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## Evolutionary placement of Methanonatronarchaeia

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1 **Subject ontology**

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6 **Evolutionary placement of *Methanonatronarchaeia***

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19 ***Methanonatronarchaeia*, a newly discovered archaeal lineage of extremely halophilic**  
20 **methanogens, were proposed to represent an evolutionary intermediate between**  
21 **archaeal methanogens and the extremely halophilic *Halobacteria*. Here, we show that**  
22 **the sistership between *Methanonatronarchaeia* and *Halobacteria* results from a tree**  
23 **reconstruction artefact and that the divergence of *Methanonatronarchaeia* is in fact**  
24 **much deeper. This sheds a new light on the adaptation to extreme halophilic lifestyle**  
25 **in archaea and on the evolution of methanogenesis.**

26  
27 Sorokin and colleagues recently reported the identification of *Methanonatronarchaeia*, a  
28 fascinating archaeal lineage of extremely halophilic, moderately thermophilic, methyl-  
29 reducing methanogens<sup>1,2</sup>. Similar to most recently discovered methanogens,  
30 *Methanonatronarchaeia* perform methanogenesis based on H<sub>2</sub> and methyl compounds, a  
31 metabolism not previously reported from hypersaline environments. Together with  
32 *Halobacteria* and *Nanohaloarchaea*<sup>3</sup>, *Methanonatronarchaeia* represent the third discovered  
33 lineage of extreme halophilic archaea and the most halophilic methanogens ever found. They  
34 have likely adapted to this lifestyle by employing a salt-in osmoprotection strategy<sup>1</sup>, unlike  
35 previously known halophilic methanogens and similarly to the two other extreme halophilic  
36 archaeal lineages<sup>4</sup>. Moreover, *Methanonatronarchaeia* rely on cytochromes for  
37 methanogenesis<sup>1</sup>, a characteristic previously thought to be restricted to the  
38 *Methanosarcinales*<sup>5</sup>. A maximum Likelihood (ML) phylogenetic analysis of a supermatrix  
39 gathering ribosomal proteins indicated *Methanonatronarchaeia* as the closest relatives to  
40 *Halobacteria* (Fig. 1A, red branches)<sup>1</sup>. They were therefore proposed to be evolutionary  
41 intermediates on the path from methanogens to extreme halophiles<sup>1</sup>. However, multiple  
42 substitutions occurring at the same site in sequences can mask the original phylogenetic  
43 signal and provoke tree reconstruction artefacts<sup>6</sup>, a phenomenon particularly evident in  
44 lineages that adapted to extreme salinity<sup>7</sup>.

45

46 To test the phylogenetic position of *Methanonatronarchaeia*, we reanalyzed the original  
47 supermatrix of ribosomal proteins used by Sorokin et al.<sup>1</sup>, through the progressive removal of  
48 the fastest evolving sites, a method that is frequently used to reduce artefacts linked to  
49 multiple substitutions<sup>7</sup>. This analysis, both by ML and Bayesian approaches including non-  
50 homogeneous evolutionary models, shows that the clustering of *Halobacteria* and  
51 *Methanonatronarchaeia* (Fig. 1B-C, red line) was recovered only when the fastest evolving-  
52 sites are included in the analysis, while the progressive removal of these sites shifted the  
53 position of *Methanonatronarchaeia* away from *Halobacteria* and to a deeper branching  
54 position at the base of the superclass 'Methanotecta'<sup>8</sup> (Fig. 1B-C, green line). This placement  
55 is also consistently and robustly recovered when *Methanonatronarchaeia* were included in  
56 two recently published supermatrices comprising a larger number of markers<sup>6</sup> (over 250  
57 conserved protein families) or a larger taxonomic sampling of the Methanotecta<sup>9</sup> (including  
58 ANME1, Syntrophoarchaeales, Methanoliparia, and a third *Methanonatronarchaeia*  
59 member). In contrast with the dataset of Sorokin et al.<sup>1</sup>, the new placement of  
60 *Methanonatronarchaeia* was robust to the removal of the fastest-evolving sites for both these  
61 supermatrices (Fig. 1D-G).

