JOURNAL OF BACTERIOLOGY, June 1999, p. 3613–3617 Vol. 181, No. 12
0021-9193/99/$04.00+0


# GUEST COMMENTARY

## Archaebacteria Then . . . Archaes Now (Are There Really No Archaeal Pathogens?)

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In 1977, Carl Woese and George Fox argued that although methanogens looked like bacteria, they had very different cell wall structures and unique methanogenesis-related coenzymes and RNase T1 digestion of methanogen 16S rRNAs generated oligonucleotides so different from those from bacterial 16S rRNAs and from eukaryotic 18S rRNAs that methanogens should be placed in a third phylogenetic urkingdom, the *Archaebacteria* (81). They conceded that "almost nothing is known regarding their molecular biology" but concluded that "there is no reason at present to consider methanogens as any closer to eubacteria than to the cytoplasmic component of the eukaryote." This started a scientific debate that has continued with unabated vigor for over 20 years. In 1977, methanogens were the only *Archaebacteria*, and not many more were predicted (81), but by 1980 the archaebacterial urkingdom had already grown to include halophiles and thermoacidophiles, and the presence of an RNA polymerase with a complex subunit configuration similar to eukaryotic RNA polymerases had been added to the list of definitive archaebacterial features (29). The 16S rRNA sequence data were by then presented in the now familiar phylogenetic tree format, and the three urkingdoms were shown arising independently from a common, less complex ancestor designated the progenote. It was tempting to comment here on the past and present arguments for and against the urkingdom concept, the number of urkingdoms, the branching relationships in the 16S rRNA phylogenetic tree, and the nature of the progenote, the universal and last common ancestors of all life (6, 9, 30, 32, 39, 49, 50, 54, 78, 81–83). But instead, from my perspective it seemed more appropriate to review how the revolutionary/heretical archaebacterial proposal has been investigated experimentally. What has been done, what were the driving forces for the research undertaken, what do we know now, what have complete archaeal genome sequences contributed, and what's next?

As the commentary's title highlights, there are language problems. In 1985, a volume of *The Bacteria* monograph series appeared with the subtitle *Archaebacteria* (83), and in my review of this book for ASM News (62), I noted the apparent contradiction between the title and subtitle and the inherent difficulty in accepting the premise that *Archaebacteria* were not *Bacteria* when they had bacteria in their name. In 1990, Carl Woese, Otto Kandler, and Mark Wheelis changed the name to *Archaea*, eubacteria became *Bacteria*, eucaryotes became *Eucarya*, and urkingdoms became domains (82). This has helped, but difficulties still remain. We used the domain terms in the first paper published after their introduction (67) but were unsure then of the singular forms and adjectives. Now it is generally accepted that a methanogen is an archaeon (although occasionally an archaeum), *Escherichia coli* remains a bacterium, but yeast has never become a eucaryum. *Eucarya* does appear in microbiology journals (with k sometimes replacing c) but almost never elsewhere, archaeal and bacterial are often used together with eucaryotic rather than with eucaryal (9), and archaes is a more easily spoken plural widely used in oral presentations and conversations. Although not chronologically accurate, the 1990 terms are used here throughout.

