; 2, strain Ch2; 3, *Hb. sodomense*. (C) 1, strain L-11; 2, *Hb. trapanicum*; 3, strain 172P1. Thin layer, Merck HPTLC plates silica gel 60, Art. 5641; solvent, chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, v/v, single development). The chromatograms were stained with α-naphthol/sulfuric acid (circled spots were sugar positive) and charred. *Rf* values differed slightly depending on the lot of the plates or temperature of the solvent. PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate-methyl ester.

Nucleotide sequences of 16S rRNA encoding genes

16S rRNA sequences of 16 different halobacteria were recently determined by Kamekura and Seno (*13, 14*, this study) and these were aligned with nine halobacterial sequences available from the sequence databases (Table 1). The sequences of *Hb. lacusprofundi* and *Hb. saccharovorum* determined here were compared to the previously published sequences of these strains derived by direct RNA sequencing (*10*) and several differences were found. A reexamination of the two datasets, in conjunction with secondary structure information (from the RDP), resolved most of these discrepancies, and the new sequences have been deposited in the sequence databases as U17364 (*Hb. saccharovorum*) and U17365 (*Hb. lacusprofundi*). In addition, a significant error in the submission of the Ch2 sequence was discovered and the corrected sequence has now been lodged with Genbank (L00922). A few corrections were also made on the previously deposited sequences.

From the aligned 16S rRNA sequences, percentage similarities were calculated (omitting positions with unknown nucleotides or gaps), and these are given in Table 3. If we accept the recent proposals (*2, 6*) to use rRNA similarity values to

Table 2. Distribution of glycolipids among extreme halophiles.

Species/Isolate name	Representative glycolipids	Core diether
Halobacterium halobium	S-TDG-1, S-TeGD	C_{20}, C_{20}
Halobacterium cutirubrum	S-TGD-1, S-TeGD	C_{20}, C_{20}
Halobacterium sp. Y12	S-TDG-1, S-TeGD	C_{20}, C_{20}
"Haloarcula sinaiiensis"	TGD-2	C_{20}, C_{20}
Haloarcula marismortui	TGD-2	C_{20}, C_{20}
Haloarcula hispanica	TGD-2	C_{20}, C_{20}
Haloarcula vallismortis	TGD-2	C_{20}, C_{20}
Haloferax mediterranei	S-DGD-1	C_{20}, C_{20}
Haloferax gibbonsii	S-DGD-1	C_{20}, C_{20}
Haloferax denitrificans	S-DGD-1	C_{20}, C_{20}
Haloferax volcanii	S-DGD-1	C_{20}, C_{20}
Halobacterium saccharovorum	S-DGD-3	C_{20}, C_{20}
Halobacterium lacusprofundi	S-DGD-3	C_{20}, C_{20}
Halobacterium sodomense	S-DGD-3	C_{20}, C_{20}
Halobacterium distributum	S-DGD-3	C_{20}, C_{20}
Ch2	S-DGD-3	C_{20}, C_{20}
Halobacterium trapanicum NRC 34021	S-DGD-5	C_{20}, C_{20}
Halobacterium trapanicum NCIMB 767	Unidentified	C_{20}, C_{25}[a]
L-11 (GSL-11)	Unidentified	C_{20}, C_{25}[a]
172P1	S_2-DGD-1	C_{20}, C_{25}[a]
B1T	S_2-DGD-1	C_{20}, C_{25}[a]
Natronobacterium magadii	None	C_{20}, C_{25}[a]
Natronococcus occultus	None	C_{20}, C_{25}[a]
Natronobacterium strain SSL1	DGD-4[b]	C_{20}, C_{25}[a]
Halococcus morrhuae	S-DGD-1	C_{20}, C_{25}[a]

[a] Both C_{20}, C_{20} and C_{20}, C_{25} diethers (*29*) are detected.

[b] See Ref. *39*).

Compiled from the previously published data and the present work. The structures of the glycolipids and C_{20}, C_{20} and C_{20}, C_{25} core diethers are depicted in Refs. *12* and *15*).

define membership within the same genus (i.e., >93–95% similarity), then the groups identified in this study by their distinctive lipid profiles can be considered different genera.

