Ch2, a Novel Halophilic Archaeon from an Australian Solar Saltern

STEWART D. NUTTALL* AND MICHAEL L. DYALL-SMITH

Department of Microbiology, University of Melbourne, Parkville, Victoria 3052, Australia

A novel halophilic archaeon, strain Ch2, was isolated from a marine solar saltern in Geelong, Australia. The fact that this organism had a *dam*-methylated genome suggested that it is closely related to the taxon that includes *Halobacterium saccharovorum*, *Halobacterium sodomense*, and *Halobacterium trapanicum*. A sequence analysis of the 16S rRNA gene (Ch2 has three copies of this gene) showed that Ch2 is phylogenetically equidistant from the genera *Haloarcula* and *Haloferax* and closely related to *H. saccharovorum*. The susceptibility of both Ch2 and *H. saccharovorum* to the recently isolated halophage HF2 supported the hypothesis that these two organisms are closely related.

Traditionally, the members of the family *Halobacteriaceae* have been classified on the basis of their biochemical and physiological properties and lipid compositions into six genera (4, 24). More recently, sequence comparisons of the small-subunit rRNAs have gained importance in classification. Using this approach, Lodwick et al. (13) suggested that up to nine halobacterial genera may be justified. The genus *Halobacterium* contains some of the best-characterized examples of these organisms, including *Halobacterium salinarum* and related strains (4). The validly named species *Halobacterium saccharovorum*, *Halobacterium sodomense*, and *Halobacterium trapanicum* form a separate taxon based on possession of *dam*-methylated genomes (12); this classification is supported by sequence data (7, 13). However, until a comprehensive taxonomic review has determined the true positions of these species, they remain species incertae sedis (4).

In a study to isolate halobacterial bacteriophages, we isolated both a phage (HF2) and a susceptible host (Ch2) from a solar saltern on Corio Bay near Geelong, Victoria, Australia. The bacterial host had many features typical of halobacteria and was easily cultivated. In this paper we describe the characterization of strain Ch2 as a new isolate and discuss its relationship to *Halobacterium saccharovorum*.

MATERIALS AND METHODS

Bacterial strains. *Haloferax* phenon K isolate Aa2.2 (6, 24), *Halobacterium saccharovorum* NCMB 2081^T (T = type strain) (23), *Halobacterium halobium* NCMB 777, and *Haloferax volcanii* NCMB 2012^T, which were obtained from the National Collection of Marine Bacteria, Aberdeen, Scotland, and *Haloarcula vallismortis* ATCC 29715^T were used as reference strains in biochemical tests and chromosomal DNA digestions. *Escherichia coli* K-12 strains DH5 α and DH5 α F' were supplied by Bethesda Research Laboratories and were used in all transformations.

Media and growth conditions. A solution containing 25% (wt/vol) artificial salt water (SW) was prepared as described previously (6). This stock solution was diluted to a concentration of 18% (vol/vol) and used to prepare both modified growth medium (MGM), which contained 18% (vol/vol) SW, 15 mM Tris-HCl (pH 7.5), 0.1% (wt/vol) yeast extract

(Oxoid), and 0.5% (wt/vol) peptone (Oxoid), and SW-glucose minimal medium, which contained 18% (wt/vol) SW, 15 mM Tris-HCl (pH 7.5), 1% (wt/vol) glucose, and 0.72 mM NaH₂PO₄. In experiments in which the magnesium ion concentration was adjusted, MgSO₄ was replaced by 0.19 M Na₂SO₄ and the MgCl₂ concentration was varied. For solid media, 15 g of agar (Difco) per liter was added. *E. coli* strains were grown and selected on standard media (1).

Anaerobic conditions were achieved by incubating cultures under a layer of oil (liquid cultures) or in anaerobic jars (5% H₂, 10% CO₂, 85% N₂). Tests for indole, for catalase and oxidase activities, and for motility were performed by standard procedures (3). Antibiotic susceptibility tests (disc method) and carbohydrate utilization tests were performed as described by Rodriguez-Valera et al. (17) except that solid MGM was used in the antibiotic assays.

