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The genome of *Nanoarchaeum equitans*: Insights into early archaeal evolution and derived parasitism

Elizabeth Waters^{††}, Michael J. Hohn[§], Ivan Ahel[¶], David E. Graham^{††}, Mark D. Adams^{††}, Mary Barnstead^{††}, Karen Y. Beeson^{††}, Lisa Bibbs[†], Randall Bolanos^{††}, Martin Keller[†], Keith Kretz[†], Xiaoying Lin^{††}, Eric Mathur[†], Jingwei Ni^{††}, Mircea Podar[†], Toby Richardson[†], Granger G. Sutton^{††}, Melvin Simon[†], Dieter Söll^{¶§¶}, Karl O. Stetter^{†§¶¶}, Jay M. Short[†], and Michiel Noordewier^{†¶¶}

[†]Diversa Corporation, 4955 Directors Place, San Diego, CA 92121; ^{††}Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182; [§]Lehrstuhl für Mikrobiologie und Archaeenzentrum, Universität Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany; ^{††}Celera Genomics Rockville, 45 West Gude Drive, Rockville, MD 20850; Departments of [¶]Molecular Biophysics and Biochemistry and ^{§§}Chemistry, Yale University, New Haven, CT 06520-8114; and ^{¶¶}Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

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The hyperthermophile *Nanoarchaeum equitans* is an obligate symbiont growing in coculture with the crenarchaeon *Ignicoccus*. Ribosomal protein and rRNA-based phylogenies place its branching point early in the archaeal lineage, representing the new archaeal kingdom Nanoarchaeota. The *N. equitans* genome (490,885 base pairs) encodes the machinery for information processing and repair, but lacks genes for lipid, cofactor, amino acid, or nucleotide biosyntheses. It is the smallest microbial genome sequenced to date, and also one of the most compact, with 95% of the DNA predicted to encode proteins or stable RNAs. Its limited biosynthetic and catabolic capacity indicates that *N. equitans*' symbiotic relationship to *Ignicoccus* is parasitic, making it the only known archaeal parasite. Unlike the small genomes of bacterial parasites that are undergoing reductive evolution, *N. equitans* has few pseudogenes or extensive regions of noncoding DNA. This organism represents a basal archaeal lineage and has a highly reduced genome.

The discovery and cultivation of *Nanoarchaeum equitans* (1), probably representing a novel archaeal kingdom, raised new questions about the evolution of the Archaea. These hyperthermophiles grow only in coculture with another archaeon, *Ignicoccus* sp., and phylogenetic analysis of their 16S rRNA sequences suggests that they diverged early in the archaeal lineage, before the emergence of the Euryarchaeota and Crenarchaeota. The unknown nature of this symbiosis and the remarkable evolutionary divergence of *N. equitans* raised the question of whether this archaeon is "primitive" or has been extensively modified by reductive evolution (2, 3).

Organisms undergoing reductive evolution usually have a surplus of pseudogenes and noncoding DNA in their genomes (4). Although these vestiges of a more complex ancestral genome implicate organisms currently undergoing reductive evolution, the process must eventually stop in a free-living organism and the evidence of gene loss may erode. The assertion that an organism branches deeply from the tree of life is even more controversial, and the potential for artifacts of phylogenetic inference and genetic exchange is well known (2). Even organisms believed to be deeply branching have acquired some genetic material by horizontal transfer (5). To determine whether the small genome of *N. equitans* is the product of reductive evolution driven by its symbiosis or whether the organism represents the "primitive" archaeal ancestor, we have sequenced its genome.

Materials and Methods

Library Construction and DNA Sequencing. *N. equitans* was grown in a 300-liter fermenter in a coculture with *Ignicoccus* sp. and the *N. equitans* cells were purified from *Ignicoccus* as described (1). The cell pellet was lysed by enzymatic and chemical digestion, followed by the isolation and purification of genomic DNA

(6–8). Genomic DNA was either digested with restriction enzymes or sheared to provide clonable fragments. Two plasmid libraries were made by subcloning randomly sheared fragments of this DNA into a high-copy number vector (≈ 2.8 kbp library) or low-copy number vector (≈ 6.3 kbp library). DNA sequence was obtained from both ends of plasmid inserts to create "mate-pairs," pairs of reads from single clones that should be adjacent to one another in the genome. Library construction, DNA sequencing, and assembly methods were essentially as described (9–11). The assembly procedures resulted in a single scaffold of four contigs comprising 489,082 base pairs. The gaps between the four contigs were then sequenced, resulting in a single circular sequence.