62  
63 Our analyses indicate that the placement of *Methanonatronarchaeia* as the methanogenic  
64 closest relatives of *Halobacteria* proposed in Sorokin et al.<sup>1</sup> is likely the consequence of a  
65 tree reconstruction artefact induced by a multiple substitution-bias which is particularly strong  
66 in their ribosomal protein dataset, but not in the other two datasets. The alternative position  
67 of the *Methanonatronarchaeia* disclosed here provides a new perspective on the evolution of  
68 this fascinating lineage. For example, it indicates that their adaptation to extreme halophily  
69 would have occurred independently from the *Halobacteria*. Moreover, following the recent  
70 proposal for the placement of *Nanohaloarchaea* as sister to the *Methanocellales*<sup>6</sup>, the salt-in  
71 strategy used for thriving in hypersaline environments would have emerged three times  
72 independently in the *Archaea*, a remarkable example of convergent evolution for adaptation  
73 to similar environments. Finally, the new placement of *Methanonatronarchaeia* is highly  
74 relevant for the evolution and diversity of methanogenesis, as their characteristics may  
75 reflect those of the methanogenic ancestor of the whole 'Methanotecta' superclass. For  
76 example, the fact that *Methanonatronarchaeia* rely on cytochromes for methanogenesis<sup>3</sup>  
77 raises the question of whether this feature may be ancestral to all Class II methanogens and  
78 was retained only in *Methanosarcinales* while *Methanomicrobiales*, *Methanocellales* and  
79 *Methanoflorentaceae* shifted secondarily to methanogenesis without cytochromes, or if  
80 instead it emerged twice independently.

81 The current pace in the acquisition of genomic data and the discovery of new lineages<sup>8,10</sup> will  
82 certainly allow to tackle these fundamental questions in the evolution and ecology of  
83 methanogens and of *Archaea* in general.

## 84 85 **References**

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107  
108 **Author contributions**

109 S.G. and C.B.A. supervised the study. M.A. and G.B. assembled the datasets and performed  
110 all analyses. All authors analysed the data and wrote the manuscript.

111  
112 **Competing interests**

113 The authors declare no competing interest.

114  
115 **Legend of Fig. 1**

116 **(A)** Schematic phylogeny of the *Archaea*, with a focus on the ‘Methanotecta’ superclass<sup>8</sup>.  
117 Dotted lines indicate two alternative branchings of *Methanonatronarchaeia*: as the sister-  
118 lineage of *Halobacteria* (red) or at the base of ‘Methanotecta’ (green).

119 **(B-G)**: Impact on the placement of *Methanonatronarchaeia* of the progressive removal of the  
120 fastest-evolving sites from the three analysed supermatrices (see Supplementary Information  
121 (SI) for details). **(B-C)**: the supermatrix of ribosomal proteins (8,072 amino acid positions)  
122 derived from Sorokin et al.<sup>1</sup>, **(D-E)**: the supermatrix, derived from Adam et al.<sup>8</sup> (40 conserved  
123 protein families, 9,228 amino acid positions), and **(F-G)**: the supermatrix derived from Aouad  
124 et al.<sup>6</sup> (258 conserved protein families, 62,398 amino acid positions).

125 The x-axis indicates the percentage of amino acid positions of the supermatrices that were  
126 kept for phylogenetic analyses during the progressive removal of the fastest evolving sites.  
127 The y-axis corresponds to bootstrap values associated to the ML trees inferred using the  
128 LG+G4 evolutionary model **(B, D, and F)** or the PMSF+LG+G4 evolutionary model **(G)**, or to  
129 posterior probabilities associated to the Bayesian trees inferred with the CAT+GTR+G4  
130 evolutionary model **(C, and E)**. The green and red lines shown on these graphs correspond  
131 to the bootstrap values and posterior probabilities supporting the two alternative placements  
132 of *Methanonatronarchaeia* as illustrated in Figure 1A. In all trees, the clustering of  
133 *Methanonatronarchaeia* with ‘Methanotecta’ was strongly supported, excluding the branching  
134 of *Methanonatronarchaeia* elsewhere in the archaeal phylogeny. For two supermatrices on  
135 panel C (86, 82, indicated by an asterisk), *Methanonatronarchaeia* branched in-between  
136 *Archaeoglobales* and ‘Ca. Methanophagales’ (ANME-1). All trees and corresponding  
137 supermatrices are provided in Supplementary Information (SI).