The sequence of 16S rRNA encoding gene of the [*Hb. saccharovorum*] obtained from Rodriguez-Valera (data not shown) clearly showed that the strain was closely related to the classical *Halobacterium salinarium* complex (only 4 bases different), thus supporting the conclusion of Tindall (*32*).

Phylogenetic tree reconstitution

From the 25 aligned sequences of 16S rRNA encoding genes, phylogenetic trees were constructed using a number of different algorithms (see 'MATERIALS AND METHODS') and representative examples are shown in Fig. 2. These support the monophyly of halobacteria currently classified in each of the genera *Haloferax*, *Haloarcula*, *Halococcus*, and *Natronococcus*. The deepest branching within the Halobacteriaceae remains uncertain, but some specific relationships between genera

Table 3. Similarities (%) of 16S rRNA encoding genes.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 (172P1)	98.6	94.4	94.0	88.4	86.8	86.9	87.3	87.0	88.8	88.8	88.8	87.9	87.9	89.0	89.2	89.1	89.3	94.0	93.4	86.9	87.5	86.8	86.7	86.3
2 (B1T)		94.7	94.4	88.8	87.3	86.9	87.3	87.1	88.9	88.9	89.0	88.1	87.6	89.1	89.3	89.1	89.4	93.8	93.7	86.8	87.5	86.8	86.5	86.2
3 (*H. trapanicum*)			99.2	88.9	88.0	87.5	88.2	87.9	89.5	89.3	89.4	88.8	88.9	89.6	89.8	89.5	89.8	93.7	93.0	87.3	87.6	87.3	86.9	87.0
4 (L-11)				88.5	87.7	87.5	87.9	88.1	89.0	88.9	89.1	88.8	89.0	89.5	89.6	89.4	89.6	93.0	92.9	86.9	87.2	87.0	86.7	86.6
5 (*H. marismo*-A)					94.4	94.1	95.9	94.5	90.0	90.0	89.9	89.0	88.8	87.9	87.6	88.1	88.1	88.6	87.7	85.8	86.3	85.6	85.3	85.5
6 (*H. marismo*-B)						98.6	97.0	99.3	87.5	87.6	87.5	86.8	86.6	87.1	86.9	87.3	87.2	87.3	87.6	86.1	86.2	86.0	85.9	85.9
7 ("*H. sinaiiensis*" major)							97.3	99.2	87.2	87.1	87.2	86.6	86.5	86.9	86.7	87.1	87.0	87.0	86.9	85.7	85.8	85.7	86.1	85.6
8 ("*H. sinaiiensis*" minor)								97.2	88.3	88.3	88.2	87.7	87.6	87.5	87.3	87.8	87.6	87.6	87.6	85.7	85.8	85.7	85.5	85.7
9 (*H. vallismortis*-A)									87.4	87.3	87.4	86.7	86.6	87.2	87.0	87.4	87.2	87.3	87.5	86.2	86.3	86.1	85.5	86.1
10 (*H. cutirubrum*)										99.7	99.6	88.2	88.2	87.7	87.7	87.6	87.9	89.2	89.8	87.2	87.9	87.3	86.5	86.9
11 (*H. halobium*)											99.5	88.2	88.3	87.7	87.7	87.6	87.9	89.0	89.9	87.2	87.8	87.3	86.4	86.8
12 (*H.* Y12)												88.1	88.1	87.9	87.7	87.7	87.9	89.1	89.8	87.2	87.8	87.2	86.5	86.8
13 (*H. morrhuae*)													99.3	88.0	87.9	87.5	88.2	87.8	88.5	85.4	85.8	85.2	84.1	84.7
14 (*H. morrhuae* 16008)														87.8	87.7	87.6	88.0	87.8	88.2	85.1	85.5	84.9	83.8	84.4
15 (*H. denitrificans*)															99.0	98.0	99.3	88.8	88.4	88.2	88.5	88.3	87.7	87.8
16 (*H. gibbonsii*)																98.0	99.0	89.0	88.3	87.9	88.2	88.1	87.5	87.5
17 (*H. mediterranei*)																	98.4	89.3	89.0	88.0	88.1	87.6	86.8	87.3
18 (*H. volcanii*)																		89.3	88.8	88.6	88.7	88.3	87.5	88.0
19 (*N. magadii*)																			91.8	86.5	86.6	85.7	86.0	85.5
20 (*N. occultus*)																				87.5	88.0	87.3	87.1	87.1
21 (*H. lacusprofundi*)																					98.3	95.2	95.0	95.0
22 (*H. saccharovorum*)																						95.6	95.2	95.5
23 (*H. sodomense*)																							97.8	98.4
24 (*H. distributum*)																								98.4
25 (Ch2)																								