PCR amplification of the 16S rRNA gene coding sequence. Genomic DNA was isolated from strain Ch2 cultures by the hexadecyltrimethyl ammonium bromide-NaCl method (1). Two oligonucleotide primers, which were complementary to opposite strands of the 16S rRNA termini (for positions see Fig. 3), were designed by using the previously published sequence of *Halobacterium salinarum* (8). Polymerase chain reaction (PCR) amplification of the Ch2 16S rRNA

FIG. 1. Effect of NaCl concentration on the growth rate of strain Ch2. The medium contained 1 g of yeast extract per liter and 5 g of peptone per liter and was buffered to pH 7.5. The culture was incubated at 37°C in an orbital shaker (100 rpm). Gen Time, generation time.

* Corresponding author.

FIG. 2. Phase-contrast photomicrographs of Ch2 cells. (A) Early-logarithmic-phase culture containing pleomorphic forms. (B) Stationary-phase culture. Long rods are the predominant morphological type. Bars = 5 µm.

gene with *Taq* DNA polymerase (Pharmacia) was performed as described by Sambrook et al. (19) by using 30 cycles consisting of 1 min at 94°C, 2 min at 50°C, and 4.5 min at 72°C.

Cloning and sequence analysis. The amplified 16S rRNA gene (length, approximately 1,500 bp) was isolated from an agarose gel, ligated into the *Sma*I site of plasmid pUC19, and introduced into *E. coli* DH5α. Several clones were studied by restriction analysis, and fragments were subcloned into

M13mp19 vectors. A universal primer and several 16S-specific primers described previously (6) were used for sequencing (20). Comparison sequences were obtained from the rRNA sequence data base (15) or from the study of Oren et al. (16) or had been determined previously by us (6, 7). These sequences were aligned by using CLUSTAL V (5) and were analyzed by using the phylogeny inference package of Felsenstein (PHYLIP, version 3.4) (2).

Southern analysis of Ch2 16S rRNA genes. Ch2 genomic

TABLE 1. Characteristics of strain Ch2 and related species

Characteristic	<i>Halobacterium saccharovorum</i> ^a	<i>Halobacterium sodomense</i> ^b	<i>Halobacterium trapanicum</i> ^b	Strain Ch2
<i>dam</i> methylation	+	+	+	+
Cell morphology	Rods	Rods	Pleomorphic	Pleomorphic
Motility	+	+	—	+
Gas vesicles present	—	—	ND ^c	—
Minimum NaCl concn required for growth (M)	1.5	0.5	ND	2.2
Mg ²⁺ concn required for growth (M)	0.005	0.005	ND	0.005
Amino acids required for growth	+	+	+	—
Oxidase	+	+	+	+
Catalase	+	+	+	+
Anaerobic growth	—	—	—	—
Indole	—	—	—	—
Carbohydrate utilization				
Glucose	+	+	+	+
Sucrose	+	+	+	+
Lactose	+	—	ND	+
Glycerol	+	+	ND	+

^a Data from references 4 and 22.

^b Data from reference 4.

^c ND, not determined.