Annotation. A set of computational methods was applied to the *N. equitans* genome. Two gene prediction programs, GLIMMER (12) and CRITICA (13), were run on the assembled sequences. The results of the two programs were merged to generate a unique set of genes. When the two programs selected different start codons for genes with the same stop codon, the longer gene was included in the set for further analysis. Additional genes were identified in the intergenic regions by using TBLASTN to compare DNA sequences with protein sequences from other archaeal genomes. The unique set of genes was then translated into amino acid sequences and subjected to BLASTP searches (with an *E* value cutoff of 10^{-10}) against the nonredundant amino acid protein database (<http://ncbi.nlm.nih.gov>) (14). The predicted protein set was searched against the InterPro database release 3.1 (15) by using software modified from the original iprscan programs provided by InterPro. The predicted protein set was also searched against the NCBI Clusters of Orthologous Groups database mid-2001 update (16). Finally, gene family analysis was performed by using the NCBI BLASTCLUST program. Protein sets from the main scaffold and small scaffolds were compared with the protein sequences from all finished genomes deposited in the GenBank by using the BLASTP program. Intein-like regions of split genes were identified by using the BLASTP program to search the InBase database (17).

tRNA genes were identified by the TRNASCAN-SE program (18) or the RNAMOTIF program by using customized motifs (19).

Abbreviations: CDS, coding DNA sequences; snoRNA, small nucleolar-like RNA.

Data deposition: This Whole Genome Shotgun project has been deposited in the DDBJ/EMBL/GenBank databases under the project accession no. AACL000000000. The version described in this paper is the first version (accession no. AACL01000000).

^{††}Present address: Department of Chemistry and Biochemistry, University of Texas, 1 University Station A5300, Austin, TX 78712.

^{¶¶}To whom correspondence may be addressed. E-mail: mick@diversa.com, karl.stetter@biologie.uni-regensburg.de, or soll@trna.chem.yale.edu.

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Ribosomal RNAs were identified by searching the genomic sequences against a set of known rRNAs with BLASTN and verified by profile alignment to multiple alignments from known rRNA sequences. Small nucleolar-like RNAs (snoRNAs) were identified by using a profile-hidden Markov model constructed from an alignment of *Pyrococcus furiosus* snoRNAs by the HMMER program (20, 21). A new model was constructed from an alignment of *N. equitans* snoRNAs and used to iteratively search for additional snoRNAs.

Preparation of Alanyl-tRNA Synthetases and Aminoacylation Assay.

The methods were adapted from Ahel *et al.* (22). *N. equitans* *alaS1* (NEQ547), *N. equitans* *alaS2* (NEQ211) and *Methanococcus jannaschii* *alaS* genes were amplified by PCR from the respective genomic DNA and cloned into the pCR2.1 TOPO vector (Invitrogen). Correct sequences were subcloned into pET11a (Invitrogen) for expression of the proteins in the *E. coli* BL21-Codon Plus (DE3)-RIL strain (Stratagene). Cultures were grown at 37°C in Luria-Bertani medium supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Expression of the recombinant proteins was induced for 3 h at 30°C by addition of 1 mM isopropyl α-D-thiogalactopyranoside before cell harvesting. Cells were resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, and 300 mM NaCl, and broken by sonication. S-100 fractions were extensively flocculated at 70°C for 45 min, and then centrifuged for 30 min at 20,000 × *g*. Supernatants were collected and stored at 4°C before use in aminoacylation assays.