Similarities $\geq 98.0\%$ are underlined.

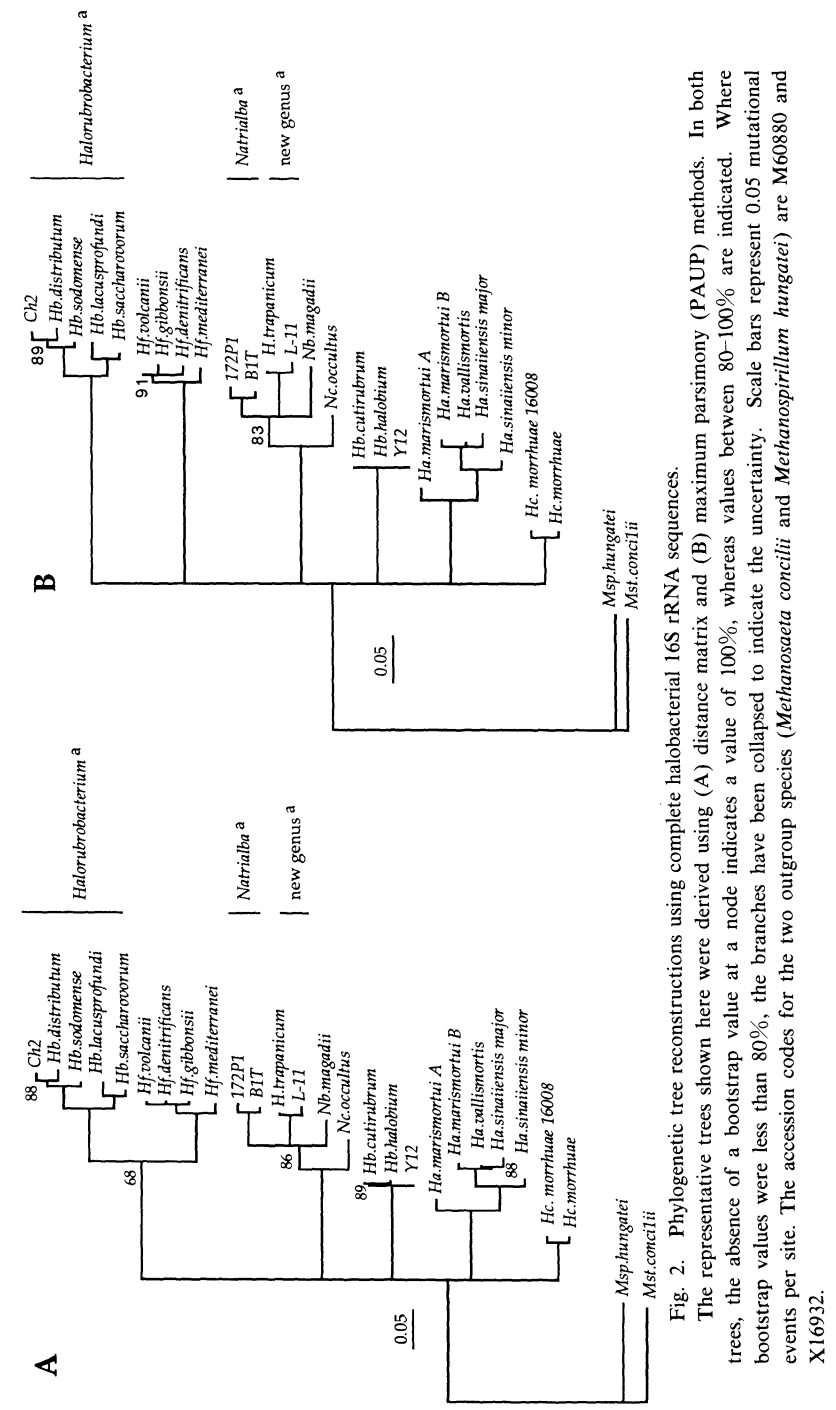

Fig. 2. Phylogenetic tree reconstructions using complete halobacterial 16S rRNA sequences.

