REVIEW ARTICLE

Model organisms for genetics in the domain Archaea: methanogens, halophiles, *Thermococcales* and *Sulfolobales*

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Abstract

The tree of life is split into three main branches: eukaryotes, bacteria, and archaea. Our knowledge of eukaryotic and bacteria cell biology has been built on a foundation of studies in model organisms, using the complementary approaches of genetics and biochemistry. Archaea have led to some exciting discoveries in the field of biochemistry, but archaeal genetics has been slow to get off the ground, not least because these organisms inhabit some of the more inhospitable places on earth and are therefore believed to be difficult to culture. In fact, many species can be cultivated with relative ease and there has been tremendous progress in the development of genetic tools for both major archaeal phyla, the *Euryarchaeota* and the *Crenarchaeota*. There are several model organisms available for methanogens, halophiles, and thermophiles; in the latter group, there are genetic systems for *Sulfolobales* and *Thermococcales*. In this review, we present the advantages and disadvantages of working with each archaeal group, give an overview of their different genetic systems, and direct the neophyte archaeologist to the most appropriate model organism.

Introduction

In his '*An Essay on Man*', the English poet Alexander Pope exhorts us to 'Know then thyself, presume not God to scan; The proper study of Mankind is Man'. The resounding success of biomedical research using model organisms gives us reason to doubt the wisdom of Pope's words. Most of our knowledge of fundamental biological processes has come from work on simple and experimentally tractable species such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans*. Over time, these basic principles have been verified in complex species such as man, but simple model organisms remain vital to further medical discoveries (Fields & Johnston, 2005). Nevertheless, there is danger in such a reductionist approach – not everything in biology can be learned from *E. coli*. In order to witness the 'grandeur in this view of life' (apologies to Charles Darwin), we must expand our repertoire of model organisms to include representatives of the domain Archaea.

The choice of model organism is critically important. It should be easy to grow, have a short generation time, and be amenable to experimental manipulation. For microbial geneticists, the minimal specification is the ability to grow in isolation on solid media. Robert Koch first recognized that a colony formed on an agar plate represents the clonal expansion of a single cell, and this unassuming mound of cells has always been the cornerstone of microbiology. The ability to generate mutants is another part of the foundation of microbial genetics. Traditionally this was carried out by random mutagenesis (forward genetics), but since the molecular biology revolution the preferred method has been targeted mutation of a specific gene (reverse genetics). The latter would not be possible without methods for transformation, selectable markers, plasmid vectors, and systems for gene knockout by homologous or site-specific recombination. Of late, reverse genetics has been made significantly easier by whole genome sequencing, and is now taken for granted in the genetic toolbox.

Archaea make up one of the three main branches of the evolutionary tree; they are as different from eukaryotes as they are from bacteria (Garrett & Klenk, 2007). The distinct status of archaea was revealed in the late 1970s, when Carl

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Woese and colleagues seized upon the emerging technology of nucleic acid sequencing to tackle the problem of prokaryotic phylogeny. Woese chose small-subunit rRNA as a molecular chronometer; rRNA is an essential component of all self-replicating organisms and shows remarkable sequence conservation. The tree he constructed showed that an unusual group of methane-producing microorganisms were not bacteria, but formed a separate domain. Woese termed these organisms 'archaebacteria', but later changed this name to archaea (Woese & Fox, 1977; Woese *et al.*, 1990). The tree suggested a closer relationship between archaea and eukaryotes, compared with bacteria, and reflected the common heritage of information-processing systems found in the archaeo-eukaryal lineage. This had been noted by Wolfram Zillig and colleagues in the 1980s, when they found that archaeal DNA-dependent RNA polymerase is strikingly similar to its eukaryotic counterpart, in terms of both complexity and subunit composition (Huet *et al.*, 1983). Genome sequencing in the 1990s confirmed that archaea are a genetic mosaic – their information processing systems show significant homology to eukaryotic counterparts, while most operational (housekeeping) functions have a bacterial aspect (Olsen & Woese, 1996; Rivera *et al.*, 1998; Yutin *et al.*, 2008).