Aminoacylation was performed in a 0.1 ml reaction at 70°C in 50 mM Hepes (pH 7.2), 50 mM KCl, 10 mM ATP, 50 µM [³H]alanine (52 Ci/ml; 1 Ci = 37 GBq), 15 mM MgCl₂, 5 mM 2-mercaptoethanol, 3 mg/ml unfractionated *M. jannaschii* tRNA, and the different alanyl-tRNA synthetases (100 nM). Aliquots of 20 µl were removed at the time intervals indicated in Fig. 2, and radioactivity was measured as described (22).

Phylogenetic Analysis. A concatenated alignment of 35 ribosomal proteins was obtained from Matte-Tailliez (23). To this alignment we added the *N. equitans*, *Methanopyrus kandleri*, and eukaryotic outgroup sequences (*Arabidopsis thaliana* and *Saccharomyces cerevisiae*). The alignment was then recalculated with CLUSTALW (24) and optimized by hand with BIOEDIT (25). The program RASA was used to evaluate the alignment for the presence of long branches (26). Alignments can be obtained by request from the authors. Maximum likelihood analysis was performed with PROML from the PHYLIP package (version 3.6a2.3) (27) by using the Jones-Taylor-Thornton model, the default program parameters, and a randomized input order of sequences with three jumbles. One hundred bootstrap resamplings were performed to assess the support for individual branches. Bayesian analysis of the data set was done with the MRBAYES software (28). Four simultaneous chains were run, using the Markov chain Monte Carlo method, for 200,000 generations after the convergence of the likelihood values, using the default settings of the program. A 50% majority-rule consensus tree was generated based on the resulting 2,000 trees and the bipartition values (percentage representation of a particular clade) were recorded at the nodes. The program PAUP* (29) was used for parsimony analysis. The alignment was sampled for 500 bootstrap replicates. Each bootstrap replicate was analyzed with 10 random addition sequence replicates with tree bisection-reconnection branch swapping and equal weighting for all sites.

Results and Discussion

The genome of *N. equitans* (GenBank accession no. AACL01000000) consists of a single, circular chromosome of 490,885 bp and has an average G+C content of 31.6%. All 61 sense codons are used, but in line with the low G+C content the third codon position is mainly A or T. We identified 552 coding

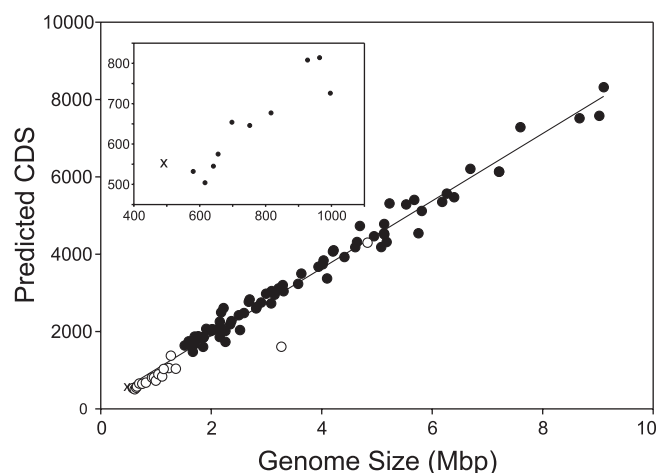


Fig. 1. Correlation between microbial genome size and the number of predicted coding DNA sequences CDS. Bacterial genomes predicted to be undergoing reductive evolution are indicated by open circles, whereas other genomes are indicated by filled circles. The *N. equitans* genome is marked by "x". (Inset) An expansion of the data from small microbial genomes with the abscissa shown in genome size units of kbp.

DNA sequences (CDS) with an average length of 827 bp. No extrachromosomal elements could be detected (either by biochemical methods or during sequencing). Despite having the smallest genome of a cellular organism sequenced to date, this archaeon has an unusually high gene density, with CDS and stable RNA sequences covering ≈95% of the genome. There is a remarkably good correlation between microbial genome size and the predicted number of CDS in a genome, an average of one gene per 1,090 bp (Fig. 1). However, the *N. equitans* genome contains even more predicted CDS than the genome of *Buchnera aphidicola* str. Sg, which is 23% larger (30). In contrast to the other small microbial genomes that are undergoing reductive evolution, *N. equitans* has little noncoding DNA and few recognized pseudogenes.

