ARCHAEAL GENETICS – THE THIRD WAY

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Abstract | **For decades, archaea were misclassified as bacteria on account of their prokaryotic morphology. Molecular phylogeny eventually revealed that archaea, like bacteria and eukaryotes, are a fundamentally distinct domain of life. Genome analyses have confirmed that archaea share many features with eukaryotes, particularly in information processing, and therefore can serve as streamlined models for understanding eukaryotic biology. Biochemists and structural biologists have embraced the study of archaea but geneticists have been more wary, despite the fact that genetic techniques for archaea are quite sophisticated. It is high time for geneticists to start asking fundamental questions about our distant relatives.**

Ever since microbiology was established by Louis Pasteur and Robert Koch, scientists have wrestled with the problem of defining the phylogenetic relationships among bacteria. Classical taxonomy, which relies on cell morphology, physiology, and pathogenicity, is useful for identifying specific microorganisms. However, it fails to establish meaningful evolutionary relationships that can be used to group species into higher taxonomical orders. Carl Woese's solution was to harness the newly-emerging techniques of nucleic acid sequencing, and use small-subunit (SSU) ribosomal RNA nucleotide sequence as a universal molecular chronometer (Box 1). When he published his findings in 1977, Woese upset the taxonomic applecart by suggesting that prokaryotes are a great deal more diverse than we had previously supposed, and that the phylogenetic structure of the prokaryotic DOMAIN should be reassessed[1].

What he found was that a group of anaerobic "bacteria", which had been studied for years due to their unique ability to generate methane, are not bacteria at all. There had been inklings that these microbes have rather "unbacterial" aspects, such as the presence of N-linked glycoproteins and a peculiar spectrum of antibiotic sensitivity. The rRNA phylogeny revealed that they are no more related to typical bacteria than they are to eukaryotes, and Woese therefore renamed this group of microorganisms *archaebacteria*[1]. In a subsequent paper, he shortened the name to Archaea and suggested that this domain be given equal footing with Bacteria and Eukarya[2]. Unsurprisingly, this proposal ran into stiff resistance.

Despite numerous attempts to square the taxonomic circle, the three-domain organisation has stood the test of time (Box 1). We now recognise that the domain Archaea is home to a great number of microbes that were previously misclassified as bacteria on account of their prokaryotic morphology. Archaea are clearly MONOPHYLETIC, and their status is underpinned by unique features such as a distinctive cell membrane utilising isoprene side-chains that are ether-linked to glycerol[3]. The SSU rRNA tree also reveals several archaeal phyla, which exhibit biological differences that underpin their taxonomic split. For example, euryarchaeota contain histones that are strikingly similar to eukaryotic homologues, whereas crenarchaeota use completely different DNA-binding proteins[4]. Further insights has come from genome sequencing projects, which have shown that archaea are a chimera of bacterial and eukaryotic features; their core metabolic functions resemble those of bacteria, whereas information processing functions have a distinctly eukaryotic flavour. One feature that appears to unite archaea is their ability to thrive in harsh and unusual environments (Box 2); it is because these organisms are so well suited to conditions that might have existed on the early (archaean) earth that Woese gave them their name. However, it would be misleading to think that all archaea are EXTREMOPHILES. Recent environmental studies have shown that archaea are much more widespread than previously thought, and might constitute as much as 20% of the total biomass[5].

As the domain Archaea has become widely accepted, so researchers have turned to these fascinating microorganisms for answers to some of biology's most pressing questions. Owing to molecular features they share with their more complex cousins, archaea have served well as a streamlined model for eukaryotes, particularly in the field of DNA replication[6] (discussed in detail later). On the other hand, the ability of

archaea to thrive at high temperature and salinity has endeared them to structural biologists, who have found thermostable and halophilic proteins to be a great boon. For example, the first crystal structure for a ribosome was obtained using the large ribosomal subunit from *Haloarcula marismortui*[7]. Archaeal enzymes are now routinely exploited as a source of high-quality structural data that can be used to predict functional interactions in eukaryotic systems, particularly in fields related to information processing such as DNA repair[8]. Exploitation of the extremophilic features of archaea for biotechnology has yet to bear fruit[9]. Of the few examples in current use, those familiar to most scientists are the thermostable enzymes used for DNA amplification by PCR (e.g. *Pfu* DNA polymerase from *Pyrococcus furiosus*). However, the potential of archaea for biotechnological applications is significant. For example, the ability of METHANOGENIC archaea to thrive under anaerobic conditions means they are ideally suited for bioremediation in anoxic sludge, such as marine coastal sediment. Furthermore, the methane they generate by anaerobic digestion of manure can be used as a fuel source. Finally, we should not underestimate the ecological impact of archaea; since methane is a powerful greenhouse gas, these organisms might be partly responsible for global warming.

With all the interesting aspects of archaea, why do so few scientists work on this domain of life? A significant factor is the perceived lack of genetic systems. Archaea, or at least the ones that are cultivable, are renowned as extremophiles, and organisms that thrive in boiling acid are not conducive to routine genetic techniques. Furthermore, when faced with sophisticated model organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*, it is understandable that scientists are reluctant to switch domain. Unfortunately, this means that numerous biochemical and structural studies on archaea are not being underpinned by *in vivo* data; *Escherichia coli* and *Saccharomyces cerevisiae* have been such successful models precisely because of the synergy of biochemistry and genetics. In this review, we aim to show that archaeal genetics is a great deal more advanced than is commonly believed. We will survey the current state of genetic systems – the difficulties in establishing genetic tools for archaea will be set out, followed by an update of methodologies in current use. We will also review the field of archaeal genomics, illustrating how data from genome projects have lead to a reappraisal of the phylogenetic status of archaea, and how the striking similarity between archaeal and eukaryotic information processing systems has stimulated a new generation of researchers to seek answers in the third domain.

Archaeal genomics – lessons from genome comparisons

While the DNA sequencing revolution of the late 1970s gave birth to the domain Archaea, it was the genome sequencing revolution of the last decade that enabled the archaeal concept to pass from adolescence to maturity. In retrospect, it was fortuitous that one of the first genome sequences to be published was of the methanogenic archaeon *Methanococcus jannaschii* (now renamed *Methanocaldococcus jannaschii*; see Table 1)[10]. The new discipline of genomics stimulated interest in these exotic microorganisms, as biologists found their genes of interest in a novel context. Archaea proved to be a mosaic of molecular features, which are encoded by two different groups of genes: a lineage coding for information processing that is eukaryotic in nature, and a lineage coding for operational (housekeeping) functions with a bacterial aspect[11]. This tidy schism is not inviolable, for LATERAL GENE TRANSFER can lead to conflicting phylogenetic signals when any one archaeal species is examined in isolation[12] (see Box 1). According to the complexity hypothesis of Jain & Rivera, informational genes are less prone to lateral transfer than operational genes, as the former are typically members of large complex systems[13]. However, the comparison of complete genome sequences has revealed that archaea are far more than a sum of their (eukaryotic and bacterial) parts[14]. More than anything else, it is the high fraction (as much as 50%) of archaeal genes with no clear function that should prompt experimental biologists to reclaim the initiative in a post-genomic era.

Transcription and translation. The revelation that archaeal and eukaryotic information processing systems are similar predates genome sequencing, and was noted in the 1980s by Wolfram Zillig and colleagues in studies of DNA-dependent RNA polymerases[15]. The core components of the archaeal and eukaryal (RNA polymerase II) enzyme more closely related to each other than to the bacterial version, and the archaeal holoenzyme contains additional subunits that have counterparts in eukaryotes but not bacteria. Like eukaryal RNA polymerase II, the archaeal enzyme requires additional basal factors for efficient promoter recognition, including TATA-box binding protein (TBP) and transcription factor B (TFB)[16]. Many archaea contain multiple homologues of TFB and/or TBP that might play distinct roles in transcription; for example, expression of one TFB is upregulated in response to heat shock of *Haloferax volcanii*[17]. Nevertheless, the basal transcription machinery in archaea is much simpler than the eukaryotic system, and therefore is more amenable to analysis. This should lead to a better understanding of the many small subunits conserved between archaeal and eukaryal RNA polymerases.

To considerable surprise, genome analysis has revealed that archaea also encode numerous homologues of bacterial transcription regulators[18]. This suggests that archaea might use a bacterial mode of transcriptional regulation where repressors binds at operator sites near the promoter and interfere directly with initiation. Indeed, such repressors have been studied *in vivo* in *Archaeoglobus fulgidus* and *Methanococcus maripaludis*[19,20]. However, other systems are more reminiscent of eukaryotic regulation. In an elegant genetic analysis of gas vesicle synthesis genes from *Halobacterium salinarum*, Felicitas Pfeifer and colleagues have shown that transcriptional activation by GvpE involves binding upstream of the TFB recognition element,

most likely leading to direct contact with the basal machinery[21]. It is noteworthy that GvpE resembles a basic leucine-zipper (bZIP) protein, a motif that is commonly found in eukaryotic regulators.

Translation in archaea has been studied much less intensively than transcription, but the message is similar[22]. The core components (such as rRNA) are eukaryal in nature, as are the levels of complexity – more than 10 initiation factors are found in archaea and eukaryotes, whereas bacteria require only 3. Similarly, translation initiation in archaea and eukaryotes uses methionine, while bacteria use N-formylmethionine. On the other hand, both bacteria and archaea use polycistronic mRNAs, and recognition of mRNA by the ribosome is often by means of a purine-rich Shine-Dalgarno sequence in the 5' untranslated region. It should be noted that a second mechanism for translation initiation is used in archaea, which operates on leaderless mRNAs and is therefore more reminiscent of the eukaryotic pathway[23]. Studies of these two mechanisms, and the circumstances under which they are used, should shed light on the origins of translation initiation.

Chromatin. The octameric nucleosome, consisting of two copies each of the histones H2A, H2B, H3 and H4, has long been considered a hallmark of the eukaryotic cell. Since prokaryotes were thought not to require such a baroque machinery for DNA compaction, it came as a considerable surprise when the laboratory of John Reeve reported that the methanogenic archaeon *Methanothermus fervidus* contains a homologue of eukaryotic histones[24]. Genome sequencing has revealed that histones are widespread amongst euryarchaeota but absent from crenarchaeota[4].

Archaeal histones dimerise to form a structure resembling the eukaryotic H3-H4 dimer, and assemble into a tetramer to bind ~60 base pair (bp) of DNA. However, archaeal histones lack the N- and C-terminal tails that are sites of regulatory posttranslational modification in eukaryotes, suggesting that chromatin remodelling is not used as a mode of gene regulation in archaea[25]. By contrast, nucleoid proteins found in crenarchaeota, such as Alba, undergo posttranslational modification; a significant proportion of Alba is acetylated at lysine residues, and deacetylation (which is mediated by Sir2) leads to transcriptional repression[26]. The lysine acetylase that acts on Alba was recently identified (S. Bell, personal communication), and should be a fruitful area of further study.

DNA recombination and repair. There is considerable interest in studying DNA recombination and repair systems in archaea, as they commonly have to contend with harsh conditions that threaten genomic stability[27]. Furthermore, these are highly complex repair processes, doubly so in eukaryotic cells, which have a specialised programme of meiotic recombination. The potential of archaeal genomics was plainly demonstrated when homology to an archaeal topoisomerase led to the identification of Spo11 as the eukaryotic enzyme responsible for double-strand breaks that are formed during meiosis[28].