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0   ATTCGGTTGATCCTGCCGAGGCCATTGCTATTGGGATCCGATTAGCC
50  ATGCTAGTCGCACGAGTTCAGACTCGTGGCGAATAGCTCAGTAACACGTG
100 GCCAAATACCTTCGGAACACAATACCTTCGGGAAATGTGTATACCAT
150 ACCACCACTGGAATGAGTGGTATGCCAAACGCTCCGGCGCAGGCTAATAA
200 CGAAGGATGTGGCTGCGGCCGATTAGGTAGACGGTGGGGTAACGGCCAC
250 CGTGCCAATAATCGGTATGGGTCTATGAGAGCTGAGACAAGATTCCGGGCC
300 CTACGGGCGCAGCAGGCGCGAAACCTTTATGAGAACCAGAGACGGAAT
350 CACTGCACGACAGTGCATAGGGGGATCCCAAGTGCACAGGCATAGCGCC
400 TGTGCTTTTCGGTACCTTAAGGCGGTACCAGAATAAGGGCTGGCAAGAC
450 CGGTGCCAGCCGCGCGGTAATACCGGCAGCCCAAGTATGGCCGATCTT
500 ATTGGGCCTAAAGCGTCCGTAGCTGGCCGCGCAAGTCCATCGGAAATCC
550 ACCTGCTCAACAGGTGGCGGCCCGGTAGAAATGCGTGGCTTGGGACCGG
600 AAGGCGCGACGGGTACGTCCGGGGTAGGAGTGAAATCCCGTAATCCTGGA
650 CGGACCGCCGATGGCGAAAGCAGTCGCGGAGAACGGATCCGACAGTGAGG
700 GACGAAAGCCAGGGTCTCGAACCAGGATTAGATACCCGGTAGTCTGGCC
750 GTAAACAATGTCTGCTAGGTGTGGCCCCACTACGAGTGGGTGCTGTGCC
800 GTAGGGAAGCCGCTAAGCAGACCGCTCGGGAAGTACGTCCGCAAGGATGA
850 AACCTAAAGGAATTGGCGGGGAGCACTACAACCGAGGAGCCTGCGGTT
900 TAATTGGACTCAACGCCGACATCTCACCAGCATCGACTGTAATAATGAC
950 GACCAGGTTGATGACCTTGCTCCGAGTTTCAGAGAGGAGGTGCATGGCCGC
1000 TACCGTGAGGCGTCTGTTAAGTCAGGCAACGAGCGAGACCGTCAGCTCG
1050 CCGCACCTTACTTGCCAGCAGTACCGCGAGGTAGCTGGGACAGTAGGG
1100 GGACCGCCGTGGCTAACCGGAGGAAGGAACGGCAACGGTAGGTCAGTA
1150 TGCCCGGAATGTGCTGGGCAACACGCGGGCTACAATGGTCGAGACAAAGG
1200 GTTCCTACTCCGAAAGGAGACGGTAATCTCAGAACTCGATCGTAGTTCTG
1250 GAGGTTGGGCTGCAACTCGCCACATGAAGCTGGATTTCGGTAGTAATCGC
1300 GTGTCAACAAGCGCGCGGTGAATACGTCCCTGCTCCTTGACACACCGCCC
1350 GTCAAAGCACCCGAGTGAGGTCCGGATGAGCGTTCCACGAACGTGCAAT
1400 CTGGCTTCGCAAGGGGCTTAAGTCGTAACAAGGTAGCCGTAGGGGAATC
1450 TGCGGCTGGATCACCTCCT

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FIG. 3. Ch2 gene sequence coding for the 16S rRNA molecule. The terminal sequences used as PCR primers and additional sequencing primers are underlined.

DNA was digested with restriction enzymes *Bam*HI, *Sal*I, and *Eco*RI. The digests were electrophoresed on 0.9% agarose gels, transferred to a nylon membrane (Zeta-Probe; Bio-Rad), and hybridized with nick-translated [³²P]dATP-labelled DNA (Bresatec, Adelaide, Australia) probes made from either the entire PCR fragment or a 362-bp *Bam*HI fragment (near the 5' end of the cloned gene) (22). Genomic DNA was examined for *dam* methylation by digestion with restriction enzymes *Dpn*I, *Mbo*I, and *Sau*3AI (12).

Strain Ch2 has been deposited in the Australian Collection of Microorganisms as strain ACM 3911.

Nucleotide sequence accession number. The Ch2 16S rRNA sequence has been deposited in the GenBank data base under accession number L00922.

RESULTS AND DISCUSSION

Ch2: source of isolate and cultural characteristics. To isolate hosts for halobacterial bacteriophages, we took samples of water from crystallization ponds containing up to 27% (wt/vol) salts at a marine saltern (Cheetham Salt, Ltd., Corio Bay, Geelong, Australia). Macroscopically, the water

FIG. 4. Southern hybridization of Ch2 chromosomal DNA digests probed with the entire PCR fragment (A) or with a 362-bp *Bam*HI fragment (B). The positions of size markers (in kilobases) are indicated on the left. Lanes B, *Bam*HI digests; lanes S, *Sal*I digests; lanes E, *Eco*RI digests.

was visibly pink, and as determined by light microscopy numerous halobacterium-like organisms were present along with small numbers of *Dunaliella* flagellates. Of the many halobacterium-like isolates that were cultured from these water samples, one isolate (Ch2) grew well in the presence of 18% SW on minimal and rich (MGM) media and was susceptible to infection by halophage HF2 (14a).