The representative trees shown here were derived using (A) distance matrix and (B) maximum parsimony (PAUP) methods. In both trees, the absence of a bootstrap value at a node indicates a value of 100%, whereas values between 80–100% are indicated. Where bootstrap values were less than 80%, the branches have been collapsed to indicate the uncertainty. Scale bars represent 0.05 mutational events per site. The accession codes for the two outgroup species (*Methanosaeta concilii* and *Methanospirillum hungatei*) are M60880 and X16932.

Table 4. DNA-DNA homologies.

Strain	% DNA homology with [^{3}H]-labeled DNA from	
	B1T	172P1
B1T	100	82
172P1	86	100
Haloarcula hispanica ATCC 33960	1	0
japonica JCM 7785	ND	0
marismortui ATCC 43049	0	14
"sinaiiensis" ATCC 33800	20	0
vallismortis ATCC 29715	ND	5
sp. GN-1	0	0
Halobacterium salinarium CCM 2148	9	0
halobium CECT 396	ND	0
Halococcus morrhuae CCM 537	0	0
Haloferax denitrificans DSM 4425	20	0
gibbonsii ATCC 33959	20	0
mediterranei CCM 3361	ND	0
volcanii NCIMB 2012	11	6
Halorubrobacterium lacusprofundi DSM 5036	0	0
saccharovorum ATCC 29252	14	0
distributum VKM B-1733	1	0

ND, not determined.

are well supported. In addition, three phylogenetically novel groups were clearly resolved.

i) It is clear from Fig. 2 that the group consisting of *Hb. saccharovorum*, *Hb. lacusprofundi*, *Hb. sodomense*, *Hb. distributum*, and Ch2 is phylogenetically separate from the *Hb. halobium*/*Hb. cutirubrum*/*Hb.* sp. Y12 group. Note that the names used for members of the latter group are all subjective synonyms for *Hb. salinarium* (see 'DISCUSSION').

ii) The group consisting of 172P1 and B1T also forms a distinct group that is highly supported by bootstrap analysis. DNA-DNA hybridization experiments (Table 4) showed that the strains 172P1 and B1T are closely related and should be considered members of the same species of a novel genus.

iii) The group consisting of *Hb. trapanicum* NCIMB 767 and strain L-11 also deserves a novel genus.

The exact relationship between *Nb. magadii* and the two novel groups (172P1/B1T and L-11/*Hb. trapanicum*) was not resolved by the distance matrix or parsimony methods (bootstrap values of 63%) where it branched either just before the node leading to L-11 and *Hb. trapanicum* in some trees, or just above the node leading to *Nc. occultus* in others. In maximum likelihood trees, *Nb. magadii* branched just outside the two non-alkaliphilic groups (data not shown).

DISCUSSION

As interest in the archaea continues to grow, a sound taxonomy is required to enable new halobacterial isolates to be rapidly and accurately classified and named. In the present study we examined representatives of all the valid genera as well as several other isolates. The results of lipid analyses and phylogenetic trees placed most of the formally named isolates into their currently recognized genera. In particular, all genera except the genus *Halobacterium* were well supported. On this basis some new genera were identified and the genus *Halobacterium* was found to be composed of three distinct groups: (i) the classical *Hb. salinarium* strains, (ii) *Hb. saccharovorum* JCM 8865, *Hb. lacusprofundi* JCM 8891, *Hb. sodomense* IFO 14740, *Hb. distributum* JCM 9100 (and strain Ch2), and (iii) *Hb. trapanicum* NCIMB 767 (and isolate L-11).