Strand exchange is the cornerstone of DNA recombination, providing the means to identify and synapse with an homologous template, and is carried out by proteins of the RecA family. The archaeal homologue (RadA) shows greater similarity to the eukaryotic sequence (Rad51) than the bacterial one (RecA). The resemblance is even more striking at the structural level, to the point where functional interactions between eukaryotic

proteins can be extrapolated from the archaeal crystal structure[8]. RadA has been shown to promote strand exchange *in vitro*[29], and a *radA* mutant of *Hfx. volcanii* has been generated that is defective in recombination and highly sensitive to DNA damage[30]. A RadA paralogue, RadB, has been identified in the genome sequences of euryarchaeota. RadB has no strand exchange activity[31], and *radB* mutants of *Hfx. volcanii* are not defective in recombination (T.A. unpublished observations). Genetic studies of RadB, which are underway in one of our laboratories (T.A.), should provide some insight into the function of eukaryotic Rad51 paralogues; these are largely of unknown function and have no counterparts in bacteria.

Other forms of DNA repair involve either excision or direct reversal of the lesion. The archaeal homologue of eukaryotic XPF (Rad1), a nuclease that recognises junctions between single and double-stranded regions of DNA, might function in excision repair. Intriguingly, crenarchaeal XPF lacks the N-terminal "helicase" domain present in the euryarchaeal and eukaryotic proteins[32]. An example of direct reversal of DNA damage is the photoreactivation system, which uses photolyase to act on pyrimidine dimers. While this enzyme is not widespread amongst archaea, it is found in HALOPHILES that are commonly exposed to solar radiation[33]. Most archaea also lack homologues of *mutS* and *mutL* genes, which encode the mismatch repair machinery that is conserved from *E. coli* to humans. In spite of this, genetic studies of *Sulfolobus acidocaldarius* have shown that archaea are just as efficient at repairing DNA damage as *E. coli*[34], suggesting that novel pathways of DNA repair have yet to be discovered. Such a repair system for THERMOPHILIC archaea has been predicted by genome sequencing and analysis[35], and awaits genetic studies.

DNA replication. Archaea and bacteria share a genomic structure, usually consisting of a single circular chromosome, but differ in the machinery used to carry out DNA replication[6]. As with other aspects of information processing, the archaeal proteins are more similar to eukaryotic homologues than bacterial ones. Since only a subset of the eukaryotic proteins are found in archaea, the latter system is considerably simpler and is therefore more amenable to dissection. The laboratory of Hannu Myllykallio used genome analysis to predict (and biochemistry to confirm) the location of the chromosomal replication origin in *Pyrococcus abyssi*[36]. This prediction was based on the observation that leading strands of replication often contain an excess of G over C nucleotides. The origin of replication is highly conserved amongst the three *Pyrococcus* species examined (*P. furiosus, P. abyssi* and *P. horikoshii*), and the identity of gene cluster located in this region is of particular interest. In addition to sequences encoding RadB (discussed above), a single gene similar to eukaryotic *cdc6* and *orc1* is found directly adjacent to the origin; *orc1* codes for a subunit of the eukaryotic origin recognition complex, suggesting that Cdc6/Orc1 acts as the initiator protein in archaea. This is indeed the case[37], and almost every archaeal chromosomal replication origin identified to date is adjacent to a *cdc6/orc1* gene[6]. The exception is intriguing: both *S. acidocaldarius* and *S. solfataricus* have three *cdc6/orc1* genes and three replication origins, but only two of these colocalize[38,39]. Even more mystifying is why some species of haloarchaea (such as *Halobacterium* species NRC-1) should require 10 distinct *cdc6/orc1* genes. This is a question that genetics is best placed to answer.

Central metabolism and energy conversion. It is commonly stated that operational genes in archaea (coding

for central metabolism, energy conversion and biosynthesis) are bacterial in origin[11]. As with the comparison between archaeal and eukaryotic informational genes, this statement is more of a soundbite than a true representation of archaea. For instance, methanogenesis is not found in any bacteria. Genome analysis of *Methanosarcina acetivorans* has revealed a surprising diversity of methanogenic pathways that utilise acetate and a variety of one-carbon compounds (acetoclastic and methylotrophic pathways, respectively)[40]. However, *M. acetivorans* is unable to reduce CO_2 using H_2 (the hydrogenotrophic pathway), as this species lacks the ferredoxin-dependent hydrogenase encoded by the *ech* operon. The pivotal role of this enzyme has been confirmed by genetics studies in *Methanosarcina barkeri*, which have demonstrated that mutants lacking Ech are unable to grow on H_2/CO_2 alone[41].

Amongst HETEROTROPHIC archaea, a significant fraction can metabolise sugars. While glycolytic pathways are well conserved in bacteria and eukaryotes, archaea use several variant enzymes that can best be explained by independent, convergent evolution[42]. Support for variant metabolic pathways in archaea has come from a number of studies, such as the prediction of a novel aconitase family by comparative genome analysis[43]. Aconitase is an essential part of the tricarboxylic acid cycle, and the canonical gene is found only in a minority of euryarchaea. Similarly, genome analysis has indicated that many archaea, such as the HYPERTHERMOPHILIC crenarchaeon *Thermoproteus tenax*, lack the enzymes for an oxidative pentose phosphate pathway (PPP)[44]. Since pentoses are essential for anabolic purposes, it is likely that archaea use a variant PPP encoded by genes with no obvious bacterial or eukaryotic homologues. Thus, while it is true that well-conserved operational genes in archaea are most similar to their counterparts in bacteria, there are many novel or variant enzymes that await discovery. Genomics can point the way, but genetics and biochemistry must work hand in hand to unravel these mysteries.

Archaeal genetics – back to basics

In the early years of archaeal genetics, the development of markers and transformation protocols were intimately linked – without a selectable phenotype it is impossible to score transformation efficiency, and vice versa. This impasse was due to the scarcity of archaeal antibiotics. Bacterial antibiotics such as ampicillin are safe for medical use because their targets, such as peptidoglycan cell walls, are not encountered in eukaryotic cells. Since these drug targets are also absent from archaea, it is not surprising that such antibiotics are ineffective in the third domain. The issue of selectable markers is discussed in a later section. Here, we summarise the methods available for introducing foreign DNA in archaeal cells.

Transformation. To circumvent the bottleneck of developing transformation protocols without genetic markers, researchers turned to unconventional methods. Cline and Doolittle assayed TRANSFECTION of *Halobacterium halobium* with naked DNA from phage ΦH[45], enabling them to develop an efficient polyethylene glycol-mediated transformation method for *Hfx. volcanii*[46], which was subsequently adapted for use in a variety of archaea including *M. maripaludis* and *Pyrococcus abyssi*[47,48]. It is only effective in species for which SPHEROPLASTS can be generated readily, usually by removing the paracrystalline glycoprotein surface layer (S-layer). By contrast, archaea with a rigid cell wall made of pseudopeptidoglycan, such as *Methanothermobacter thermautotrophicus*, have been recalcitrant to transformation. Although it is possible to remove the cell wall enzymatically using pseudopeptidoglycan endopeptidase, the protoplasts fail to regenerate (J. Chong, personal communication).

Other transformation protocols have been used with varying success (see table in Box 3). Electroporation is a versatile technique and can be used for *Methanococcus voltae* and *S. solfataricus*[49-51], but is inefficient in species such as *M. acetivorans*[52]. Furthermore, it is not universally applicable; *P. abyssi* cannot be transformed by this method[48], and electroporation is out of the question for halophilic archaea, which cannot tolerate salt concentrations of <1 M NaCl. Heat-shock after treatment with $CaCl_2$, a method commonly used for *E. coli*, can be used with some archaea but is not efficient. It is noteworthy that in *Thermococcus kodakaraensis*, $CaCl_2$ treatment is not essential for DNA uptake[53]; this is reminiscent of natural transformation, which has been observed in *M. voltae*[54]. Efficient transformation of *Methanosarcina* species is only possible using a liposome-mediated protocol, which yields $>10^7$ transformants per microgram of DNA. The drawback of this method is that the requisite cationic liposomes are expensive.

Other gene transfer mechanisms. Once the DNA is safely inside an archaeal cell, it can be transferred to its neighbours by various means. Phage-mediated TRANSDUCTION is a mainstay of *E. coli* genetics, and similar phenomena have been reported in *Methanobacterium thermoautotrophicum* Marburg[55] and *M. voltae*[56]. In the latter case, the jury is still out; there is no evidence that gene transfer in *M. voltae* is mediated by viral particles[56], and the observation that the transfer agent is resistant to DNase does not rule out alternative routes. For example, bidirectional genetic exchange has been observed during cell mating in *Hfx. volcanii*[57], which involves cell-to-cell contact or fusion rather than transduction, and is actually stimulated by DNase

treatment[58]. Similar cell mating has also been seen in *S. acidocaldarius* and *S. solfataricus*. In the former, chromosomal marker exchange between two AUXOTROPHIC mutants can be measured by the appearance of stable genetic recombinants[59]. In the latter, conjugative plasmids such as pNOB8 have been shown to propagate throughout the culture using a cell-to-cell contact-dependent mechanism[60]. The kinetics of cell mating in *Sulfolobus* differs from that in *Hfx. volcanii*, as it does not require that cell-to-cell contacts are stabilised by growth on solid media[58,61].

Restriction/modification systems. From the perspective of foreign DNA, the inside of a cell can be a hostile environment. Restriction/modification systems are widespread amongst prokaryotes, and archaea are no exception. Enzymes that recognise 5'-CTAG-3' are common, having been identified initially in *Methanobacterium thermoformicicum*, where they are plasmid-encoded[62]. Genome analysis has revealed putative CTAG methylases in many species (restriction endonucleases are virtually impossible to identify by sequence homology), and DNA isolated from *Hfx. volcanii* is resistant to cleavage at NCTAGN' sites, suggesting modification[46]. *Hfx. volcanii* also has a restriction system that recognises adenine-methylated GATC sites (which occur frequently in vectors based on *E. coli* plasmids), resulting in DNA fragmentation followed by plasmid loss or chromosomal integration by recombination[63]. This can be circumvented by passaging the DNA through an *E. coli dam*$^-$ strain that is deficient in GATC methylation[64]. Other restriction/modification systems have been documented, such as the *Sua*I enzyme of *S. acidocaldarius* that recognises 5'-GGCC-3'[65].

Antibiotics. While most bacterial antibiotics are ineffective in archaea, several exceptions have been exploited to develop selectable markers for archaeal genetics (see Table 2). Novobiocin is a potent inhibitor of DNA gyrase (*gyrB*), an enzyme present in both bacteria and archaea. To develop a vector for halophilic archaea, the laboratory of Mike Dyall-Smith isolated a novobiocin-resistant mutant of *Haloferax* strain Aa2.2[66] that mapped to the *gyrB* gene[67]. Puromycin is another drug that is effective in both bacteria and archaea, and has been shown to inhibit growth in *M. voltae* and other methanogens[68]; it is the most widely used antibiotic for this group of archaea. The resistance marker (puromycin transacetylase) is a bacterial gene from *Streptomyces alboniger*. Owing to differences between bacterial and archaeal gene regulation, it is transcribed using an *M. voltae* promoter[69]. A similar approach was used to generate a construct for neomycin resistance in *M. maripaludis* (using APH3'I and APH3'II genes from Tn*903* and Tn*5* respectively[70]).

