Phylogenetic relationships within the order *Halobacteriales* inferred from 16S rRNA gene sequences

André-Denis Girard Wright†

Correspondence

André-Denis Girard Wright
andre-denis.wright@csiro.au

CSIRO Livestock Industries, Centre for Environment and Life Sciences, Private Bag 5,
Wembley, Western Australia 6913, Australia

Phylogenetic relationships within the halophilic archaea were inferred from comparisons of the 16S rRNA gene sequences from 61 strains, representing 18 genera with validly published names within the order *Halobacteriales*. Trees produced using distance-matrix (least-squares and neighbour-joining) methods affirm with strong bootstrap support that the members of the order *Halobacteriales* are a monophyletic group. Ten genera were supported as monophyletic groups [*Haloarcula*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halorubrum*, *Halosimplex* (multiple sequences from a single strain), *Natrialba*, *Natrinema*, *Natronococcus* and *Natronorubrum*] and eight genera were represented by a single strain (*Halobacterium*, *Halobaculum*, *Halogeometricum*, *Halomicrobium*, *Halorhabdus*, *Halosimplex*, *Natronobacterium* and *Natronomonas*). The genus *Haloterrigena* was always paraphyletic, and the phylogenetic placement of and sister groups to *Halobacterium* and *Natronomonas* could not be resolved. Both treeing methods failed to resolve the deep branching patterns within the order *Halobacteriales* and the relationships between the major clades. Additional representation from the currently monospecific genera and/or the use of other macromolecules may be required to resolve the relationships within the order *Halobacteriales*.

The order *Halobacteriales* consists of a large group of aerobic microbes which live and grow in hypersaline (i.e. brine) environments such as the Great Salt Lake and the Dead Sea. Members of this order are the most halophilic organisms known to exist, thriving in environments 10 times saltier than seawater; hence, collectively they are referred to as the 'extreme halophiles'. High salinity is toxic to most cells. However, the extreme halophiles are well adapted to their hypersaline environment, as evidenced by their predominance in these habitats, requiring at least 1.5 M NaCl for growth and 3.5–4.5 M NaCl for optimal growth (Grant *et al.*, 2001). To prevent dehydration and maintain osmotic balance with their surroundings, the extreme halophiles have a high intracellular concentration of salts, as high as the NaCl concentration in their immediate environment.

The extreme halophiles are very diverse, existing as cocci, rods, flat discs (*Halococcus*), squares (*Haloferax*), rectangles (*Halobacterium*) and triangles (*Haloarcula*) (Grant *et al.*, 2001). They are either Gram-negative or Gram-positive, and some are motile. Some extreme halophiles are chemoheterotrophs that can grow on sugars, carboxylic acids, alcohols and amino acids, while others are photosynthetic autotrophs

that use a special pigmented membrane protein called bacteriorhodopsin (Grant *et al.*, 2001; Oren, 2002) instead of chlorophyll to photosynthesize. Bacteriorhodopsin uses all light except for violet light, making these cells appear purple. The red coloration of hypersaline soda lakes is due to the abundance of carotenoid pigments found in the cell membranes of other halobacteria (Oren, 1994).

Limited phenotypic data often do not provide a clear means of differentiating the extreme halophiles or determining their taxonomic affinities with each other. Deciphering the evolutionary history of the extreme halophiles is essential to understand their adaptation to extreme environments and for the exploration of their novel metabolic activities. For this report, I tested the monophyly of and the phylogenetic relationships within the order *Halobacteriales* using 16S rRNA gene sequences from 61 extreme halophiles whose names have been validly published. This examination, involving 18 different genera, represents an extensive phylogenetic analysis of the extreme halophiles.

The 61 halophilic archaeal 16S rRNA gene sequences examined in this paper were downloaded from the GenBank database. Because the extreme halophiles are closely related to the methanogenic archaea (i.e. methanogens) and some thermoacidophiles (i.e. *Thermoplasma*), 12 methanogens and two thermoacidophiles were used as the outgroup (Watrous & Wheeler, 1981) to the extreme halophiles. A

†Present address: CSIRO Livestock Industries, Queensland Bioscience Precinct, 306 Carmody Rd, St. Lucia, Queensland 4067, Australia.

Abbreviations: LS, least-squares; NJ, neighbour-joining.

total of 75 sequences were globally aligned using the Dedicated Comparative Sequence Editor (DCSE) program (de Rijk & de Wachter, 1993) and further refined by eye.