Functional roles could be assigned to two-thirds of the annotated genes. Among the CDS of unknown function, only 18.3% have homologs in other organisms, whereas the remaining ones are unique to *N. equitans* (see Supporting Data Set 1, which is published as supporting information on the PNAS web site, www.pnas.org). Predicted protein sequences from 39 genes grouped into 37 clusters of archaeal genome signature proteins, proteins that are believed to function uniquely in the Archaea (31). Three-quarters of these signature clusters include both euryarchaeal and crenarchaeal homologs and may be fundamental to the archaeal cell type.

Genes encoding single copies of 5S, 16S, and 23S rRNA and 38 tRNAs were identified along with at least 14 sno-like RNAs. These noncoding RNAs exhibit much higher G+C content (65–80%) than the rest of the genome and are readily identified by their base composition, as observed for other AT-rich hyperthermophiles (32). The rRNA genes were not found in an operon. Gene clusters (putative operons), although less common in archaea than in bacteria, are rarely conserved between *N. equitans* and other archaeal genomes. Even ribosomal proteins that are clustered together in bacterial, euryarchaeal, and crenarchaeal genomes are dispersed in this genome (33).

Unlike its *Ignicoccus* host, which gains energy by using hydrogen to reduce elemental sulfur, *N. equitans* has no genes to support a chemolithoautotrophic physiology. However the genome does encode two enzymes for amino acid oxidative deamination: a branched-chain amino acid aminotransferase (NEQ190) and glutamate dehydrogenase (NEQ077). There are

To shed light on the phylogenetic relationship of *N. equitans* among the Archaea, we concatenated and aligned the amino acid sequences of 35 ribosomal proteins. *N. equitans* was placed with high support at the most deeply branching position within the Archaea in the maximum likelihood, maximum parsimony, and Bayesian trees (Fig. 3), suggesting that the Nanoarchaeota diverged early within the Archaea. This result is consistent with the small subunit rRNA phylogeny reported previously (1).

N. equitans is the first obligate archaeal symbiont, thus far cultured only in association with *Ignicoccus* sp. Two 16S rRNA sequences from Uzon Caldera (Kamchatka, Russia) and Yellowstone National Park (U.S.) exhibited 83% sequence similarity to *N. equitans*, and therefore represent a distinct group within the Nanoarchaeota (52). Light microscopy and fluorescence *in situ* hybridization reveal that these novel Nanoarchaeota are tiny cocci-like *N. equitans* attached to other archaeal species (M.J.H., unpublished data). No free-living Nanoarchaeota have been detected. Therefore, symbiosis may be widespread or even ubiquitous within the Nanoarchaeota. The minimal gene complement of *N. equitans* implies that this organism behaves parasitically: it must derive lipids, nucleotides, amino acids, cofactors, and possibly energy from its host. Although *N. equitans* has considerable proteolytic capacity for peptide degradation and may release ammonia from the oxidative deamination of amino acids, it is unclear whether these cells ever benefit their chemolithoautotroph hosts under environmental conditions. In contrast, *Ignicoccus* cells grow at least as well in pure culture as

in symbiosis with *N. equitans*. Too high a burden of *N. equitans* cells inhibits *Ignicoccus* growth. This evidence suggests that *N. equitans* has a parasitic behavior.

Although *N. equitans* diverged relatively early in the archaeal lineage, it is not primitive (53). It contains complete versions of the modern archaeal-genre replication, transcription and translation systems, including a number of archaeal-specific innovations. Its genome encodes a significant number of archaeal signature proteins. Its few biosynthetic, DNA repair and RNA modification genes are characteristically archaeal. But this genome lacks the genes for central metabolism, primary biosynthesis and bioenergetic apparatus that are expected to have been present in an archaeal ancestor. As a highly modified, derived organism, *N. equitans* does not fit the stereotype of a microbial parasite undergoing genomic degradation. It has a highly compact genome with few pseudogenes or long regions of noncoding DNA. Consequently, we suggest that this microbe is a derived, but genomically stable parasite that diverged anciently from the archaeal lineage. The complexity of its information processing systems and the simplicity of its metabolic apparatus suggests an unanticipated world of organisms to be discovered.

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