Historically, the genus *Halobacterium* was comprised of three well-studied species (*Hb. salinarium* (type species), *Hb. cutirubrum*, *Hb. halobium*) and others (*Hb. marismortui*, *Hb. trapanicum*) (Bergey's Manual of Determinative Bacteriology, 7th ed., 1957). In the 8th ed. (1974) *Hb. cutirubrum* was made the subjective synonym of *Hb. salinarium*. In 1980 Fox et al. showed that 16S rRNA oligonucleotide catalogues for representative strains of the three species were all identical (*4*). Finally, Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984) proposed considering *Hb. cutirubrum* and *Hb. halobium* as subjective synonyms of *Hb. salinarium* (type strain NRC 34002), the latter epithet having priority since 1922. Later, Gutierrez et al. (*9*) included *Hb. salinarium* NRC 34002 and *Hb. cutirubrum* NRC 34001 in a DNA hybridization study, and reported 98% homology between the two strains. Such a high degree of homology, together with the identical rRNA oligonucleotide catalogues, indicates that the two strains are essentially identical (*34*). Recently, two species names, *Hb. salinarium* (*halobium*) strain R1 and *Hb. salinarium* R1, were used in an article proposing a novel species *Halococcus salifodinae*, and the 16S rRNA sequence for *Hb. halobium* strain R1 was adopted as that for the type species of *Hb. salinarium* (*1*).

We propose that the first of these groups, i), retain the genus name *Halobacterium*, and the other groups ii) and iii) should be removed from the genus *Halobacterium* and be designated as novel genera.

For the second group, the name *Halorubrobacterium* is proposed. The name is similar to that proposed by Grant and Ross (*8*), i.e., "*Halorubrum*," for a group consisting of *Hb. saccharovorum* NCIMB 2081, *Hb. sodomense* ATCC 33755, and *Hb. trapanicum* NRC 34021 based on thermal stability of 16S rRNA/DNA hybrids. Adoption of this proposal was not pursued, probably because of misleading lipid profiles resulting from incorrectly identified cultures of *Hb. saccharovorum*. Kushwaha et al. at The University of Ottawa reported that [*Hb. saccharovorum*] obtained from the NRC (culture collection number not cited) showed a polar lipid pattern characteristic of the classical *Hb. salinarium* complex (*17*). Their

data led to the inclusion of [*Hb. saccharovorum*] in the genus *Halobacterium* by Torreblanca et al. (*36*). Lanzotii et al. (*18*) then reported the glycolipid of *Hb. saccharovorum* NCIMB 2081 was S-DGD-1, characteristic of the genus *Haloferax*. The turmoil was resolved in part by Tindall (*32*), who compared the polar lipid profiles of the original isolate from L. I. Hochstein and the strain used in the work by Kates's group. The glycolipid of the authentic *Hb. saccharovorum* co-chromatographed with those of *Hb. sodomense* (S-DGD-3, Ref. *37*)) and *Hb. lacusprofundi* on TLC, thus suggesting that Lanzotii et al. (*18*) may have used a misidentified culture. In this study we determined the 16S rRNA sequences of the authentic *Hb. saccharovorum* and the strain used by Kates's group; our data also clearly demonstrated that the latter strain was a misidentified (mislabeled) one which belongs to the classical *Halobacterium salinarium* complex.

In this study we analyzed polar lipids and the 16SrRNA sequence of *Hb. distributum* VKM B-1733, a valid species of the genus *Halobacterium*. In the original description the authors (*16*) claimed that it was distinguished from *Ha. vallismortis*, *Hb. saccharovorum* and any other known extreme halophile by a greater variability of cell shape and size, and by forming resting forms having a multilayer cell wall (halocysts). Our analysis, however, showed that the strain had a glycolipid, S-DGD-3, the same as that of *Hb. saccharovorum* (data not shown in Fig. 1), and the 16S rRNA sequence was closely related to the members of the new genus *Halorubrobacterium*.

Devereux et al. (*2*) and Fry et al. (*6*) have proposed that a similarity of less than 98% in 16S rRNA sequences was considered evidence for separate species, and a similarity less than 93–95% indicates that they should be in different genera. However, sequence similarities of greater than 98% for 16S rRNA sequence comparisons should not be considered sufficient evidence that two organisms belong to the same species (*21*). Based on these criteria and physiological characteristics, the authors propose maintaining the five strains of the second group as five separate species.