By contrast, the gene for pseudomonic acid resistance originates in archaea, and was generated by mutagenesis of the isoleucyl-tRNA synthetase gene from *Methanosarcina barkeri*[71]. Similarly, resistance to mevinolin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was isolated from a spontaneous *hmgA* mutant of *Hfx. volcanii*[72]. However, pseudomonic acid is not available commercially, and mevinolin is difficult to obtain as it is licensed as a cholesterol-lowering drug; by inhibiting the conversion of acetyl-CoA to mevalonic acid, mevinolin prevents the synthesis of cholesterol in humans and isoprenoid lipid side-chains in archaea. There are additional drawbacks to these antibiotics: spontaneous resistance can arise at high frequency due to gene amplification, and plasmids bearing mevinolin or novobiocin markers

suffer from instability due to homologous recombination with the chromosome (both these markers were derived from *Haloferax* species and are virtually identical to the chromosomal sequence of *Hfx. volcanii*). This instability can be alleviated by using markers from distantly related species, such as the mevinolin-resistant *hmgA* mutant allele from *Haloarcula hispanica*[73].

Auxotrophic selectable markers. Plasmid instability due to recombination could also be prevented by deleting the homologous chromosomal gene. Although this is not possible for mevinolin and novobiocin-resistance markers (both *hmgA* and *gyrB* are essential), it is feasible for genes in amino acid biosynthesis and other metabolic pathways where auxotrophic strains can easily be complemented. For example, a *leuB* deletion mutant defective in leucine synthesis can be grown on complete media, unless selection for a plasmid-encoded *leuB* marker is required, in which case leucine-deficient media is used. Such an approach has been adopted in yeast genetics, as few bacterial antibiotics are effective against eukaryotic cells. In addition to leucine, strains auxotrophic for histidine, proline, tryptophan and thymidine have been isolated in several species (see Table 2 for details). The principal drawback with auxotrophic markers is that they cannot be developed easily in obligatory AUTOTROPHS, which includes most methanogens. However, many *Methanosarcina* and *Methanococcus* species are facultative autotrophs that readily take up amino acids, and are therefore compatible with auxotrophic markers[74]. To enable full exploitation of auxotrophic markers, it is best if the organism can be grown on chemically defined (minimal) medium, as is the case for *P. abyssi*[75], *Hfx. volcanii*[58] and *M. maripaludis*[76].

Gene knock-out systems. Auxotrophic markers for uracil biosynthesis (*ura3*, *pyrE* or *pyrF* genes) are the most useful, as they can be COUNTER-SELECTED using 5-fluoroorotic acid (5-FOA); *ura*$^-$ cells are resistant to this compound due to their inability to convert 5-FOA to the toxic analogue 5-fluorouracil. Such markers can be implemented in organisms that grow poorly on minimal medium, as complex media deficient in uracil (such as casamino acids) can be used. This has enabled the establishment of gene knock-out systems in *Halobacterium*[77,78], *Hfx. volcanii*[79,80], and *T. kodakaraensis*[53] (see Table 2). The salient features of these systems are that the uracil marker can be reused (see Figure 1b), and that transformation is carried out using circular DNA (transformation using linear DNA is inefficient in some species). Uracil-auxotrophic mutants have also been isolated in *P. abyssi*[48,81], *S. acidocaldarius*[82] and *S. solfataricus*[83], but gene knock-out systems have yet to be developed. A trivial problem is that Gelrite, the gelling agent used in solid media for hyperthermophiles, contains trace uracil[84] (see Box 3). More seriously, in the widely used *S. solfataricus* P1 and P2 strains, gene-targeting constructs fail to recombine with the chromosome[83]. While it is possible that recombination is suppressed in *S. solfataricus* due to active transposable elements[84], *Halobacterium* also suffers from active transposition but is proficient for recombination[85]. Moreover, a different isolate of *S. solfataricus* is capable of homologous recombination, and has been used for gene knock-out experiments (using selection for lactose utilisation)[86].

Counter-selectable markers have recently been developed for methanogens, using the purine salvage enzyme hypoxanthine phosphoribosyltransferase encoded by the *hpt* gene; mutants are resistant to the toxic base

analogues 8-aza-2,6-diaminopurine and 8-azahypoxanthine[87]. Unlike uracil/5-FOA systems, gene knock-out with *hpt* requires an additional marker, for positive selection of plasmid integration. Puromycin and neomycin-resistance markers have been used with *hpt*, to construct a Δ*proC* mutant of *M. acetivorans*[88] and alanine-utilisation mutants of *M. maripaludis* respectively[89].

Random mutagenesis. As mentioned earlier, homology to bacterial or eukaryotic enzymes can be used to predict the function of only half the proteins encoded by archaeal genomes. If we are to elucidate the function of the remaining half, we must move beyond targeted gene knock-outs. Random mutagenesis provides the means to uncover genes and reaction pathways that are unique to archaea. UV radiation and chemical mutagenesis (using ethyl methanesulphonate) have been used to isolate auxotrophic mutants of *Hfx. volcanii*[58], *M. voltae*[54] *M. maripaludis*[90], and *P. abyssi*[91]. Since these mutations are difficult to map, transposon insertion mutagenesis has been attempted. *In vitro* transposition was used to study the *nifH* gene of *M. maripaludis*[92], although mutagenesis was not random as a defined target (rather than the whole genome) was used. The laboratory of Bill Metcalf has developed an elegant *in vivo* transposition system for *M. acetivorans* using a modified version of *mariner*-family transposon *Himar1*, which carries a puromycin-resistance marker as well as features that allow facile cloning of transposon insertions[93]. Unfortunately, this system is restricted to methanogens, as eukaryotic or bacterial transposons cannot function in the hypersaline interior of halophiles, or at the high temperatures demanded by hyperthermophiles. Synthetic transposons based on insertion sequences from *H. salinarum* have been constructed for use in *Hfx. volcanii*, but have had little success[94].

Random insertional mutagenesis is possible without transposition, so long as the species is proficient for homologous recombination. In this approach, recombination between a truncated version of the gene and its chromosomal copy leads to an insertion/disruption mutation (Figure 2B). A targeted version of insertional mutagenesis has been used in *M. voltae*, to characterise genes encoding flagellins and hydrogenases[95-97]. For random mutagenesis, a genomic library of small fragments (less than a full-length gene) is used to target recombination. As with the *in vivo* transposition system for *M. acetivorans*, insertion/disruptions are easily cloned by cutting and self-ligating genomic DNA fragments from the mutant, followed by introduction into *E. coli* (Figure 2B). Such random insertional mutagenesis has been used to isolate acetate auxotrophs of *M. maripaludis*[98].

Plasmid vectors. There are two basic flavours of vectors for archaeal genetics: INTEGRATING PLASMIDS and SHUTTLE PLASMIDS (see Figure 2A and online resources). As their name implies, integrating (or suicide) plasmids must integrate into the host chromosome, as they do not have an origin of replication for archaea. They are usually employed in gene knock-out or insertion/disruption mutagenesis, where efficient homologous recombination is paramount. In strains where this is not possible, such as *S. solfataricus* P1 and P2 isolates, vectors based on the SSV1 virus have been used that integrate into the chromosome by site-specific recombination[83]. SSV1 is also capable of stable replication as a circular plasmid, and this faculty has permitted the construction of recombinant shuttle vectors for *S. solfataricus*[51,99]. An intriguing feature of

SSV1 (and derived vectors) is that it spreads efficiently in cultures without lysis of the host cells[50]. This conjugative behaviour eliminates the need for efficient transformation. Self-spreading is also seen with pNOB8, another plasmid of *Sulfolobus*[60], but vectors derived from pNOB8 impose a significant burden on the host cell and have not been widely used[100].

Shuttle vectors for other species are more conventional, and use replication origins cannibalised from plasmids indigenous to the host. For example, the shuttle vector in Figure 2A (pTA230) uses the origin from pHV2, a naturally occurring 6.4 kb *Hfx. volcanii* plasmid. As it is non-essential, *Hfx. volcanii* could be cured of pHV2 by using ethidium bromide, thus generating the widely used strain WFD11[46]; a strain cured of pHV2 without using ethidium bromide (DS70) has since been isolated[73]. The WFD11 strain enabled the laboratory of Ford Doolittle to develop shuttle vectors for halophilic archaea, using the pHV2 origin and a mevinolin-resistance marker[72]. To date, it remains the most commonly used replicon for use in *Hfx. volcanii*. While pHV2-based plasmids are capable of replication in *Halobacterium*, a number of additional shuttle vectors have been derived from plasmids pGRB1[101] and pHH1[63]. Interestingly, plasmids based on pHV2 and pHH1 fail to replicate in recombination-deficient *radA* mutants of *Hfx. volcanii*[30], but pHK2 replicons[66] do not have this problem.

Shuttle vectors for methanogens are less common. The most useful replicon is based on the naturally occurring plasmid pC2A from *M. acetivorans*. The laboratory of Bill Metcalf has developed a series of pC2A derivatives, using puromycin-resistance as a selectable marker, and demonstrated that they are capable of transforming a variety of *Methanosarcina* species[52]. Shuttle vectors for use in *M. maripaludis* have been derived from the cryptic plasmid pURB500. Early incarnations suffered from instability in *E. coli* hosts, most probably due to the high A+T content of the replicon (~70%)[102]; stable vectors featuring a gene expression cassette for *M. maripaludis* and a *lacZ* gene for blue/white screening in *E. coli* have since been constructed[103]. Plasmids for use in hyperthermophilic euryarchaea are similarly rare. Thus far, only shuttle vectors based on the small pGT5 plasmid of *P. abyssi* strain GE5 have been developed[104]. They can be stably propagated in *P. abyssi* strain GE9 (which is devoid of pGT5), as well as the crenarchaeote *S. acidocaldarius*[48,105,106], indicating that mechanisms of plasmid replication are conserved between the major archaeal phyla.

Analysis of gene expression. Few compounds have had a greater impact on microbial genetics than X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), a chromogenic substrate that is converted by β-galactosidase into an insoluble blue dye. Originally developed by Julian Davies and Jacques Monod for studies of the *lac* operon of *E. coli*, it has since been put to 1001 uses. In archaea, as in many other organisms, it has been employed as a phenotypic reporter for gene expression (Table 3). The *E. coli* genes can be used directly in methanogens: *lacZ* has been used to monitor gene expression in *M. maripaludis*[20] and the β-glucuronidase gene *uidA* has been used similarly in *M. voltae* and *M. acetivorans*[88,107]. However, since methanogens are strict anaerobes and oxygen is necessary for blue colour development from X-gal, replica-plating is often essential if viable cells are to be recovered; this is not necessary for *M .maripaludis*, which

can tolerate short exposure to oxygen (J. Leigh, personal communication). While such problems are not encountered with halophiles (they are aerobic), *E. coli lacZ* is not active in the high salt concentrations found in the haloarchaeal cytosol (up to 5 M KCl). The laboratory of Mike Dyall-Smith therefore isolated a β-galactosidase gene *bgaH* from *Haloferax alicantei* (now called *Haloferax lucentensis*) that develops a blue colour from X-gal[108]. Moreover, it is functional in *H. salinarum* and *Hfx. volcanii* (which lacks detectable β-galactosidase activity), and has been used as a reporter gene for transcription analyses in both species[109,110]. A similar approach has been taken for thermophilic archaea, using a thermostable β-galactosidase from *S. solfataricus* encoded by *lacS*[111,112]. A mutant strain of *S. solfataricus* is available, where *lacS* has been inactivated by transposition of an insertion element[113], and was recently used by the laboratory of Christa Schleper to develop a sophisticated gene reporter system[83].