Over time, many halophilic and thermophilic microorganisms have found their home in the archaeal domain. Several of these species had been studied long before the third domain of life was proposed. For example, bacteriorhodopsin had been discovered in *Halobacterium salinarum* in 1971 (Oesterhelt & Stoeckenius, 1971), Thomas Brock had isolated *Sulfolobus acidocaldarius* from acidic mud ponds in Yellowstone National Park in 1972 (Brock *et al.*, 1972), and in the 18th century Alessandro Volta had unwittingly unearthed methanogenic archaea in the swamps of northern Italy. What appeared to bind together all these exotic microorganisms was their love of habitats that had previously been considered uninhabitable. However, cultivation-independent analyses of microbial biodiversity have since revealed that archaea are surprisingly abundant in 'normal' environments (DeLong, 1998; Karner *et al.*, 2001; Robertson *et al.*, 2005). Unfortunately these archaea are not fit for genetics, because with the exception of the recently isolated *Nitrosopumilus maritimus* (Konneke *et al.*, 2005), they cannot yet be cultured in isolation (Hugenholtz *et al.*, 1998; Schleper *et al.*, 2005). We are therefore left with four groups of archaea for which genetic systems have been developed: methanogens and halophiles (both euryarchaea), as well as thermophilic euryarchaea (*Thermococcales*) and crenarchaea (*Sulfolobales*) (Fig. 1).

Each of these archaeal groups has its own unique selling point. Haloarchaea are renowned for the comparative sophistication of their genetic systems, the development of which was made possible by early work on transformation protocols. In addition, haloarchaea are easy to cultivate because they grow at moderate temperatures. Methanogenic archaea are also mesophilic, but unlike haloarchaea, their cytoplasm is not hypersaline. This has permitted the direct adaptation of many tools from bacterial genetics to methanogens; bacterial antibiotics remain the exception, their targets are generally not found in archaea. Thermophilic archaea of kingdoms Euryarchaeota and Crenarchaeota have long been of interest to biochemists and structural biologists, owing to their thermostable enzymes. They offer significant potential for biotechnology, and for researchers wishing to use a multidisciplinary approach that combines genetics with biochemistry.

In this review, we offer guidance to microbiologists who wish to convert to the third domain, and reassure them that archaeal genetics is not difficult or unusual. The first step on this road is to choose the most appropriate model organisms. We deal in turn with each archaeal group, highlighting their advantages and disadvantages in terms of the scientific questions that can be addressed, and the tools available to answer these questions. Our hope is that more microbiologists will work on archaea, and those who already do so will venture beyond the safe environs of biochemistry and structural biology. Only when genetics has found a place in every archaeal laboratory will the third domain of life rank alongside its eukaryotic and bacterial cousins.

Methanogens

Introduction to methanogens, an ecologically and biochemically distinctive group

In 1977, a collaboration between the laboratories of Carl Woese and Ralph Wolfe resulted in the finding that the methanogens were 'only distantly related to typical bacteria' (Fox *et al.*, 1977). Thus, the methanogens became the first known Archaea. They are now known to comprise five orders of the *Euryarchaeota*: *Methanococcales*, *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales*, and *Methanopyrales* (Liu & Whitman, 2008). Genetic tools are available for certain species of the first two orders.

The methanogens are those organisms that generate methane as a catabolic end-product (Wolfe, 1996). Biological methanogenesis occurs in a variety of anaerobic habitats, including marine and freshwater sediments, rice paddies, bioreactors and sewage sludge digesters, landfills, animal digestive tracts, and hydrothermal vents (Wolfe, 1996). Most of these habitats contain an anaerobic ecosystem in which methanogenesis is the final step in the decomposition of organic matter. However, in habitats such as hydrothermal vents the substrates for methanogenesis, H_2 and CO_2, are presumably of geochemical origin. Much of the methane that is generated is reoxidized to CO_2 or becomes sequestered in