On solid MGM at 37°C, Ch2 produced dark red- or orange-pigmented colonies (diameter, 1.75 to 2.5 mm) within 14 days, and in liquid MGM (shaken at 100 rpm at 37°C) Ch2 had a doubling time of 5 to 6 h during the logarithmic growth phase. Ch2 grew optimally in the presence of NaCl concentrations of 2.2 to 2.7 M but poorly in the presence of NaCl concentrations of 2.0 M or less (Fig. 1). Actively growing Ch2 cells were pleomorphic; the shapes included short rods and cup-shaped forms (Fig. 2A). Stationary-phase cultures contained predominantly long rods (5 by 0.5 μm) (Fig. 2B).

Biochemical and physiological characterization. Restriction enzymes *Sau*3AI and *Dpn*I, but not *Mbo*I, cut Ch2 genomic DNA (data not shown), indicating that this organism had a *dam*-methylated genome. A similar modification occurs in (and is limited to) the *Halobacterium* taxon consisting of *Halobacterium saccharovorum*, *Halobacterium sodomense*, and *Halobacterium trapanicum* (4, 12). The possession of such a modified genome placed Ch2 in this group. Further evidence of the relationship between Ch2 and this *Halobacterium* taxon came from studies performed with the recently isolated halophage HF2. The host range of HF2 was con-

FIG. 5. Phylogenetic tree of the archaeobacterial extreme halophile group. *M. thermophilum* is the closest methanogen. The values in circles are the parsimony bootstrap percentages (100 repetitions). Bar = 1 nucleotide change per 100 bp.

fined to Ch2 and *Halobacterium saccharovorum*, although plating efficiencies on *Halobacterium saccharovorum* were low ($<10^{-9}$). Host range mutants that exhibited increased plating efficiencies on *Halobacterium saccharovorum* could be readily isolated (14a). Such halophage typing provided a ready and simple means for identifying strain Ch2.

The physiological and biochemical features that characterize isolate Ch2 and distinguishing it from the other *dam*-methylated halobacterial species are shown in Table 1. The

relatively high NaCl concentration required for growth, its ability to grow without added amino acids, its motility, and its morphological characteristics distinguish Ch2 from the other species in this taxon.

Ch2 was not able to grow under anaerobic conditions with or without nitrate present as an electron acceptor. Ch2 was susceptible to novobiocin (MIC, 0.0075 $\mu\text{g/ml}$), mevilonic acid, and bacitracin and resistant to ampicillin, tetracycline, and kanamycin. Although the ability to grow in the absence

TABLE 2. Similarity matrix

Taxon	% Similarity to ^a :									
	<i>Haloferax volcanii</i>	Phenon K isolate Aa2.2	Strain Ch2	<i>Halobacterium lacusprofundi</i>	<i>Halobacterium saccharovorum</i>	<i>Haloarcula vallismortis</i>	<i>Haloarcula marismortui</i>	<i>Halobacterium salinarium</i>	<i>Halococcus morrhuae</i>	<i>M. thermophilum</i>
<i>Haloferax volcanii</i>										
Phenon K isolate Aa2.2	99.8									
Strain Ch2	87.3	87.2								
<i>Halobacterium lacusprofundi</i>	87.3	87.0	93.8							
<i>Halobacterium saccharovorum</i>	86.9	86.6	94.0	97.5						
<i>Haloarcula vallismortis</i>	86.9	86.6	85.4	84.7	85.2					
<i>Haloarcula marismortui</i>	88.5	88.2	84.1	84.4	84.8	98.0				
<i>Halobacterium salinarium</i>	87.1	86.9	86.0	85.6	86.0	86.5	87.3			
<i>Halococcus morrhuae</i>	87.3	87.2	83.0	82.3	82.2	85.3	86.6	87.8		
<i>M. thermophilum</i>	74.6	74.4	73.9	72.8	70.3	74.3	74.8	76.0	73.6	