The phylogenetic software package PHYLIP (version 3.62c) (Felsenstein, 2004) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura two-parameter correction model (Kimura, 1980). Distance-matrix trees were then constructed using the Fitch and Margoliash least-squares (LS) method (Fitch & Margoliash, 1967) and the neighbour-joining (NJ) method (Saitou & Nei, 1987). Support values for internal branches for LS and NJ data were bootstrap resampled independently 1000 times.

When Grant & Larsen (1989) proposed a new taxonomic structure for the extreme halophiles, six genera were recognized. To date, the names of 18 genera have been validly published (Schoop, 1935; Elazari-Volcani, 1957; Tindall *et al.*, 1984; Torreblanca *et al.*, 1986; Kamekura & Dyll-Smith, 1995; Oren *et al.*, 1995, 2002; McGenity & Grant, 1995; Kamekura *et al.*, 1997; McGenity *et al.*, 1998; Montalvo-Rodriguez *et al.*, 1998; Ventosa *et al.*, 1999; Xu *et al.*, 1999; Wainø *et al.*, 2000; Hezayen *et al.*, 2002; Vreeland *et al.*, 2002). Despite the geographical and habitat diversity from which each of these halophiles was isolated and the evolutionary pressure to adapt to their extreme environments, distance-matrix treeing methods strongly supported (i.e. 100 %) the conclusion that the extreme halophiles were a monophyletic group (Fig. 1). Monophyly of the order *Halobacteriales* was also supported by a recent study of five diverse halophiles using a low-pass shotgun sequencing approach (Goo *et al.*, 2004). In that study, strong similarities in characteristics associated with environmental exposure to UV radiation and hypersalinity were also affirmed (Goo *et al.*, 2004).

This treatise also closely resembled previous studies by Grant *et al.* (2001) and Boucher *et al.* (2004), where they examined 16S rRNA gene sequences from a large representation of extreme halophiles, including uncultivated and unknown strains. In comparison, this study used only taxa whose names were validly published, did not include any environmental clones or unknown strains and was derived from an independent alignment. As part of this study, pairwise distance data also revealed that the greatest genetic divergence within the order *Halobacteriales* was 18.9 %, between *Halococcus morrhuae* and *Halorubrum distributum* JCM 9100^T. In addition, the mean genetic divergence over all possible pairs of halophilic archaeal 16S rRNA gene sequences was 12.4 % (variance = 0.14 %; SD = 0.38 %). In comparison, the greatest genetic divergence within the methanogenic archaea was 34.2 %.

The LS and NJ distance-matrix methods produced nearly identical trees, except for an unsupported clade in the NJ tree consisting of three distinct branches: the *Haloarcula*–*Halomicrobium* branch, the *Halosimplex*–*Halorhabdus* branch and the *Natronomonas* branch. As in previous

examinations (Grant *et al.*, 2001; Boucher *et al.*, 2004), this study was unable to resolve the deep branching patterns within the extreme halophiles. As a result, a large polytomy was created. The LS tree revealed a polytomy consisting of six major lineages, whereas the NJ tree revealed a polytomy consisting of eight major lineages. The difference in the number of major lineages was because of unresolved relationships amongst the *Natronomonas* clade, the *Halorhabdus*–*Halosimplex* clade and the *Halomicrobium*–*Haloarcula* clade. These unresolved branches may reflect both 'hard' and 'soft' polytomies. A hard polytomy results from the absence of data to resolve branching dichotomously and may be interpreted as multiple speciation events occurring at the same time. On the other hand, a soft polytomy reflects uncertainty resulting from the incongruence among two or more fully resolved, equally parsimonious cladograms. Additional representation from the eight genera represented by a single strain (*Halo-bacterium*, *Halobaculum*, *Halogeometricum*, *Halomicrobium*, *Halorhabdus*, *Halosimplex*, *Natronobacterium* and *Natronomonas*) and/or the use of other macromolecules may be required to resolve the relationships among the extreme halophiles.