Phenotypic markers other than β-galactosidase have been used (see Table 3). For example, the salt-resistant trehalase gene *treA* from *Bacillus subtilis* is functional in *M. voltae*, and its activity can be assayed in cell lysates[114]. Recently, a modified derivative of green fluorescent protein (GFP) was developed that is soluble and active in the high salt cytosol of *Hfx. volcanii* [115]. However, researchers are increasingly turning to *ersatz* genetics. Genome sequence data has led to the development of archaeal microarrays, enabling studies of the response to UV radiation in *Halobacterium* sp. NRC-1[116], a characterisation of the central metabolism of *Hfx. volcanii*[117], and the identification of chromosomal replication origins in *Sulfolobus*[39]. It is noteworthy that the latter study was only possible because the cell cycle of *Sulfolobus* has been studied in some detail, resulting in a variety of means for synchronising cell cultures [118]. No doubt further microarray studies will be published in the near future, but if researchers are to make full use of such modern technology, they must first confront basic aspects of archaeal cell biology such as the cell cycle. Without bread-and-butter genetics, we will continue to operate without a solid foundation of knowledge about these fascinating organisms.

Future directions

Since the field was last reviewed[119,120], there has been considerable progress in development of tools for archaeal genetics. Gene knock-out systems in particular have made possible the systematic dissection of pathways that operate in this domain of life (Figure 1). However, there is much work to be done. For example, *S. solfataricus* P1 and P2 strains stubbornly refuse to integrate foreign DNA into the chromosome by homologous recombination, thereby limiting the scope of genetics in this important organism. The way forward might be to use a different isolate of *S. solfataricus* that is proficient for recombination[86]. With the exception of SSV1, archaeal viruses have yet to be harnessed for genetic purposes. Gene transfer by phage-mediated transduction would speed up the construction of archaeal mutant strains. Gene expression systems with tightly regulated promoters are sorely lacking. Heat-inducible chaperonin promoters are available for *Hfx. volcanii* and *S. solfataricus*[83,121], but the use of heat-shock to induce transcription is far from desirable. In this respect, progress is being made in *M. acetivorans*, where an acetate-inducible overexpression system has been developed (K. Sowers, personal communication). An improved method of gene regulation would also enable the development of archaeal two-hybrid systems, since yeast or bacterial two-hybrid systems are seldom of any value for analysing interactions between halophilic or thermophilic proteins. Finally, the genome sequences of several key archaea, including *Hfx. volcanii*, *M. voltae* and *S. acidocaldarius*, have yet to be published (Table 1). No doubt, this will be rectified in the near future.

Above all, more researchers should be working on archaea. Neophyte "archae-ologists" can find an entertaining introduction to the subject in *The Surprising Archaea* by John Howland[122]. There are lab manuals containing detailed protocols for methanogens[123], thermophiles[124] and halophiles[125]; for the latter, the excellent "HaloHandbook" is available online (see Online Links). Above all, researchers thinking of switching to archaea (and those who are merely curious) should remember that there is no single model organism for this entire domain. The wide range of habitats colonised by archaea is testament to their diversity, which is reflected at the molecular level by the bewildering array of metabolic and energy conversion mechanisms they employ. Nevertheless, there is a core of functions related to information processing that unites and defines archaea, and it is here that they share a common heritage with eukaryotes. Exciting discoveries await those who take the third way.

Box 1 | **Archaeal taxonomy and the impact of lateral gene transfer**

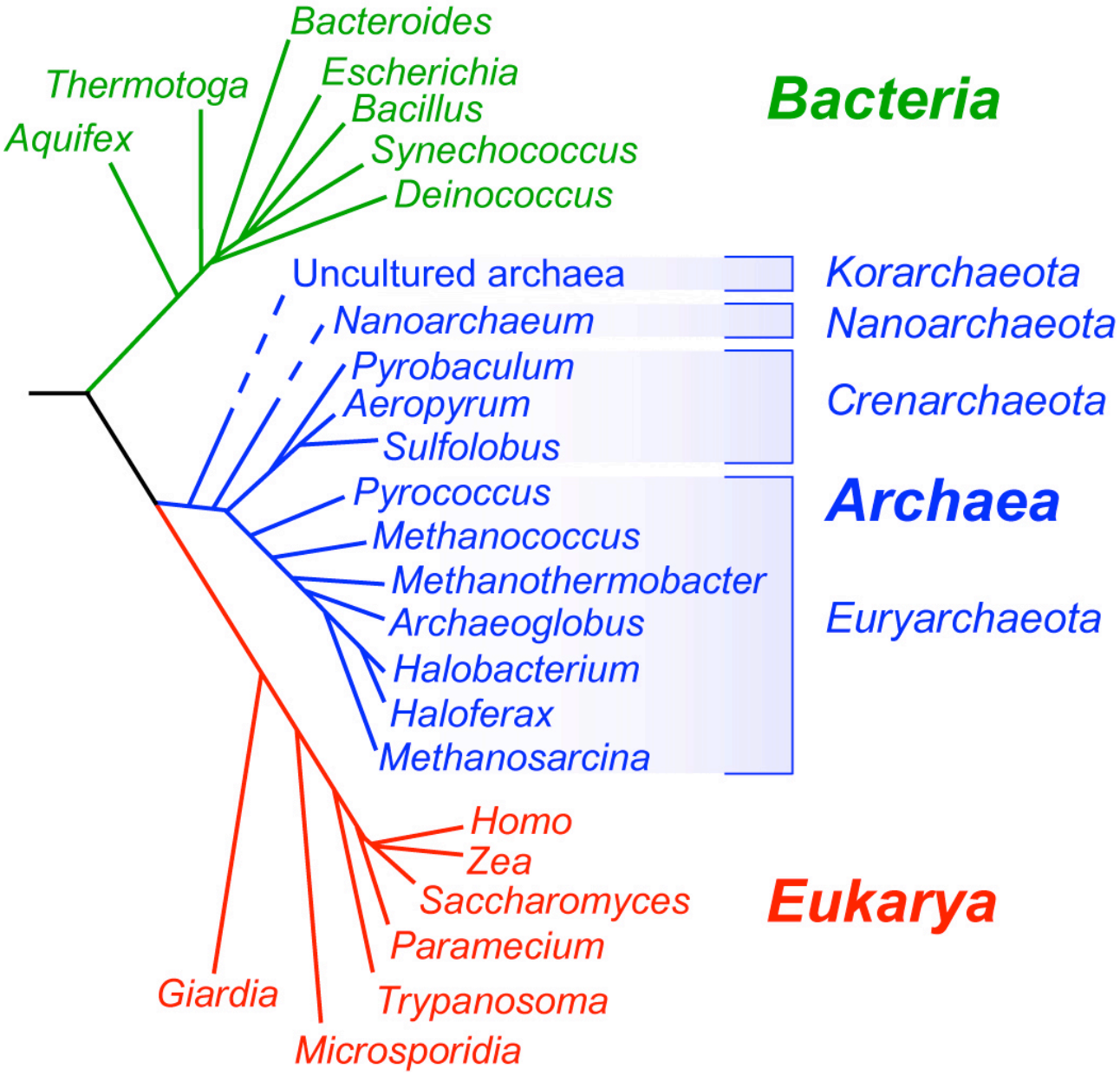
Archaeal taxonomy. The idea of using amino acid sequences as a tool for molecular phylogeny was first proposed by Francis Crick in 1958[126], but had to wait until the molecular biology revolution of the 1970s, when Carl Woese revisited the problem of prokaryotic taxonomy[1]. His choice of small-subunit (SSU) ribosomal RNA sequence as a molecular chronometer was visionary[127]. As an essential component of all self-replicating organisms, rRNA shows remarkable sequence conservation; different parts of the molecule exhibit varying rates of base substitution, allowing both coarse and fine-scale phylogenetic analyses. Furthermore, rRNA is abundant and easy to isolate, which has proved essential to CULTIVATION-INDEPENDENT STUDIES (Box 2).

The rRNA tree (below) reveals that the domain Archaea comprises several phyla. Euryarchaeota is the most diverse group, including all known methanogens and halophiles, in addition to thermophilic and PSYCHROPHILIC species. Members of Crenarchaeota are renowned as hyperthermophiles (all temperature record-breaking species belong to this phylum), but include the psychrophile *Cenarchaeum symbiosum*. Of the remaining phyla, Nanoarchaeota has one known member (*Nanoarchaeum equitans*[128]), and to date Korarchaeota are indicated only by environmental DNA sequences[129]. Owing to the paucity of identified species, the positions of these phyla within the rRNA tree are uncertain (indicated by dashed branches).

Lateral gene transfer (LGT). Unfortunately, life is not as simple as the rRNA tree suggests. It is commonly assumed that only eukaryotes indulge in sex, whereas prokaryotes rely on vertical inheritance (coupled with prodigious reproductive powers) to meet new environmental challenges. In reality, prokaryotes are highly promiscuous, and the role of LGT as a driving force in prokaryotic evolution has been grossly underestimated[12]. The fraction of "foreign" (mostly bacterial) genes in archaea might be as high as 20–30%[130]. Most are the result of orthologous replacement or acquisition of a paralogous gene. Consequently, archaeal genomes resemble fine-scale genetic mosaics[131]. Occasionally, LGT can lead to a novel gain-of-function event. It has been suggested that the switch from an anaerobic to aerobic lifestyle by the (methanogenic) ancestor of haloarchaea was facilitated by LGT of respiratory chain genes from bacteria[132]. Archaea, like bacteria, often group co-regulated genes in operons, and this arrangement would promote co-inheritance by LGT.

The degree to which archaea are "polluted" by bacterial genes depends on the species examined, and LGT might account for some variation in archaeal genome sizes (see Table 1). For example, *Methanosarcina mazei* has a bloated genome of 4.10 Mbp, 30% of which is bacterial in origin[133]. Metabolically diverse methanogenic and halophilic archaea are most promiscuous, having motive, means and opportunity for LGT: their motive is to acquire genes for novel metabolic functions, the opportunity arises because methanogens and halophiles often cohabit with bacteria, and the means is their ability to engage in cell mating, as demonstrated for *Haloferax volcanii*[57].

LGT has serious consequences for taxonomy. Phylogenetic trees based on individual genes differ significantly from the rRNA tree[134], and the notion of a "core" of never-exchanged genes has not passed muster[131]; such is the extent of LGT, that no one gene is unique to either archaea or bacteria. Even the gold standard of rRNA can be corrupted when multiple copies of the SSU rRNA gene are present, as in haloarchaea[135]. Nevertheless, as more archaeal genomes are sequenced and analysed, so a genomic signature is emerging that defines this domain in a holistic way[14].