^a Levels of similarity for members of the extreme halophile group of the archaeobacteria based on 16S rRNA sequence data. The values on the lower left are percentages derived by using the nucleotide substitution model of Jukes and Cantor (9); the values on the upper right were calculated by using the Kimura model (10).

of amino acids is more a characteristic of the genera *Haloarcula* and *Haloferax* (4), overall our results suggested that Ch2 should be placed in the *dam*-methylated taxon of the genus *Halobacterium*. To confirm this and definitively establish the taxonomic position of Ch2, the sequence of the 16S rRNA was determined.

Cloning, sequencing, and analysis of the 16S rRNA gene. A PCR analysis of the Ch2 16S rRNA gene region yielded a single band (length, approximately 1,500 bp) which was cloned and sequenced. To confirm the identity of the clones and to eliminate the possibility of contamination (11), the sequence of a characteristic (i.e., variable) region of the Ch2 rRNA (positions 404 to 430) was determined by direct rRNA sequencing by using reverse transcriptase (6). The cloned DNA and directly determined rRNA sequences were the same.

The 1,469-bp gene sequence corresponding to the full-length 16S rRNA is shown in Fig. 3. The final sequence was derived from at least two independently isolated clones. Three ambiguities between clones were detected; these ambiguities were most likely due to the inherent error rate found with *Taq* DNA polymerase (18) and were resolved by reference to additional clones.

The halobacteria possess variable numbers of genes encoding rRNAs, varying from one to four (21). In addition, the genome of *Halobacterium marismortui* contains two heterogeneous genes encoding 16S rRNAs (14). To determine the copy number of the Ch2 16S rRNA gene, we probed genomic digests with the entire PCR product or with a 362-bp *Bam*HI fragment from the 5' end of the sequence (Fig. 4). The *Bam*HI and *Sal*I lanes clearly showed the presence of three bands on different restriction fragments hybridizing with the probe. Although not clear in Fig. 4, the *Eco*RI digest also contained three bands (one at about 5 kb and two large, closely spaced bands running near the upper limit of the resolution of the gel). Since the 16S rRNA sequence contains no internal *Sal*I or *Eco*RI sites, the presence of three bands indicates that three separate 16S rRNA genes are present in the Ch2 genome. No rRNA gene heterogeneity was observed in the sequences of several clones.

Halobacterial phylogeny. The position of Ch2 within the halobacterial taxon was investigated. All available archaeal 16S rRNA sequences were used, and an eubacterial thermophile (*Thermotoga maritima*) was used as an outgroup. Figure 5 shows the halophile clade and the closest methanogen (*Methanogenium thermophilum*), as well as the topology and branch lengths obtained by neighbor-joining methodologies. Maximum-parsimony approaches gave identical topologies. The levels of similarity between species are shown in Table 2.

Ch2 clusters with *Halobacterium saccharovorum* and *Halobacterium lacusprofundi* and is approximately equidistant from the type species of the genera *Haloferax* and *Haloarcula*. When parsimony confidence bootstrapping was used, the members of this clade clustered together all of the time (Fig. 5). The bootstrap values also revealed the possibility that the branching order of the genera *Haloferax* and *Haloarcula* and the genera *Haloarcula* and *Halobacterium* may be reversed. The clade containing Ch2 and *Halobacterium saccharovorum* is clearly separate from the clades containing the previously described genera and is separated from the type species of the genus *Halobacterium* by at least one bifurcation. These data justify grouping Ch2 with *Halobacterium saccharovorum* and support the suggestion of Lodwick et al. (13) that a new genus level classification is

required. Unfortunately, the taxonomy of halobacteria is currently in such a state that large-scale revision is necessary (13), and this fact precludes us from proposing any formal taxonomic status for isolate Ch2. We designate strain Ch2 a new halobacterial isolate and suggest that when halobacterial taxonomy has been clearly resolved, this isolate should be included as a member of an appropriate genus level group.

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