In 1977, I didn't read **the** 1977 paper (81); I was working at the Max Planck Institut für Molekular Genetik in West Berlin on phage infection of minicells. However, in 1979, I moved to The Ohio State University (OSU) and occupied a laboratory next door to Jim Frea, a cultivator of methanogens, and shortly thereafter Paul Hamilton, a beginning OSU graduate student, brought the 1979 review of methanogens by Balch et al. (5) to my attention. Paul argued that if methanogens were as different as the review claimed then looking at their genes should reveal interesting novelties. Apparently, no one had cloned a protein-encoding archaeal gene, and so with methanogen cell paste provided by Jim Frea, Paul set out to clone methanogen genes by complementing *E. coli* auxotrophs. He was almost immediately successful. Methanogen genomic DNAs shotgun cloned into *E. coli* complemented *E. coli* arginine, proline, histidine, and purine auxotrophs, and the complementation was independent of the orientation of cloning. This seemed a very surprising result that not only confirmed that methanogens and, by inference, all *Archaea* used the standard genetic code but also that *E. coli* RNA polymerase recognized archaeal promoters. Without sequencing the cloned DNA, we submitted this work for publication, but it was (correctly) rejected as premature. Later, after almost 3 years of Maxam-Gilbert sequencing, we wrote the "cloned and sequenced" paper that provided evidence for archaeal operons, ribosome binding sites, and a methanogen insertion sequence element (34), and Jordan Konisky's group had also documented complementation of bacterial auxotrophs by cloned methanogen genes (18, 84). However, there was still an uncomfortable suspicion that all methanogen cultures might be contaminated with anaerobic *Bacteria* and that the complementing genes were cloned from bacterial contaminants, but cloning from single-colony isolates and exchanges of cultures finally eliminated this concern. The intergenic regions in the cloned methanogen DNAs were very AT rich and contained sequences that conformed to the consensus sequences for the AT-rich −10 and −35 elements of *E. coli* promoters. We therefore assumed, but never proved, that these sequences functioned fortuitously as promoters directing transcription of the cloned methanogen genes in *E. coli* and also in *Bacillus subtilis* (55).

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The first report of a cloned and sequenced archaeal protein-encoding gene was actually published in 1981 (25). Gobind Khorana and colleagues cloned the *bop* gene that encodes bacterio-opsin in *Halobacterium halobium* as a cDNA following reverse transcription of purified mRNA. However, to obtain synthesis of recombinant bacterio-opsin in *E. coli*, they constructed an entirely synthetic *bop* gene (24). *bop* transcription in *H. halobium* was shown to initiate only 2 bp upstream from the ATG translation-initiating codon (20), the first example of the now frequent observation of transcription initiating at or close to the site of translation initiation in nonmethanogenic *Archaea*. How translation of the resulting leaderless mRNAs is initiated remains an open question, and although most methanogen mRNAs do have 5′ leaders with sequences appropriately positioned to function as ribosome binding sites, this function has not yet been experimentally documented.

Through the 1980s, archaeal research was reported regularly at international symposia, and review volumes from these meetings provide accurate historical perspectives of the then state of the art (42, 43, 63). Cell envelope structures, analogs but not homologs of bacterial peptidoglycan, and membrane lipids that contained ether rather than ester linkages were described in detail. Lipids with ether linkages in *Bacteria* and *Eucarya* have also occasionally been reported, but lipids with branched isoprenoid side chains attached to the glycerol backbone in the sn-2 and sn-3 configuration remain unique to *Archaea* (56). Antibiotic sensitivity and resistance profiles were determined to probe for the presence of target structures and to identify antibiotic resistance genes and phenotypes for use in genetics. Consistent with having different cell envelope structures and hinting at differences in DNA replication, transcription, and translation, *Archaea* were found to be resistant to almost all antibacterial antibiotics, and sensitivity to aphidicolin and diphtheria toxin argued for the presence eucarya-like targets. The number of archaeal genes cloned and sequenced increased, but when Jim Brown surveyed the literature to write a comprehensive review in 1988, there were still only 46 methanogen, 13 halophile, and 6 *Sulfolobus* protein-encoding archaeal gene sequences available to evaluate (10). A larger number of archaeal tRNA-, rRNA-, and 7S RNA-encoding sequences were known, and the presence of introns in some of these stable RNA-encoding genes (41, 46) seemed a persuasive argument for an archaeal-eucaryal relationship that excluded *Bacteria*. However, archaeal mRNAs lacked introns (still the case), did not have a 5′ cap structure, and had only very short 3′ poly(A) tails (11). *Archaea* have since been shown to contain fibrillarin homologs (3) and small nucleolar RNA-like molecules (60), and common features in the maturation and removal of introns from stable RNAs in *Archaea* and *Eucarya* have been established (47, 76), but archaeal genome sequences provide no hints of an archaeal spliceosome (12, 44, 48, 70).