Box 2 | **The ecological distribution of archaea – not just extremophiles**

The archaea are notorious for inhabiting some of the most forbidding places on earth, and thrive under conditions that few bacteria and no eukaryotes would tolerate. Some of these habitats are illustrated in the figure. Methanogens are strict anaerobes that inhabit a variety of anoxic habitats such as swamps (a) and sewage plants, and have a unique ability to generate energy by reducing carbon dioxide to form methane[136]. This was first noted by the Italian physicist Alessandro Volta, who found that "combustible air" is produced in lakes and bogs. Halophilic archaea grow readily in hypersaline ponds (b) and salt lakes such as the Dead Sea. Unlike bacteria, which maintain an osmotic balance using organic compatible solutes such as betaine, archaea accumulate inorganic salts (mainly K^+) in the cytoplasm. While this allows them to grow in saturating NaCl solutions, it requires that proteins of haloarchaea be adapted to function in high salt. ACIDOPHILES and ALKALIPHILES grow, as their names suggest, at extremes of pH; acidophiles are often thermophiles and a significant fraction of alkaliphiles are also halophiles. Thermophiles such as *Pyrolobus fumarii* are found near deep-sea hydrothermal vents (c), where temperatures exceed 100°C, and *Sulfolobus solfataricus* populates hot springs such as those in Yellowstone National Park (d). There are numerous molecular adaptations to thermophily, but the most striking is reverse gyrase, an enzyme that introduces positive supercoils in DNA and thereby protects it from unwinding[137]. At the opposite end of the spectrum, psychrophilic archaea thrive in permanently cold conditions such as the seawater or dry lakes in the Antarctic. However, it is misleading to believe that all archaea have been damned to such a hellish existence. Cultivation-independent methods such as ENVIRONMENTAL GENOME SHOTGUN SEQUENCING have indicated that mesophilic archaea are remarkably commonplace[138] and might represent more than 20% of microbial cells in the oceans[5]. Thus, classical microbiology, with its emphasis on pure culture, is inadequate at determining microbial diversity.

It has been suggested that our inability to culture mesophilic archaea might account for our inability to detect pathogenic archaea[139]. Although indicators of archaeal population density (such as methane levels) have been found to correlate with diseases such as chronic periodontitis[140], there is not one single example of an archaeon being directly responsible for a human malady. Lateral gene transfer between archaea and bacteria might have contributed to bacterial pathogenesis, where it has led to the emergence of novel virulence genes in the latter[141]. Although pathogenic archaea might exist, it is also possible that an intrinsic feature prevents them from posing a threat to vertebrates. For instance, archaea have unique cell membranes with isoprene side-chains[3], and liposome adjuvant prepared from archaeal membranes elicit a significantly greater immune response than liposomes prepared from bacterial membranes[142]. Thus, an archaeal pathogen would be an easy target for the immune system.

Panel A is a woodcut from the book by Alessandro Volta *Lettere sull'Aria Inflammabile nativa delle Paludi*, and is reproduced with permission from Cold Spring Harbor Laboratory Press; Panel B kindly provided by Richard Shand, Northern Arizona University, USA; Panel C kindly provided by Michel Gouillou, French Research Institute for the Exploitation of the Sea; Panel D kindly provided by Malcolm White, University of St Andrews, UK.

Box 3 | **Genetics needs a solid (media) foundation**

The primary requirement for any genetic system is the ability to obtain clonal cultures. Robert Koch first realised the potential of solid media for pure culture methods, by noting that different colonial forms breed true and therefore represent the clonal expansion of a single cell. Thus, he laid the cornerstone for microbiology and microbial genetics. Two associates of Koch developed the necessary technology at the end of the 19th century: Richard Petri invented the eponymous dish and Walter Hesse (more accurately, his wife Fannie) adopted agar as a gelling agent. To this day, little has changed.

Archaeal genetics is no exception – growth on solid media is essential before techniques such as transformation can be developed (see Table). The culture of haloarchaea is trivial, as they are aerobic and most grow at 35–45°C and neutral pH. Provided sufficient salt is present in the media, there is little to distinguish halophilic methods from those used with *E. coli*[125]. Handling methanogens is trickier, primarily because they are obligate anaerobes and require an environment with a reducing potential of less than –330 mV. Efficient cultivation has only been possible since 1950, when the "Hungate" technique for preparing and dispensing chemically-prereduced media into stoppered tubes was introduced[123]. Another innovation due to Hungate was the use of roll tubes, where agar is spun horizontally to coat the inner surface of the vessel[143]. A refined version of the Hungate technique is still in use today[144], although the introduction of the anaerobic glove box has allowed the use of conventional Petri dishes.

Gellan gum (also known as Gelrite) has been instrumental in the establishment of genetics for hyperthermophiles, which grow above the gelling temperature of agar[124]. Gelrite is a deacetylated polysaccharide produced by *Pseudomonas elodea*, and solidifies in the presence of divalent cations to form a matrix that is stable at temperatures as high as 120°C. The major disadvantage of Gelrite is that it contains trace nucleic acids, which can interfere with selection for uracil PROTOTROPHY[53,84]. Transformants are therefore grown in selective liquid medium (deficient in uracil) before plating on Gelrite. However, given the importance of selection on solid media to the development of genetics, a more elegant solution will no doubt be found.

Transformation or gene transfer method	**Species** (see Table 1)	**References**	**Notes**
Polyethylene glycol (PEG)	Har Hbt Hvo Mma Pab	[45,47,48,145,146]	Requires spheroplast formation by removal of S-layer (usually by treatment with EDTA, but not for *Methanococcus maripaludis*).
Electroporation	Mac Mvo Sso	[49-52]	Not universally applicable in archaea.
Liposomes	Mac Mba Mvo	[52,114]	Very efficient, but expensive.
$CaCl_2$ & heat shock	Mvo Pfu Sac Tko	[53,54,105]	Not very efficient.
Cell mating/conjugation	Hvo Sac	[57,59]	Chromosomal marker exchange requires stable cell contact for *Haloferax volcanii*, but not *Sulfolobus acidocaldarius*.
Virus/plasmid-mediated conjugation	Sso	[50,51,60,99,100]	Mediated by self-spreading vectors based on SSV1 virus or pNOB8 plasmid.
Transduction	Mth Mvo	[55,56]	Mth refers to *Methanobacterium thermoautotrophicum* Marburg.

Figure 1 | **Gene knockout methods used in archaeal genetics.**
a | Direct replacement of gene with selectable marker, by recombination between linear DNA comprising flanking regions of the gene and a chromosomal target. In some archaeal species, recombination using linear DNA is less efficient than circular DNA.
b | The pop-in/pop-out method uses circular DNA and selection for transformation to uracil prototrophy. Thus, a *ura*$^-$ strain must be used[53,77,79]. Intramolecular recombinants that have lost the plasmid are counter-selected using 5-fluoroorotic acid (5-FOA), which is converted to toxic 5-fluorouracil in *ura*$^+$ (but not *ura*$^-$) cells. Unless the mutant has a readily screened phenotype, the deletion must be verified by Southern blotting.
c | Variant of the pop-in/pop-out method for gene deletion, where the gene is replaced with a marker allowing direct selection[80].
d | Combination of gene replacement (with *ura* marker) and pop-in/pop-out method, suitable for generating point mutants[77]. Counter-selection with 5-FOA ensures that the *ura*-marked gene deletion is replaced with the desired mutation.

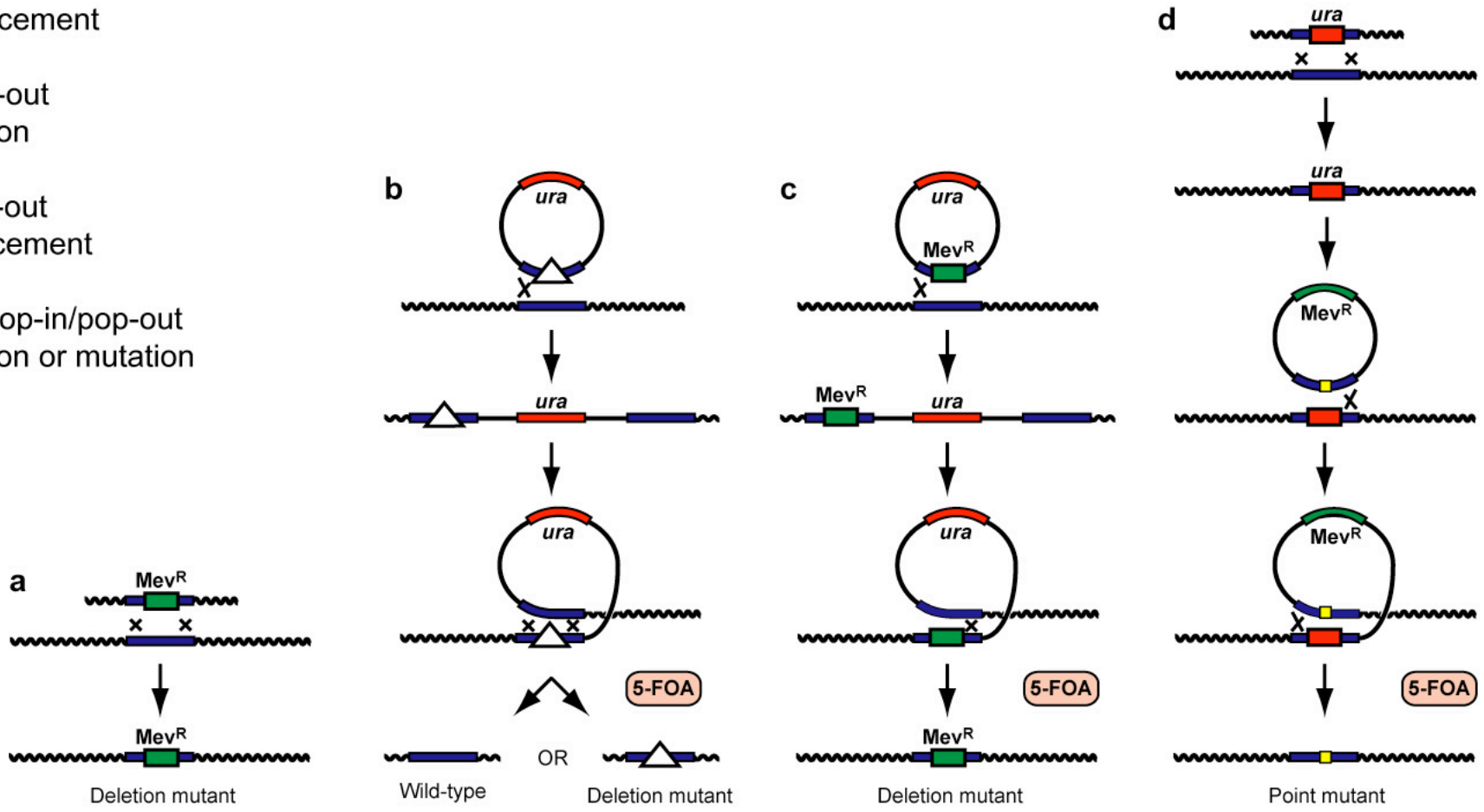
Figure 2 | **Plasmid vectors.**
a | Typical integrative and shuttle plasmid vectors for archaeal genetics (in this case, *Haloferax volcanii*), with relevant features[80]. A table of commonly used plasmids is available in the online resources.
b | Random insertional mutagenesis using small-fragment library. Recombination between an integrative plasmid carrying a small (internal) fragment of a gene and the chromosome leads to disruption of the gene. Rapid identification of the mutant gene is possible by using DNA sequencing primers (light blue) directed to plasmid sequences. The insertion can be cloned directly from genomic DNA by cutting with an enzyme (e.g. *Eco*RI) to liberate the plasmid and some surrounding sequence, which is self-ligated and used to transform *E. coli*.