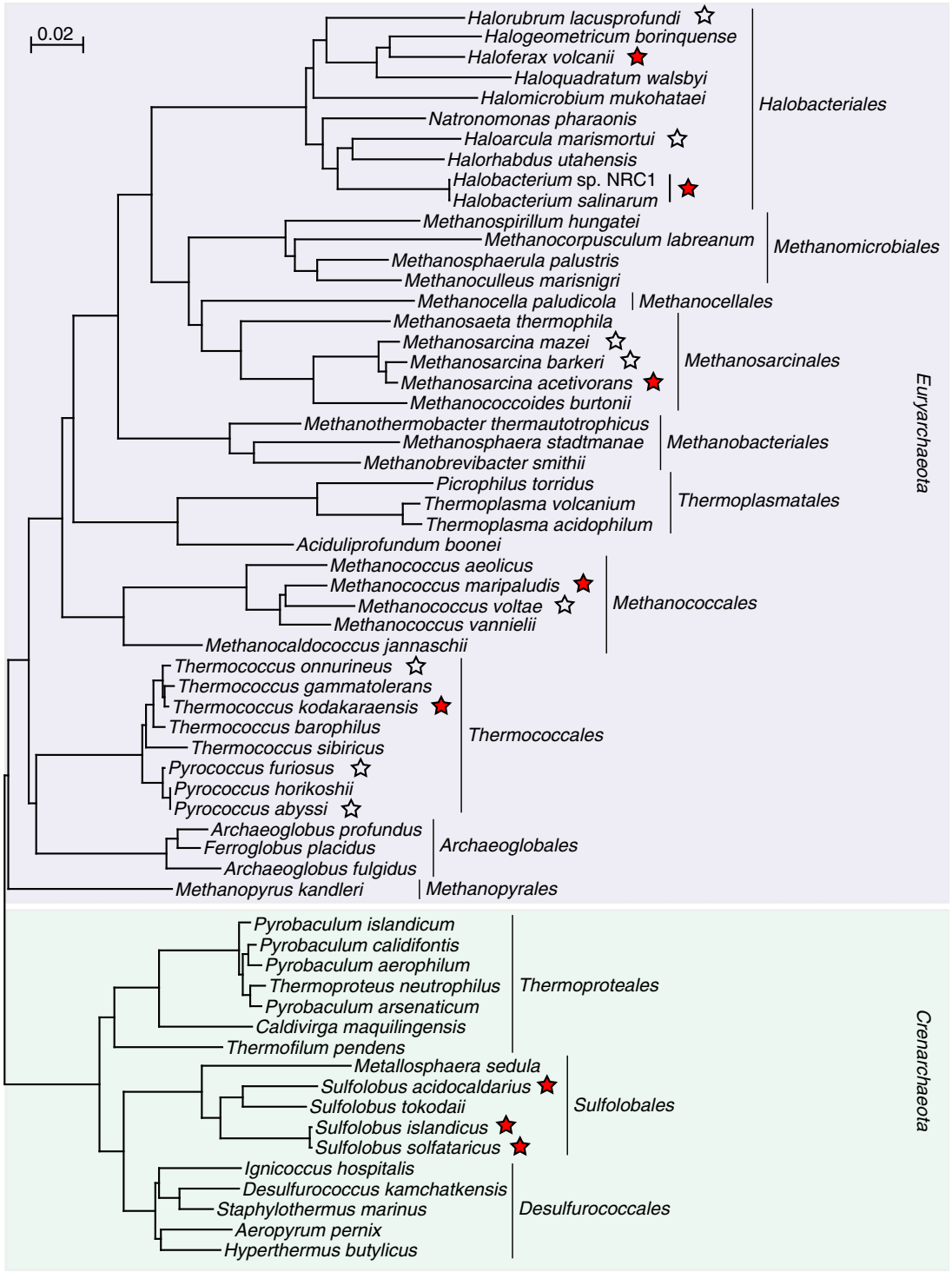

Fig. 1. Phylogenetic tree showing key archaeal species with genetic systems. Phylogenetic tree based on 16S rRNA gene sequences of selected archaeal species whose genome sequences are available. Organisms indicated with solid red stars are key species that have been the focus of genetic development; open stars indicate species where genetics has been applied or where there is potential for genetics. Sequence alignments were performed using Clustal W and the tree was constructed by neighbor joining; branches with bootstrap values of $< 50\%$ are not shown.