There is no doubt that the third group comprising *Hb. trapanicum* NCIMB 767 and strain L-11 form a novel genus. Judged from the identity of the polar lipid profiles and a very high similarity of the 16S rRNA encoding genes (99.2%), they probably constitute a single species. One of the authors, M. K., obtained strain L-11 from F. J. Post in May 1985. In a later article (*28*) they changed the strain name to GSL-11 after the Great Salt Lake from which it was isolated, and have shown that it possesses a C_{20}, C_{25} core diether. Our TLC also suggested that both L-11 and *Hb. trapanicum* NCIMB 767 possess C_{20}, C_{25} diether as judged from double spots of phosphatidylglycerol (PG, Fig. 1C).

There remains, however, some confusion regarding the identity of different cultures of *Hb. trapanicum*. Previous studies on the distribution of dam-methylation in halobacterial genomic DNA revealed that only four isolates (*Hb. saccharovorum* NCIMB 2081, *Hb. sodomense* ATCC 33755, *Hb. trapanicum* NRC 34021, and Ch2) within the family Halobacteriaceae possess dam-methylated

chromosomes (*20,24*). The results of the present study show no close relationship between *Hb. trapanicum* NCIMB 767 and the genus *Halorubrobacterium* (which contains the other three isolates, as well as *Hr. lacusprofundi*) but it is not certain that the strains of *Hb. trapanicum* used in this and the previous study were identical. Strains NCIMB 767 and NRC 34021 are supposed to be the same, but the results from Ross and Grant (*29*), and more recently Tindall (*34*), have indicated that this is not the case. Trincone et al. (*38*) determined the structure of the major glycolipid (S-DGD-5, Ref. *15*)) from *Hb. trapanicum* NRC 34021 and found that it had an *Rf* value smaller than that of S-DGD-1 from *Haloferax mediterranei*. Our results showed the glycolipids from *Hb. trapanicum* NCIMB 767 possessed *Rf* values larger than that of S-DGD-1 in the same solvent system, thus suggesting that the two strains are not the same. Unfortunately, the remaining confusion regarding these strains (and the unknown glycolipid structure of strain NCIMB 767) delays the formal proposal of a new genus comprising *Hb. trapanicum* NCIMB 767 and L-11.

The two isolates 172P1 and B1T also deserve a novel generic status based on possession of the novel glycolipid S_2-DGD-1, and on the sequences of 16S rRNA genes. We propose the generic name *Natrialba*. DNA/DNA hybridization experiments (Table 4) showed that they have more than 80% homology, indicating they constitute a single species (*30*). The two strains possess C_{20}, C_{25} core diether lipids as shown by chemical analyses (*22*) and also by the double spots of PG and PGP-Me (phosphatidylglycerophosphate methyl ester) on TLC.

The topology of the deeper branches within the halobacteria was not resolved by our phylogenetic reconstruction, but some specific relationships between genera were supported (e.g., *Natrialba* and natronobacteria). The lipid data (Table 2) also support some of these deeper relationships, for example, the closely related genera comprising the natronobacteria, *Natrialba*, and L-11/*Hb. trapanicum* NCIMB 767 all have C_{20}, C_{25} core diether lipids.

Comparison of Table 2 and Fig. 2 shows that the membrane lipids (particularly polar lipids) remain very good markers for phylogenetic groupings at the genus level. Even if a laboratory is not equipped for DNA sequencing, the lipid profiles are relatively easy to obtain using TLC. Many strains can be analyzed on the same TLC plate and the authenticity of isolates checked by comparing these to recognized members of established genera.

This study has solved several problems and established a clear picture of the halobacterial taxonomy and phylogeny.

Emended description of the genus Halobacterium

Halobacterium (Hal.o.bac.te'ri.um. Gr. n. *halo*, *halos* the sea, salt; Gr. n. *bakterion* a small rod; M. L. neut. n. *Halobacterium* salt bacterium).

When grown under optimum conditions, the cells are rod shaped. Resting stages are not known. Gram negative. Most strains are strictly aerobic and motile.