a Gene replacement

b Pop-in/pop-out gene deletion

c Pop-in/pop-out gene replacement

d Two-step pop-in/pop-out gene deletion or mutation



	a	b	c	d
Advantages	Simple, any strain can be used Direct selection	Circular DNA transformation Marker can be reused	Circular DNA transformation Direct selection	Direct selection Markers can be reused
Disadvantages	Linear DNA transformation Marker cannot be reused	Requires *ura*⁻ strain No direct selection	Requires *ura*⁻ strain Marker cannot be reused	Requires *ura*⁻ strain Linear DNA transformation

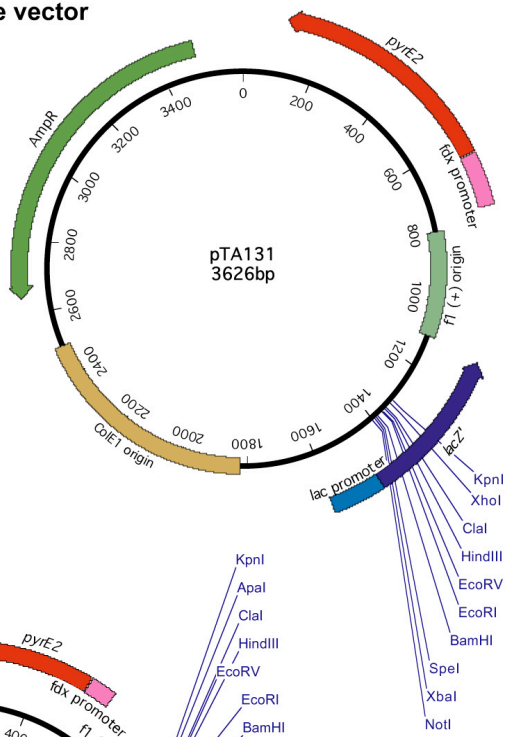
Features of integrative vector

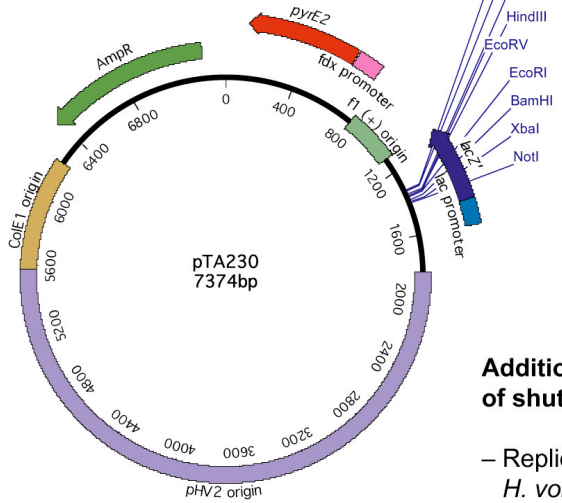
– Selectable marker for *H. volcanii* (*pyrE2*)

– Replication origin for *E. coli* (ColE1 origin)

– Selectable marker for *E. coli* (AmpR)

– Multiple cloning site

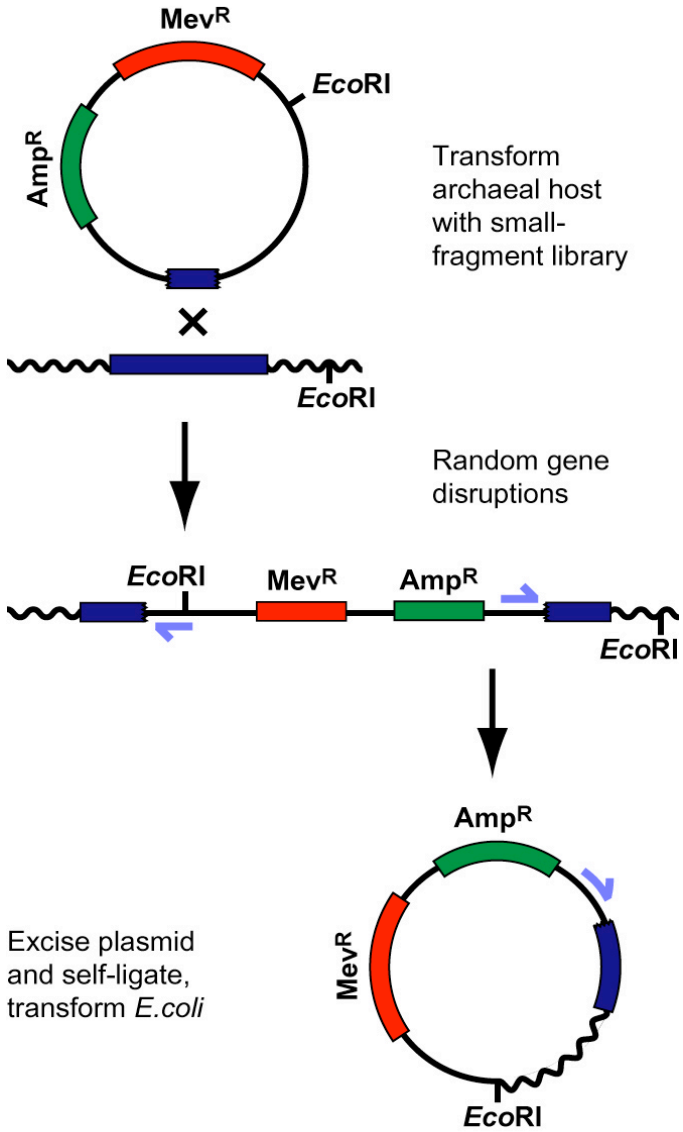




Additional features of shuttle vector

– Replication origin for *H. volcanii* (pHV2 origin)

a



b

Species	Abbreviation	Genome	Date	Phylum	Growth characteristics and optimal temperature	Genetics	Sequence
Methanocaldococcus jannaschii		1.66	1996	Eury.	Hyperthermophilic methanogen, anaerobic, 85°C		NCBI
Archaeoglobus fulgidus		2.18	1997	Eury.	Hyperthermophile, sulphate-reducing, anaerobic, 83°C		NCBI
Methanothermobacter thermautotrophicus	Mth	1.75	1997	Eury.	Methanogen, anaerobic, 65°C	+	NCBI
Pyrococcus horikoshii		1.74	1998	Eury.	Hyperthermophile, anaerobic, 96°C		NCBI
Aeropyrum pernix		1.67	1999	Cren.	Hyperthermophile, aerobic, 95°C		NCBI
Halobacterium sp. NRC-1*	Hbt	2.57	2000	Eury.	Halophile, aerobic, 42°C	+++	NCBI
*Halobacterium salinarum**	Hbt	~2.5	2000†	Eury.	Halophile, aerobic, 42°C	+++	MPG
Thermoplasma acidophilum		1.56	2000	Eury.	Thermoacidophile, aerobic, 59°C		NCBI
Thermoplasma volcanium		1.58	2000	Eury.	Thermoacidophile, aerobic, 60°C		NCBI
Pyrococcus abyssi	Pab	1.77	2001	Eury.	Hyperthermophile, anaerobic, 96°C	++	NCBI
Pyrococcus furiosus	Pfu	1.91	2001	Eury.	Hyperthermophile, anaerobic, 96°C	++	NCBI
Pyrolobus fumarii		1.85	2001†	Cren.	Hyperthermophile, aerobic 106°C		
Sulfolobus solfataricus	Sso	2.99	2001	Cren.	Thermoacidophile, aerobic, 80°C	+++	NCBI
Sulfolobus tokodaii		2.69	2001	Cren.	Thermoacidophile, aerobic, 80°C	+	NCBI
Ferroplasma acidarmanus		1.87	2002§	Eury.	Acidophile, anaerobic, 42°C		ORNL
Methanopyrus kandleri		1.69	2002	Eury.	Hyperthermophilic methanogen, anaerobic, 98°C		NCBI
Methanosarcina acetivorans	Mac	5.75	2002	Eury.	Methanogen, anaerobic, 35°C	+++	NCBI
Methanosarcina barkeri	Mba	4.86	2002§	Eury.	Methanogen, anaerobic, 35°C	+++	ORNL
Methanosarcina mazei	Mmz	4.10	2002	Eury.	Methanogen, anaerobic, 37°C	+++	NCBI
Pyrobaculum aerophilum		2.22	2002	Cren.	Hyperthermophile, nitrate-reducing, aerobic, 100°C		NCBI
Hyperthermus butylicus		1.67	2003†	Cren.	Hyperthermophile, sulphate-reducing, anaerobic, 100°C		
Methanogenium frigidum		~2.5	2003§	Eury.	Psychrophilic methanogen, anaerobic, 15°C		UNSW
Nanoarchaeum equitans		0.49	2003	Nano.	Symbiotic hyperthermophile, anaerobic, 90°C		NCBI
Sulfolobus acidocaldarius	Sac	2.23	2003†	Cren.	Thermoacidophile, aerobic, 80°C	++	
Haloarcula marismortui	Har	4.27	2004	Eury.	Halophile, aerobic, 37°C	+	NCBI
Haloferax volcanii	Hvo	4.03	2004†	Eury.	Halophile, aerobic, 45°C	+++	UMBI; TIGR
Methanococcoides burtonii		2.56	2004§	Eury.	Psychrotolerant methanogen, anaerobic, 23°C		ORNL
Methanococcus maripaludis	Mma	1.66	2004	Eury.	Methanogen, anaerobic, 37°C	+++	NCBI
Methanococcus voltae	Mvo	~1.9	2004†	Eury.	Methanogen, anaerobic, 37°C	+++	
Natronomonas pharaonis		2.75	2004†	Eury.	Haloalkaliphile, aerobic, 40°C		
Picrophilus torridus		1.55	2004	Eury.	Acidophile, aerobic, 60°C		NCBI
Thermococcus kodakaraensis	Tko	2.09	2004†	Eury.	Hyperthermophile, anaerobic, 85°C	++	

Thermoproteus tenax		~1.84	2004§	Cren.	Hyperthermophile, anaerobic, 86°C		[44]
Acidianus brierleyi		~1.9		Cren.	Thermoacidophile, aerobic, 70°C		
Halobaculum gomorrense		~2.7		Eury.	Halophile, aerobic, 37°C	+	
Haloquadratum walsbyi		~3.18		Eury.	Halophile, aerobic, 40°C		
Halorubrum lacusprofundi		~2.6		Eury.	Psychrotolerant halophile, aerobic, 30°C	+	
Natrialba asiatica		~3.1		Eury.	Halophile, aerobic, 37°C	+	
Sulfolobus metallicus		~1.9		Cren.	Thermoacidophile, aerobic, 80°C		
Cenarchaeum symbiosum				Cren.	Symbiotic psychrophile, aerobic, 10°C		
Methanococcus thermolithotrophicus				Eury.	Thermophilic halotolerant methanogen, anaerobic, 62°C		
Methanosaeta concilii				Eury.	Methanogen, anaerobic, 37°C		
Methanosarcina thermophila				Eury.	Thermophilic methanogen, anaerobic, 50°C		
Methanosphaera stadtmanae				Eury.	Methanogen, anaerobic, 37°C		
Methanospirillum hungateii				Eury.	Methanogen, anaerobic, 37°C		

Table 1 | **Archaea with sequenced genomes or ongoing genome projects.**

Abbreviations are given for species described in other tables; Har refers to *Haloarcula* species and Hbt refers to *Halobacterium* species. * *Halobacterium* sp. NRC-1 and *Halobacterium salinarum* genome sequences essentially identical. Genome size given in mega base pairs (Mbp) or not determined (no entry). Date indicates either completion or publication of genome sequence (no entry indicates ongoing genome project); § genome sequence published but remains incomplete; † genome sequence complete but not published. Archaeal phyla comprise Euryarchaeota (Eury.), Crenarchaeota (Cren.) and Nanoarchaeota (Nano.). Genetics: + potential for genetics, such as growth on solid media; ++ rudimentary genetics, such as transformation and selectable markers; +++ advanced genetics, including shuttle vectors, gene replacement, reporter genes. Where possible, websites hosting genome sequence data are given; for abbreviations and URLs see Online Links.