All trees supported the monophyly of 10 genera: *Haloarcula* (100 %), *Halobiforma* (100 %), *Halococcus* (100 %), *Haloferax* (100 %), *Halorubrum* (100 %), *Halosimplex* (100 %), *Natrialba* (86 % LS, 60 % NJ), *Natrinema* (77 %), *Natronococcus* (65 %) and *Natronorubrum* (68 % LS, 87 % NJ). Moreover, all trees revealed a large, strongly supported clade (100 %) consisting of 18 species, representing seven different genera (*Halobiforma*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus* and *Natronorubrum*). In addition, *Haloterrigena thermotolerans* PR-5^T was always more closely related to species belonging to the genus *Natrinema* than to *Haloterrigena turkmenica* VKM B-1734^T. Even the genetic distance between *Htg. thermotolerans* PR-5^T and *Htg. turkmenica* VKM B-1734^T ($d = 3.7$ %) was greater than the divergence between *Htg. thermotolerans* PR-5^T and *Natrinema pellirubrum* NCIMB 786^T ($d = 2.3$ %) and the divergence between *Nnm. pellirubrum* NCIMB 786^T and *Natrinema versiforme* XF10^T ($d = 2.7$ %). However, paraphyly of the genus *Haloterrigena* was not unexpected, given the assertion of Tindall (2003) that the assignment of strains GSL-11 and JCM 9743 to the species *Htg. turkmenica* may be unreliable as a result of errors in the original dataset.

Within the *Haloarcula* clade, the sequence of *Haloarcula vallismortis* strain A did not pair with the sequence deposited under GenBank accession no. D50851, representing an unidentified strain of *Har. vallismortis*. Instead, *Har. vallismortis* A was closer to operon *rrnB* of *Haloarcula marismortui* ATCC 43049^T, whereas the unidentified strain of *Har. vallismortis* was closer to *Haloarcula hispanica* ATCC 33960^T. Examination of their 16S rRNA gene sequences revealed that *Har. vallismortis* A was 99.6 % similar to operon *rrnB* (i.e. 6 bp difference) and 98.8 % similar to

Har. marismortui ATCC 43049^T was more closely related to *Har. vallismortis* strain A than to operon *rrnC* of *Har. marismortui* ATCC 43049^T. In addition, two of the three 16S rRNA gene sequences of *Har. hispanica* ATCC 33960^T (*rrnA* and *rrnB*) were 2.4% divergent from each other, whereas an additional operon of *Har. hispanica* ATCC 33960^T (Arahal *et al.*, 1996) was 2.3% divergent from *Har. hispanica* ATCC 33960^T operon *rrnA* and 4.2% divergent from *Har. hispanica* ATCC 33960^T operon *rrnB*. In all trees, the *rrnB* operon of *Har. hispanica* ATCC 33960^T branched first, and the *rrnA* operon of *Har. hispanica* ATCC 33960^T was more closely related to *Haloarcula argentinensis* arg-1^T. Furthermore, Vreeland *et al.* (2002) identified three operons (A, B, C) of the 16S rRNA gene of *Hsx. carlsbadense* ATCC BAA-75^T. However, Boucher *et al.* (2004) reported that operon B was a chimera of the other two operons. So, only operons A and C were analysed with two additional operons (*rrnA*, *rrnB*), available from GenBank, from *Hsx. carlsbadense* ATCC BAA-75^T. Pairwise comparisons of these four operons indicated that operon A was 99.7% similar to operon *rrnB* (i.e. 5 bp different) and that operon C was 98% similar to operon *rrnA* (19 bp different). However, operon A was 6.2% divergent from operon C and operon *rrnA* was 6.9% divergent from operon *rrnB*. This intragenomic variability was sometimes greater than the divergence between some halophiles. Given this high variability, intragenomic heterogeneity is likely to have a significant impact on the phylogenetic reconstruction of the halophiles. However, the decision of which 16S rRNA gene paralogue is 'correct' must await further examination.

Based on the GenBank database, the number of 16S rRNA gene sequences for the halobacteria has more than doubled over the past 3 years. This increased level of interest in the extreme halophiles probably stems, in part, from an increase in their potential biotechnological uses (Galinski & Tindall, 1992; Rodríguez-Valera, 1992; Ventosa & Nieto, 1995; Oren, 2002). The extreme halophiles have evolved proteins that are able to function in high-ionic-strength solutions (see Oren, 1999, 2000) and their highly evolved, complex set of biosynthetic and metabolic pathways could be exploited to enable other organisms to function under salty conditions. Deciphering the evolutionary history of the extreme halophiles is essential to understand their adaptation to extreme environments and for the exploration of their novel metabolic activities.