Oxidase and catalase positive. Extremely halophilic with growth occurring in media containing 3.0–5.2 M NaCl.

Chemoorganotrophic. Amino acids are required for growth. Major glycolipid components of cell membrane are S-DGD-1 and S-TeGD, some strains have S-DGD-5 instead.

The mol% G+C of the major component of the DNA is 66–71% and that of the minor component, 57–60%.

Type species is *Halobacterium salinarium* (Harrison and Kennedy 1922) Elazari-Volcani 1957.

The emended genus *Halobacterium* contains *Hb. salinarium* and *Hb. trapanicum.* Further consideration is required for taxonomical position of the latter species.

Descriptions

Halorubrobacterium gen. nov.

Halorubrobacterium (Hal.o.rub.ro.bac.te'ri.um. Gr. n. *halo, halos*, the sea, salt; L. adj. *ruber*, red; Gr. n. *bakterion*, a small rod; M. L. neut. n. *Halorubrobacterium*, red-colored salt bacterium).

Gram-negative rods or pleomorphic forms. Cell dimensions are 0.5–1.2×2.0–12.0 μm. Motile, non-spore forming. Gas vacuoles not present. Colonies are round, smooth, forming a red-pink, non-diffusible pigment. Strictly aerobic. Oxidase-catalase positive. Growth occurs in media containing between 1.5 M to saturated (5.3 M) NaCl, at a temperature range of 4–56°C.

Isolated from hypersaline habitats (salt ponds, salterns). The G+C content of the DNA is 65.3–71.2 mol% (buoyant density method). Structure of the glycolipid (S-DGD-3) is 2,3-di-O-phytanyl-1-O-[2-HSO_3-α-mannopyranosyl-(1→4)-α-glucopyranosyl]-*sn*-glycerol.

Type species is *Halorubrobacterium saccharovorum.*

Halorubrobacterium saccharovorum comb. nov.

The properties of the type strain are the same as those published previously (*35*). Type strain is ATCC 29252 (NCMB 2081; DSM 1137).

Halorubrobacterium sodomense comb. nov.

The properties of the type strain are the same as those published previously (*26*). Type strain is ATCC 33755 (strain RD 26).

Halorubrobacterium lacusprofundi comb. nov.

The properties of the type strain are the same as those published previously (*5*). Type strain is UQM 3107 (ACAM 34).

Halorubrobacterium distributum comb. nov.

The properties of the type strain are the same as those published previously (*16*). Type strain is VKM B-1733 (JCM 9100).

Table 5. Growth characteristics of isolates B1T and 172P1.

		B1T	172P1
Growth	Opt. temp.	35–40°C	30–40°C
	Max. temp.	52°C	50°C
	Opt. pH	7.5–7.8	6.6–7.0
	pH rage	5–10	6–8
NaCl concn.	Opt. concn.	3.5 M	4.0 M
	Max. concn.	Saturation	Saturation
	Min. concn.	2.0 M	2.0 M
Addition of Mg^{2+}		Not required	Not required
Oxygen relationship		Aerobic	Aerobic
Catalase		+	+
Oxidase test		+	+
Acid from	Glucose	+ (no gas)	− (no gas)
	Arabinose	+ (no gas)	− (no gas)
	Xylose	+ (no gas)	− (no gas)
	Maltose	+ (no gas)	− (no gas)
	Starch	+ (no gas)	− (no gas)
Reduction of nitrate		+	+
Denitrification		−	−
Indole formation		+	+
H_2S production		+	−
Ammonia formation		+	ND
Acetylmethylcarbinol		−	−
Methyl red test		−	−
Urease		+	−
Degradation of	Starch	+	−
	Casein	+	+
	Arginine	−	−
Utilization of	$(NH_4)_2SO_4$	−	−
	Citrate	−	−
Heat stability (85°C, 30 min)		−	−
Form and size		Coccobacilli or rods (0.3–0.5 × 1–1.5 μm)	Coccobacilli or rods (0.5–0.7 × 1–3 μm)
Motility		Motile	Motile
Gram stain		Negative	Negative
Spore		−	−
Agar colony		Small, smooth, (1–2 mm diam.)	Convex, smooth, (2–3 mm diam.)
Agar slope		Translucent, glistening, pale yellow	Translucent, white to pale yellow
Stab		No growth	No growth
Gelatin liquefaction		+	+

ND, not determined.