With the energy crises, interest in pollutant bioremediation, and discovery of hyperthermophiles, applied goals were added in the 1980s to archaeal research. The Department of Energy (DOE), Gas Research Institute, and Environmental Protection Agency provided support in the United States to investigate methanogens with an eye to improving methanogenesis as the terminal step in anaerobic waste biodegradation and for biogas production, and the Office of Naval Research established a 5-year program to investigate *Archaea* and survival mechanisms in extreme environments. With this financial support, studies of the ecology, biochemistry, and molecular biology of methanogens and of extremophiles (as they are now known) progressed well, adding data to a foundation that now supports regular Gordon and Keystone conferences on *Archaea* and the molecular biology and metabolism of C1 compounds and international symposia on thermophily and extremophiles. The anticipated practical developments have however been limited, primarily by the recalcitrance of *Archaea* to genetics and genetic engineering. Transformation systems are now established for representative halophiles, methanogens, and hyperthermophiles, but there is still very little real genetics. Such fanciful 1980's ideas as constructing a photosynthetic methanogen with pollutant-biodegrading ability and facile genetic engineering of temperature and salt tolerance into industrial microorganisms remain remote and unrealistic.

Although recognized early as an archaeal feature (29), the complex, eucarya-like subunit composition of archaeal RNA polymerases was not extended into a functional homology until the 1990s. Archaeal promoters were then found to contain a TATA-box element, and Michael Thomm's group established that archaeal transcription initiation required two transcription factors in addition to the RNA polymerase which, subsequently, were shown to be structural and functional homologs of eucaryal TATA-binding protein (TBP) and transcription factor IIB (21, 35, 51, 61, 65, 71, 73). Based on the eucaryal paradigm, it appears that promoter-specific archaeal transcription factors must interact with regulatory DNA sequences and/or with an archaeal RNA polymerase-containing preinitiation complex to regulate transcription initiation, although some *Archaea* contain several TBPs (65, 71) which could function in a manner analogous to that of alternative sigma factors in *Bacteria*. The discovery of histones in *Archaea* in 1990 added another unambiguously eucaryal feature (67). Archaeal histones lack the N- and C-terminal regulatory extensions of their eucaryal homologs but do form the histone fold (72) and wrap DNA into structures, archaeal nucleosomes (58), that are very similar to the structure at the center of the eucaryal nucleosome formed by the histone $(H3 + H4)_2$ tetramer (52, 59). Not all *Archaea* have histones, and those that do not have a variety of other "histone-like" DNA binding proteins. Apparently many unrelated small DNA binding proteins can solve the genome packaging problem for bacterial (69) and archaeal (68) procaryotes, but only the histone-wrapping-DNA-into-nucleosomes solution remained effective when genome size increased substantially with eucaryal development. Investigations of heat shock and stress responses have identified archaeal homologs of bacterial and eucaryal chaperonins (75), and archaeal hsp60s with sequences related to the eucaryal cytosolic TCP1 chaperonins form proteasomes (designated thermosomes) with subunits similarly assembled into hexadecameric rings. However, as is the case for archaeal transcription initiation complexes and nucleosomes, archaeal proteasomes appear to be reduced, simplified versions of their eucaryal homologs. They contain only α and β subunits, whereas eucaryal TCP1 proteasomes contain at least eight different subunits (53, 85).

In 1994, the DOE again boosted archaeal research by promoting and supporting archaeal genome sequencing, and now four archaeal genome sequences have been published (12, 44, 47, 70) and several more are nearing completion. What was learnt immediately? That methanogens, absolute autotrophs that conserve energy and synthesize themselves in toto from just three gases ($CO_2$, $H_2$, and $N_2$) and salts have genomes less than 40% the size of the *E. coli* genome. Based on the differences in the two methanogen genome sequences (12, 70), as little as 1.2 Mbp may be sufficient to encode a fully independent, autotrophic cell. Genome neighborhoods and operon organizations are not well conserved, and although most components of the central dogma machinery (DNA, RNA, and protein synthesis and modification activities) do have se-

quences more closely related to their eucaryal than bacterial counterparts, most archaeal metabolic enzymes have sequences more similar to bacterial than eucaryal enzymes (66, 70). There is evidence for interspecies gene transfer having occurred on a large scale (4) and support for the progenote concept (81) in which the universal ancestor was not an individual but rather a genetically intermingling community. The small-subunit rRNA tree is no longer viewed as reflecting the early evolution of whole organisms but rather "in its deep branches,. . .is merely a gene tree" (80).