Acknowledgements

The author thanks Dr Lucy Skillman (CSIRO Livestock Industries, Perth, Australia) and the anonymous reviewers for critical comments on previous versions of the manuscript.

References

- Arahal, D. R., Dewhirst, F. E., Paster, B. J., Volcani, B. E. & Ventosa, A. (1996). Phylogenetic analyses of some extremely halophilic archaea isolated from Dead Sea water, determined on the basis of their 16S rRNA sequences. *Appl Environ Microbiol* **62**, 3779–3786.
- Baliga, N. S., Bonneau, R., Facciotti, M. T. & 12 other authors (2004). Genome sequence of *Haloarcula marismortui*: a halophilic archaeon from the Dead Sea. *Genome Res* **14**, 2221–2234.
- Boucher, Y., Douady, C. J., Sharma, A. K., Kamekura, M. & Doolittle, W. F. (2004). Intragenomic heterogeneity and intragenomic recombination among haloarchaeal rRNA genes. *J Bacteriol* **186**, 3980–3990.
- de Rijk, P. & de Wachter, R. (1993). DCSE, an interactive tool for sequence alignment and secondary structure research. *Comput Appl Biosci* **9**, 735–740.
- Elazari-Volcani, B. (1957). Genus XII. *Halobacterium*. In *Bergey's Manual of Determinative Bacteriology*, 7th edn, pp. 207–212. Edited by R. S. Breed, E. G. D. Murray & N. R. Smith. Baltimore: Williams & Wilkins.
- Felsenstein, J. (2004). PHYLIP – Phylogeny Inference Package documentation files, version 3.62c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, Washington, USA.
- Fitch, W. K. & Margoliash, E. (1967). Construction of phylogenetic trees. *Science* **155**, 279–284.
- Galinski, E. A. & Tindall, B. J. (1992). Biotechnological prospects for halophiles and halotolerant microorganisms. In *Molecular Biology and Biotechnology of Extremophiles*, pp. 76–114. Edited by R. A. Herbert & R. J. Sharp. Glasgow: Mackie.
- Goo, Y. A., Roach, J., Glusman, G., Baliga, N. S., Deutsch, K., Pan, M., Kennedy, S., DasSarma, S., Ng, W. V. & Hood, L. (2004). Low-pass sequencing for microbial comparative genomics. *BMC Genomics* **3**, 1–19.
- Grant, W. D. & Larsen, H. (1989). Extremely halophilic archaeobacteria. Order *Halobacteriales* ord. nov. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 2216–2218. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.
- Grant, W. D., Kamekura, M., McGenity, T. J. & Ventosa, A. (2001). Class III. *Halobacteria* class. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, p. 294. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.
- Hezayen, F. F., Tindall, B. J., Steinbuchel, A. & Rehm, B. H. A. (2002). Characterization of a novel halophilic archaeon, *Halobiforma haloterrestris* gen. nov., sp. nov., and transfer of *Natronobacterium nitratireducens* to *Halobiforma nitratireducens* comb. nov. *Int J Syst Evol Microbiol* **52**, 2271–2280.
- Kamekura, M. & Dyll-Smith, M. L. (1995). Taxonomy of the family *Halobacteriaceae* and the description of two new genera *Halorubrobacterium* and *Natrialba*. *J Gen Appl Microbiol* **41**, 333–350.
- Kamekura, M., Dyll-Smith, M. L., Upasani, V., Ventosa, A. & Kates, M. (1997). Diversity of alkaliphilic halobacteria: proposals for transfer of *Natronobacterium vacuolatum*, *Natronobacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., respectively, as *Halorubrum vacuolatum* comb. nov., *Natrialba magadii* comb. nov., and *Natronomonas pharaonis* comb. nov., respectively. *Int J Syst Bacteriol* **47**, 853–857.
- Kimura, M. (1980). A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- McGenity, T. J. & Grant, W. D. (1995). Transfer of *Halobacterium saccharovorum*, *Halobacterium sodomense*, *Halobacterium trapanicum* NRC 34041 and *Halobacterium lacusprofundi* to the genus *Halorubrum* gen. nov., as *Halorubrum saccharovorum* comb. nov., *Halorubrum sodomense* comb. nov., *Halorubrum trapanicum* comb.