Halorubrobacterium coriensis sp. nov.

Halorubrobacterium coriensis (co.ri.e'ns.is. L. adj. *coriensis*, from corio; referring to the place of origin, a saltern along Corio Bay, Geelong, Australia).

The properties of the type strain are the same as those published previously for the strain Ch2 (*24*). Type strain is ACM 3911.

Natrialba gen. nov.

Natrialba (Na.tri.a'lba. L. n. natrium, sodium; L. adj. *alba*, white; L. fem. n. *Natrialba*, sodium white; referring to the high sodium ion requirement, and the pigmentless colonies of these isolates).

Gram-negative rods, 0.5–1.0×1.0–5.0 μm in cell dimensions. Motile, non-spore forming. Gas vacuoles not present. Colonies are round, smooth. Does not form visible amounts of bacterioruberins, red-pink pigments, in the whole range of NaCl concentration and temperature which supports growth. Content of bacterioruberins was less than 0.1% of that of *Hb. cutirubrum* NRC 34001 as estimated by absorption at 500 nm of neutral lipid fractions. Cultivation under illumination does not cause pigmentation, while intensities of the pigmentation of the strain L-11 and *Hb. trapanicum* NCIMB 767 were enhanced upon illumination during cultivation on agar slopes.

Strictly aerobic. Oxidase-catalase positive. Growth occurs in media containing between 2.0 M to saturated (5.3 M) NaCl. Isolated from hypersaline habitats (salt ponds, beach sands). The G+C content of the DNA is 60.3–63.1 mol% (T_m method). Structure of the glycolipid (S_2-DGD-1) is 2,3-di-O-phytanyl- or phytanyl-sesterterpenyl-1-O-[2,6-$(HSO_3)_2$-α-mannopyranosyl-(1→2)-α-glucopyranosyl]-*sn*-glycerol.

Type species is *Natrialba asiatica.*

Table 6. Summary of the major distinguishing features between genera of neutrophilic extreme halophiles.

	Haloarcula	*Halobacterium*	*Halococcus*	*Haloferax*	*Halorubrobacterium*	*Natrialba*
Cell shape	Irregular rods, triangles, rectangles	Rods	Cocci	Irregular rods, discs	Rods	Rods
Gram stain	Negative	Negative	Variable	Negative	Negative	Negative
Motile	D	+	−	D	+	+
Glycolipid present	TDG-2	S-TGD-1 S-TeGD	S-DGD-1	S-DGD-1	S-DGD-3	S_2-DGD-1
PGS present	+	+	−	−	+	−
C_{20}, C_{25} lipid	−	−	+	−	−	+
Color of colony	Orange or red	Red/Pink	Red	Pink/Red	Red	White/Pale yellow
Lysis in water	+	+	−	+	+	+

Alkaliphilic extremely halophilic genera (growing only at pH≥8.5), *Natronobacterium* and *Natronococcus*, were not included in this table. D, different reactions in different species.

Natrialba asiatica sp. nov.

Natrialba asiatica (a.si.a'ti.ca. L. fem. adj. *asiatica*, from asia; referring to the geographical region from which these organisms were isolated).

The properties are the same as the description for the genus. Detailed characterization of the two strains (B1T and 172P1) are given in Table 5. Both strains contain C_{20}, C_{20} and C_{20}, C_{25} core diether lipids. The strain 172P1 does not require vitamins for growth at 37°C. The requirement for NaCl is partly replaceable by KCl; growth is obtained in a medium containing 6% NaCl and 12% KCl, but no growth in 20% KCl.

The strain 172P1 was isolated in Japan from beach sands with granular salts attached, and B1T from solar salts produced in Taiwan. The type strain is 172P1, which has been deposited in the Japan Culture Collection as JCM 9576.

Table 6 summarizes the major distinguishing features between genera of neutrophilic extreme halophiles.

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