Where next in archaeal research? An entirely new family of DNA polymerases has been discovered (14) which will be studied in detail, together with novel topoisomerases, including a relative of enzymes involved in eucaryal meiotic recombination (7, 28). Reassembling an active archaeal RNA polymerase from individual subunits (19, 27) and establishing an in vitro transcription system that exhibits regulation are now urgent projects. Based on in vivo results, archaeal transcription factors will be identified and characterized that participate in the hydrogen and metal regulation of methane gene expression (36, 64), ammonia regulation of *nif* and *glnA* expression (17), substrate induction of catabolic enzymes (1, 23), biosynthetic pathway expression, gas vacuole synthesis, prophage induction (71), and the heat shock response (16, 40, 74). Progress has already been made in determining how some UGA codons direct selenocysteine incorporation in *Archaea* (79), but now it appears that UAG can also function as a sense codon in *Methanosarcina* species, promising another intriguing archaeal novelty (13). Determining how macromolecules, cofactors, and metabolites maintain their structure and functional integrity under extremophile growth conditions remains a major research challenge, and the obvious potential of extremophiles for biotechnological applications ensures their continued intense investigation. Ribulose bisphosphate carboxylase/oxygenase (RubisCo) was recently documented in hyperthermophilic, anaerobic *Archaea* (77), and enzymes and cofactors previously considered unique to methanogenesis were found in methylotrophic *Bacteria* (15). Research to determine what these surprisingly "misplaced" enzymes do is undoubtedly already well underway. The apparent lack of lysyl-tRNA synthetase genes in some archaeal genome sequences provoked experiments that have dramatically advanced amino acyl-tRNA synthetase biochemistry and phylogenetics (37, 38), and pursuing the still-missing cysteinyl-tRNA synthetase genes should similarly soon provide new insights (45). Archaeal cell cycle studies have only recently begun (8), but the presence of a combination of bacterial *ftsZ* and *minD* homologs (31) (back to minicell research?) and eucaryal *cdc* homologs (12, 44, 48, 70) promises an interesting mixture of features. Identifying an archaeal origin of DNA replication would be a valuable first step (26).

Finally to the commentary title's parenthetical question. Support from the more mission-oriented U.S. funding agencies has played a vital role in developing archaeal research in the United States, and a European community initiative has similarly supported a consortium of ~40 research groups to investigate extremophile biology, predominantly archaeal projects, explicitly aimed at biotechnology development (2). Recently, following the Martian meteorite excitement, the National Science Foundation established a Life in Extreme Environments (LExEN) initiative and the National Aeronautics and Space Administration founded the astrobiology program. Funding for environmental and ecological studies of *Archaea* should therefore now increase, and hopefully this will expedite the isolation and characterization of the many globally important (paradoxically) nonextremophile *Archaea* that currently defy laboratory cultivation (22, 57). But what about an archaeal animal, plant, or insect pathogen that might encourage more interest and support from the National Institutes of Health? Methanogens live as intracellular symbionts (33) and inhabit the rumen and lower digestive tracts of animals and insects, so they know how to interact with a host, but do they never cause problems? As *Archaea* are innately resistant to almost all clinically and agriculturally used antibiotics, surely there should be an occasional opportunistic archaeal infection, but where are the reports? Could *Archaea* be such clever pathogens that we don't recognize them as the true causative agents of disease?

Archaeal research in my laboratory at The Ohio State University has been supported by grants from the Department of Energy, Environmental Protection Agency, Gas Research Institute, Office of Naval Research, National Science Foundation, National Institutes of Health, and North Atlantic Treaty Organization.

I thank the students and postdoctoral scientists who have contributed to the work and ideas discussed and G. Dilworth, E. Eisenstadt, P. Harriman, R. Rabson, and A. Venosa for their long-term interest, help, and support.

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