- nov., and *Halorubrum lacusprofundi* comb. nov. *Syst Appl Microbiol* **18**, 237–243.
- McGenity, T. J., Gemmell, R. T. & Grant, W. D. (1998).** Proposal of a new halobacterial genus *Natrinema* gen. nov., with two species *Natrinema pellirubrum* nom. nov. and *Natrinema pallidum* nom. nov. *Int J Syst Bacteriol* **48**, 1187–1196.
- Montalvo-Rodriguez, R., Vreeland, R. H., Oren, A., Kessel, M., Betancourt, C. & Lopez-Garriga, J. (1998).** *Halogeometricum borinquense* gen. nov., sp. nov., a novel halophilic archaeon from Puerto Rico. *Int J Syst Bacteriol* **48**, 1305–1312.
- Mylvaganam, S. & Dennis, P. P. (1992).** Sequence heterogeneity between the two genes encoding 16S rRNA from the halophilic archaeobacterium *Haloarcula marismortui*. *Genetics* **130**, 399–410.
- Oren, A. (1994).** The ecology of extremely halophilic archaea. *FEMS Microbiol Rev* **13**, 415–440.
- Oren, A. (1999).** Bioenergetic aspects of halophilism. *Microbiol Mol Biol Rev* **63**, 334–348.
- Oren, A. (2000).** Life at high salt concentrations. In *The Prokaryotes. An Evolving Electronic Resource for the Microbiological Community*, release 3.1. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer-Verlag. <http://141.150.157.117:8080/prokPUB/index.htm>
- Oren, A. (2002).** Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J Ind Microbiol Biotechnol* **28**, 56–63.
- Oren, A., Gurevich, P., Gemmell, R. T. & Teske, A. (1995).** *Halobaculum gomorrense* gen. nov., sp. nov., a novel extremely halophilic archaeon from the Dead Sea. *Int J Syst Bacteriol* **45**, 747–754.
- Oren, A., Elevi, R., Watanabe, S., Ihara, K. & Corcelli, A. (2002).** *Halomicrobium mukohataei* gen. nov., comb. nov., and emended description of *Halomicrobium mukohataei*. *Int J Syst Evol Microbiol* **52**, 1831–1835.
- Rodríguez-Valera, F. (1992).** Biotechnological potential of halobacteria. In *The Archaeobacteria: Biochemistry and Biotechnology*, pp. 135–147. Edited by M. J. Danson, D. W. Hough & G. G. Lunt. London: Biochemical Society.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schoop, G. (1935).** *Halococcus litoralis*, ein obligat halphiler Farbstoffbildner. *Dtsch Tierarztl Wochenschr* **43**, 817–820 (in German).
- Tindall, B. J. (2003).** Taxonomic problems arising in the genera *Haloterrigena* and *Natrinema*. *Int J Syst Evol Microbiol* **53**, 1697–169.
- Tindall, B. J., Ross, H. N. M. & Grant, W. D. (1984).** *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaeobacteria. *Syst Appl Microbiol* **5**, 41–57.
- Torreblanca, M., Rodríguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M. & Kates, M. (1986).** Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* **8**, 89–99.
- Ventosa, A. & Nieto, J. J. (1995).** Biotechnological applications and potentialities of halophilic microorganisms. *World J Microbiol Biotechnol* **11**, 85–94.
- Ventosa, A., Gutierrez, M. C., Kamekura, M. & Dyal-Smith, M. L. (1999).** Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to *Haloterrigena turkmenica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 131–136.
- Vreeland, R. H., Straight, S., Krammes, J., Dougherty, K., Rosenzweig, W. D. & Kamekura, M. (2002).** *Halosimplex carlsbadense* gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate. *Extremophiles* **6**, 445–452.
- Wainø, M., Tindall, B. J. & Ingvorsen, K. (2000).** *Halorhabdus utahensis* gen. nov., sp. nov., an aerobic, extremely halophilic member of the Archaea from Great Salt Lake, Utah. *Int J Syst Evol Microbiol* **50**, 183–190.
- Watrous, L. E. & Wheeler, Q. D. (1981).** The out-group comparison method of character analysis. *Syst Zool* **30**, 1–11.
- Xu, Y., Zhou, P. & Tian, X. (1999).** Characterization of two novel haloalkaliphilic archaea, *Natronorubrum bangense* gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov., sp. nov. *Int J Syst Bacteriol* **49**, 261–266.