Selection	Type	Marker	Species	References	Notes
Alcohol dehydrogenase	A	*adh*, *adh-hT*	Pfu Sso	[106,147]	Alcohol dehydrogenase *adh-hT* gene from *Bacillus stearothermophilus*.
Anisomycin	A	Ani^R (23S rRNA)	Hbt	[148]	Mutant 23S rRNA gene, used for mutation of chromosomal rRNA gene but not developed as selectable marker
Bleomycin	A	ShBle	Hvo	[149]	Bleomycin-resistance ShBle gene from *Streptoalloteichus hindustanus*
Hygromycin B	A	*hph*	Sso	[51,150]	Mutated (thermostable) version of the hygromycin B phosphotransferase (*hph(mut)*) gene from *Escherichia coli*.
Mevinolin	A	Mev^R (*hmg*)	Har Hbt Hvo	[63,72,73,145]	Use of heterologous MevR gene prevents recombination between chromosomal *hmg* gene and Mev^R.
Neomycin	A	Neo^R (APH3')	Mma	[70]	Geneticin not inhibitory to *Methanococcus maripaludis*.
Novobiocin	A	Nov^R (*gyrB*)	Hvo Sac	[66,82]	Inhibits DNA gyrase, NovR over-expresses DNA gyrase B subunit.
Pseudomonic acid	A	PA^R (*ileS*)	Mba Mac Mth	[71,151]	Mutant isoleucyl-tRNA synthetase *ileS* gene from *Methanosarcina barkeri* Fusaro. Pseudomonic acid not commercially available.
Puromycin	A	Pur^R (*pac*)	Mac Mba Mma Mmz Mvo	[52,69,97,152,153]	Puromycin-resistance *pac* gene from *Streptomyces alboniger*, widely used marker for methanogenic archaea.
Thiostrepton	A	Ths^R (23S rRNA)	Hbt	[148]	Mutant 23S rRNA gene, used for mutation of chromosomal rRNA gene but not developed as selectable marker
Trimethoprim	A	*hdrA*	Hvo	[154]	Trimethoprim not widely used as selectable marker.
8-aza-2,6-diaminopurine (8ADP) or 8-aza-hypoxanthine	C	*hpt*	Mac Mma	[88,89]	Used in conjunction with puromycin or neomycin as positive selectable marker.
Uracil/5-fluoroorotic acid (5-FOA)	X/C	*ura3, pyrE*, *pyrF*	Hbt Hvo Pab Sso Tko	[48,53,77,79-81,83,84]	Very useful marker. Isolation of spontaneous 5-FOA-resistant mutants easy, allowing system to be implemented widely[53,82,84]
Histidine	X	*hisA*, *his-1*	Mvo Hvo	[155,156]	*his-1* gene of *Haloferax volcanii* not developed as marker.
Lactose	X	*lacS* (+*lacTr*)	Sso	[86]	*lacS* gene in host strain disrupted by transposon or deleted.
Leucine	X	*leuB*, *leuA*	Hvo Mma	[76,80]	*leuB* and *leuA* genes in host strains deleted. *M. maripaludis leuA* not yet developed as marker.
Proline	X	*proC*	Mac	[88]	*E. coli proC* gene also functions as marker.
Thymidine	X	*hdrA, hdrB*	Hvo	[80,154]	*hdrB* gene in host strain deleted. Complex media based on yeast extract are deficient in thymidine.
Tryptophan	X	*trpA*, *trpE*	Hvo Tko	[53,80]	*trpE* mutants (tryptophan auxotrophs) of *Thermococcus kodakaraensis* isolated, but no selectable marker developed.

Table 2 | **Selectable markers used in archaeal genetics.**
Selectable marker type: antibiotic (A); counter-selectable (C); auxotrophic or similar (X). Species abbreviations, see Table 1. In this table, Mth refers to *Methanobacterium thermoautotrophicum* Marburg.

Reporter	Host	Type	References	Notes
bgaH	Hbt Hvo	Colour (X-gal)	[108-110]	β-galactosidase from *Haloferax alicantei*. Wild-type *Haloferax volcanii* lacks detectable β-galactosidase activity.
lacS	Sso	Colour (X-gal)	[83,111-113]	Thermostable β-galactosidase from *Sulfolobus solfataricus*.
lacZ	Mma	Colour (X-gal)	[20]	*lacZ* gene from *E. coli*. Colour development requires exposure to oxygen.
uidA	Mac Mvo	Colour (X-gluc)	[88,107]	*uidA* gene from *E. coli*. Colour development requires exposure to oxygen.
treA	Mvo	Enz.	[114]	Trehalase gene *treA* from *Bacillus subtilis*, enzyme insensitive to moderate salinity.
GFP	Hvo	Fluor.	[115]	Modified variant of green fluorescent protein (GFP), soluble in *Hfx. volcanii* due to reduced hydrophobicity.
hdrA	Hvo	Res.	[157]	Ferredoxin (*fdx*) promoter of *H. salinarum* analysed in *Hfx. volcanii* using trimethoprim. Dihydrofolate reductase (*hdrA*) is competitively inhibited by trimethoprim.

Table 3 | **Phenotypic markers and reporter genes used in archaeal genetics.**

Species abbreviations, see Table 1. Marker types: blue colouration upon exposure to chromogenic indicator (Colour), 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) or 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc); enzymatic assay from cell lysate (Enz.); fluorescent reporter protein (Fluor.); resistance to antibiotic (Res.).

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Competing interests statement

The authors declare that they have no competing financial interests.

Autobiographies

Thorsten Allers has a long-standing interest in genetic recombination. He received his PhD training in the lab of David Leach at the Institute of Cell and Molecular Biology in Edinburgh, UK, where he became acquainted with *Escherichia coli*. His post-doctoral work was in the laboratory of Michael Lichten at the National Cancer Institute in Maryland, USA, where he trained in genetics of *Saccharomyces cerevisiae*. After switching domain for the third time, he joined the Institute of Genetics in Nottingham, UK. His lab uses *Haloferax volcanii* to study DNA recombination and repair in archaea. For more information on the author visit http://www.nottingham.ac.uk/genetics/allers

Moshe Mevarech received his Ph.D. training in the lab of Henryk Eisenberg at the Polymer Department in the Weizmann Institute of Science, Rehovot, Israel. He did his post-doctoral training first with Kan L. Agarwal and then with Robert Haselkorn at the University of Chicago before taking a faculty position at the Department of Molecular Microbiology and Biotechnology in Tel-Aviv University, Israel. For many years, the interests of his lab have focused on applying genetic and biochemical approaches to study halophilic archaea.

Online summary

- Archaea comprise the third domain of life, alongside bacteria and eukaryotes. The domain Archaea was proposed in 1977 by Carl Woese, as a result of phylogenetic studies using ribosomal RNA sequences as a molecular chronometer.
- Archaea are renowned as extremophiles, but environmental studies suggest that they thrive in all habitats. However, to date no pathogenic archaea have been found.
- Bacteria and archaea share a prokaryotic morphology, and have comparable pathways for central metabolism and energy conversion. On the other hand, information processing pathways in archaea and eukaryotes use similar enzymes. However, the archaeal systems are much simpler.
- Lateral gene transfer between archaea and bacteria is common, and might be responsible for some evolutionary innovations.
- As much as 50% of the genes found in archaeal genomes might encode novel proteins with no obvious counterparts in bacteria or eukaryotes.
- Archaeal proteins have proved invaluable to biochemists and structural biologists, but genetic studies of archaea are still comparatively rare.
- Genetic techniques for archaea are more advanced than is commonly believed. A wide range of archaeal species can be transformed using integrative and shuttle vectors, carrying a variety of selectable markers. Methods for mutagenesis and gene knock-out are available, as are reporter genes such as β-galactosidase.

Glossary

ACIDOPHILE
Organism that requires a low pH for growth, typically less than pH 3. Contrast with alkaliphile.

ALKALIPHILE
Organism that requires a high pH for growth, typically greater than pH 10. Contrast with acidophile.

AUTOTROPH
Organism that can synthesise its own macromolecules from simple, inorganic molecules such as carbon dioxide, hydrogen and ammonia. Contrast with heterotroph.

AUXOTROPH
Mutant that requires nutrients not needed by wild-type strains for growth on minimal medium. Contrast with prototroph.

COUNTER-SELECTABLE MARKER
A marker which if present leads to cell death under selective conditions, usually by conferring sensitivity to an antibiotic or by promoting the synthesis of a toxic product from a non-toxic precursor.

CULTIVATION-INDEPENDENT STUDY
Method for determining environmental biodiversity without the need to obtain microbiologically-pure cultures, using sequences retrieved from environmental samples to construct a molecular phylogenetic survey. For example, environmental genome shotgun sequencing.

DOMAIN
Highest level of taxonomic division, comprising Archaea, Bacteria and Eukarya. In declining order, other levels include: kingdom, phylum, class, order, family, genus, and species.

ENVIRONMENTAL GENOME SHOTGUN SEQUENCING
High-throughput sequencing and computational reconstruction of genomic DNA fragments extracted from environmental samples, in order to assess microbial diversity in a cultivation-independent manner.

EXTREMOPHILE
Organism that requires extreme environments for growth, such as extremes of temperature, salinity or pH, or a combination thereof.

HALOPHILE
Organism that requires high concentrations of salt for growth, typically greater than 1M NaCl.

HETEROTROPH
Organism that requires complex organic molecules such as amino acids and sugars, from which to build macromolecules and derive energy. Contrast with autotroph.

HYPERTHERMOPHILE
Organism that requires extremely high temperatures for growth, typically greater than 80°C. Compare with thermophile.

INTEGRATIVE VECTOR
Plasmid vector incapable of replication in archaeal host, which therefore must integrate into the host chromosome by homologous or site-specific recombination. Contrast with shuttle vector.

LATERAL GENE TRANSFER
Horizontal transfer of genes between unrelated species, as opposed to vertical inheritance within a species.

METHANOGEN
Anaerobic organism that generates methane by reduction of carbon dioxide, a variety of one-carbon compounds or acetic acid.

MONOPHYLETIC
A natural taxonomic group consisting of species that share a common ancestor.

PROTOTROPH
Organism capable of growth on minimal medium containing a carbon source and inorganic compounds. Contrast with auxotroph.

PSYCHROPHILE
Organism capable of growth at permanently low temperatures, typically less than 10°C. Contrast with thermophile.

SPHEROPLAST
Cell denuded of the vast majority of its cell wall or surface layer, usually by chemical or enzymatic treatment. Also known as protoplast.

SHUTTLE VECTOR
Plasmid vector capable of replication in both *Escherichia coli* and archaeal host. Contrast with integrative vector.

THERMOPHILE
Organism that requires high temperatures for growth, typically greater than 60°C. Contrast with psychrophile.

TRANSDUCTION
Transfer of host genes between archaeal or bacterial species, using a virus as a vector.

TRANSFECTION
Infection of a host cell by naked DNA or RNA isolated from a virus.

Online links

Genome sequence data

Archaeal genomes at National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/genomes/static/a_g.html

Halobacterium salinarum genome at Max Planck Gesellschaft (MPG): http://www.halolex.mpg.de/

Draft *Haloferax volcanii* genome at The Institute for Genomic Research (TIGR): http://tigrblast.tigr.org/ufmg/index.cgi?database=h_volcanii|seq

Halophile genomes at University of Maryland Biotechnology Institute (UMBI): http://zdna2.umbi.umd.edu/~haloweb/

Draft archaeal genomes at Oak Ridge National Laboratory (ORNL): http://genome.ornl.gov/microbial/

Draft genomes of psychrophilic archaea at University of New South Wales (UNSW): http://psychro.bioinformatics.unsw.edu.au/genomes/

Genomes OnLine Database (GOLD): http://www.genomesonline.org/

Strain repositories

Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ): http://www.dsmz.de/

National Collections of Industrial, Food and Marine Bacteria (NCIMB): http://www.ncimb.co.uk/

American Type Culture Collection (ATCC): http://www.atcc.org/

Japan Collection of Microorganisms: http://www.jcm.riken.go.jp/

Others

HaloHandbook: http://www.microbiol.unimelb.edu.au/micro/staff/mds/HaloHandbook/

Gordon Research Conference (GRC) on Archaea: Ecology, Metabolism & Molecular Biology: http://www.grc.org/programs/2005/archaea.htm

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Online Resources

Vector	Host	Archaeal replicon	Archaeal marker(s)	Size (kbp)	References	Notes
pWL102, pWL104	Har Hbt Hvo	pHV2	Mev^R	10.5, 8.7	[1-4]	pHV2 plasmid from *Haloferax volcanii*. Laboratory strains WFD11 and DS70 are cured of pHV2, enabling use of this replicon for shuttle vectors.
pWL204	Hvo	pHV2	Mev^R	10.4	[5]	Expression vector based on pWL102 with $tRNA^{Lys}$ promoter.
pMDS99	Hvo	pHV2	Mev^R	7.3	[6]	Mev^R marker from *Haloarcula hispanica*.
pUBP2	Har Hbt Hvo	pHH9 (pHH1)	Mev^R	12.3	[2,3]	pHH9 is a deletion derivative of pHH1.
pNG168	Hbt	pNRC100	Mev^R	8.9	[4]	Polylinker in *lacZ'* for blue/white screening in *Escherichia coli.*
pMPK54, 56	Hbt	pGRB1	Mev^R	8.6, 5.4	[7,8]	pMPK56 is more stable than pMPK54, but does not have an *E. coli* replicon or marker.
pNOB102	Har Hbt	pNOB1	Mev^R	9.1	[9,10]	pNOB1 from *Natronobacterium* sp. strain AS7091, see also pNOB103.
pMLH3	Hvo	pHK2	Mev^R & Nov^R	11.3	[11]	Dual-marker plasmid.
pMDS20	Hvo	pHK2	Nov^R	10	[11-13]	Derived from pMDS10, see also pMDS1, 2, 8, 9, and 11.
pWL-Nov	Hvo	pHV2	Nov^R	9.4	[14]	Derived from pWL102.
pBR-Nov, pUC-Nov	Hvo	–	Nov^R	5.7, 5.1	[14]	
pHRZ	Hbt	ND	Ani^R Ths^R	12.6	[15]	
pHRZH	Hbt	–	Ani^R Ths^R	14.4	[15]	Derived from pHRZ.
pMPK408	Hbt	–	*ura3*	4.1	[16,17]	Vector for gene knockout with 5-FOA, requires *Halobacterium* Δ*ura3* host.
pGB70	Hvo	–	*pyrE2*	3.3	[18]	Vector for gene knockout with 5-FOA, requires *Hfx. volcanii* Δ*pyrE2* host.
pTA131, 132, 133, 192	Hvo	–	*pyrE2 trpA*, *leuB*, *hdrB*	3.6, 3.9, 4.1, 3.7	[19]	pTA131 vector for gene knockout with 5-FOA. Require *Hfx. volcanii* Δ*pyrE2*, Δ*trpA*, Δ*leuB* or Δ*hdrB* hosts respectively. Polylinker in *lacZ'* for blue/white screening in *E. coli*.
pTA230, 231, 232, 233	Hvo	pHV2	*pyrE2 trpA*, *leuB*, *hdrB*	7.4, 7.7, 7.8, 7.4	[19]	Shuttle vectors derived from pTA131, 132, 133, 192, similar host requirement.
pMIP1	Mvo	–	Pur^R	7.4	[20]	Also contains *hisA* sequences for integration into *Methanococcus voltae* genome.
pMEB.2	Mma	–	Pur^R	5.0	[20,21]	pUC18 with *pac* cassette for Pur^R.
pKAS102	Mma	–	Pur^R	12.1	[22]	Derived from pMIP1, with 4.7 kb insert of *Methanococcus maripaludis argH* gene, for integration into genome.
pWAY1, 2	Mmz	–	Pur^R	5.5	[23]	

pUC::*pac*	Mvo	–	Pur^R	5.0	[24,25]	pUC BM21 with *pac* cassette for Pur^R.
pJK29, 41	Mac Mba	–	Pur^R	3.2, 3.5	[26]	Polylinker in *lacZ′* for blue/white screening in *E. coli.* Require *E. coli pir* host for replication using *ori*R6K origin. See also pJK31, 33, 35, 37, and 39.
pWLG13, 18	Mma	–	Pur^R	4.4, 7.5	[27]	Contains P_{hmvA} promoter for gene expression in *M. maripaludis*. pWL18 contains polylinker in *lacZ′* for blue/white screening in *E. coli.* See also pWLG11, 12A, 14
pWM307, 311, 321	Mac Mba	pC2A	Pur^R	8.2, 8.7, 8.9	[28,29]	pWM311 contains polylinker in *lacZ′* for blue/white screening in *E. coli*. See also pWM309, 313, 315, and 317, 319.
pDLT44	Mma	pURB500	Pur^R	12.7	[30]	Not very stable in *E. coli*.
pWLG30	Mma	pURB500	Pur^R	12.7	[27]	Derived from pWLG14. Contains P_{hmvA} promoter for gene expression in *M. maripaludis*. See also pWL30+lacZ (contains *lacZ′* for blue/white screening in *E. coli*)
pRCZ113	Mma	–	Pur^R	16	[31]	Transcriptional reporter construct using *lacZ*, includes *M. maripaludis argH* gene for integration into genome.
Miptre	Mvo	–	Pur^R	8.7	[32]	Transcriptional reporter construct using *treA*, includes *M. voltae hisA* gene for integration into genome.
pMudpur	Mma	–	Pur^R	7.3	[33]	Contains Mud transposon for insertional mutagenesis.
pWM381	Mac	–	Pur^R	7.5	[34]	Mini-mariner delivery plasmid for *in vivo* transposon mutagenesis in *Methanosarcina acetivorans*. See also pWM379, 383, 385.
pPAC60	Mvo	–	Pur^R	4.7	[35]	Vector for insertional mutagenesis, includes *hmvA* promoter to drive transcription of genes downstream of insertion.
pMP44	Mac	–	*hpt* Pur^R	4.2	[36]	Counter-selectable plasmid for marker-less gene deletion in *M. acetivorans*. Requires *E. coli pir* host for replication using *ori*R6K origin.
pCRPrtNeo	Mma	–	*hpt* Neo^R	6.5	[37]	Counter-selectable plasmid for marker-less gene deletion in *M. maripaludis*.
pJLA5, 6	Mma	–	Neo^R	5.6, 5.8	[38]	
pHisuid	Mvo	–	*hisA*	6.6	[39]	Integration of plasmid reconstitutes *hisA* gene, requires *M. voltae hisA* host. Also contains *hmvA* promoter linked to *uidA* glucuronidase gene of *E. coli*. See also pHisuid-*vhc* and -*frc*.
pPB31–35	Mac	pC2A	PA^R	11.3	[29,40]	Polylinker in *lacZ′* for blue/white screening in *E. coli.* Require *E. coli pir* host for replication using *ori*R6K origin.
pJK89	Mac	pC2A	*proC*	9.1	[36]	Requires *M. acetivorans* Δ*proC* host.
pKMSD48	Sso	SSV1	–	18.5	[41]	Derived from SSV1 virus with insertion of pBluescript II SK+. No selectable marker, transformants form viral plaques on lawn of sensitive cells.
pEXSs	Sso	SSV1	*hph*	6.4	[42]	Replicon based on SSV1 viral autonomously replicating sequence, copy number in *Sulfolobus solfataricus* increased by exposure of cells to

						mitomycin C or UV. Not widely used.
pEXADH	Sso	SSV1	*hph adh-hT*	7.7	[43]	Dual-marker plasmid derived from pEXSs.
pEX*lacOP*	Sso	SSV1	*hph lacS-lacTr*	11.7	[44]	Derived from pEXSs. Contains *lacTr-lacS* operon, requires *S. solfataricus* GθW Δ*lacS*-Δ*lacTr* host.
pSSV64	Sso	SSV1	*pyrEF*	20.1	[45]	Derived from pKMSD48, requires *S. solfataricus pyrE* or *pyrF* mutant host.
pJM03	Sso	SSV1	*pyrEF lacS*	21.8	[45]	Derived from pSSV64, requires *S. solfataricus lacS pyrE*/*F* host.
pNOB8::*lacS*	Sso	pNOB8	*lacS*	43	[46]	pNOB8 is self-spreading conjugative plasmid, selectable marker not necessary, *lacS* used as phenotypic marker. pNOB8 imposes severe growth defect on host strain.
pRN1-pUC18	Sso	pRN1	–	8.0	[47]	Used to develop transformation protocols for *S. solfataricus*.
pRN1::*lacS*	Sso	pRN1	*lacS*	7.1	[48]	*lacS* used as a phenotypic marker. pRN1-based plasmids not widely used.
pDMI1	Sac	23S rRNA intron	–	3.7	[49]	Replicon uses self-spreading mobile intron from 23SrRNA gene of *Desulfurococcus mobilis*. Selectable marker not necessary.
pCSV1	Pfu Sac	pGT5	–	6.1	[49]	pGT5 plasmid from *Pyrococcus furiosus*, replicates in both euryarchaeota and crenarchaeota.
pAG21	Pfu Sac	pGT5	*adh*	6.5	[50]	See also pAG1 and 2, less stable in *E. coli* than pAG21 due to high copy number.
pYS2	Pab	pGT5	*pyrE*	6.4	[51]	*pyrE* marker from *Sulfolobus acidocaldarius*. Requires *pyrE* mutant of *Pyrococcus abyssi* GE9 strain (devoid of pGT5).
pUDT2	Tko	–	*pyrF*	6.0	[52]	Plasmid for deletion of *trpE* gene from genome by double crossover. Used to develop gene knockout system for *Thermococcus kodakaraensis*.

Commonly used plasmid vectors for archaeal genetics

Host species abbreviations, see Table 1. Replicon not determined (ND); integrative vector (–). Markers, see Table 2.

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