methane hydrates. However, a significant amount of methane is emitted into the atmosphere where it becomes a major greenhouse gas (Liu & Whitman, 2008). The product of methanogenesis is also of obvious importance as a fuel.

Methanogenesis is a kind of anaerobic respiration where single-carbon (C-1) units, most notably CO_2, serve as the electron acceptor. Thermodynamics dictates that methanogenesis will occur only when more favorable electron acceptors are absent. Hence, methanogenesis is most prevalent when sulfate, nitrate, oxidized metals, and, especially, oxygen are absent. Because CO_2 is the only electron acceptor that does not owe its abundance to photosynthesis, methanogenesis is favored as an early metabolism on earth, predating photosynthesis and other forms of respiration (Kasting & Siefert, 2002).

Substrates for methanogenesis are relatively restricted (Whitman *et al.*, 2001). Nearly all species in the orders *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, and *Methanopyrales* are hydrogenotrophic, using H_2 and CO_2. Many of these species can also use formate. In contrast, the *Methanosarcinales* is comprised of methylotrophic species, which use methyl compounds such as methanol and methylamines; some can use H_2 and CO_2 as well. In addition, *Methanosarcina* and *Methanosaeta*, members of the *Methanosarcinales*, use acetate. Most recognized species of methanogens are mesophilic, but hyperthermophilic and psychrotolerant species are also well known. To date, genetic tools have been developed only for certain mesophilic species.

The methanogens are biochemically distinctive. The enzymes and unique coenzymes of methanogenesis are known thanks largely to the work of Ralph Wolfe, Rolf Thauer, Godfried Vogels, and Gerhard Gottschalk. Our understanding of how methanogenesis is coupled to energy conservation has been slower to develop. As for all respirers, energy conservation is fundamentally chemiosmotic. A methyl transfer step plays a central role in most methanogenic pathways and directly drives the export of sodium ions. Other components of the energy conservation apparatus appear to differ in the methylotrophic and hydrogenotrophic methanogens. Methylotrophic methanogens have cytochromes and a proton-translocating electron transport chain, which they use to conserve energy in the last, exergonic step in methanogenesis. These components are lacking in hydrogenotrophic methanogens, making it unclear how these organisms achieve a net positive gain in energy conservation, because the first step in methanogenesis from CO_2 is endergonic. A recently proposed mechanism involving electron bifurcation, where exergonic electron flow directly drives endergonic electron flow, could explain this conundrum (Thauer *et al.*, 2008).

Why study methanogens?

The methanogenic pathway itself has captured the curiosity of many for decades. Recently genetic approaches have begun to fill some gaps in our knowledge left from biochemical approaches, as mentioned below. In addition, methanogens have been chosen for many studies of the molecular biology and physiology of Archaea. *Methanocaldococcus jannaschii* became the first species of Archaea to be subjected to genome sequencing in 1996 (Bult *et al.*, 1996), and many studies followed. Genetic tools have not been developed for *M. jannaschii*, but many questions can be addressed using the genetic tools for its relatives in the genus *Methanococcus* (Tumbula & Whitman, 1999). Methanogens are models for archaeal replication (Walters & Chong, 2009), transcription, regulation (Geiduschek & Ouhammouch, 2005), osmoregulation (Spanheimer & Muller, 2008), and protein structure. The role of methanogens in nature leads directly to questions of syntrophy, the associations between organisms that facilitate the transfer of nutrients (Shimoyama *et al.*, 2009). The discovery that close relatives of the *Methanosarcinales* as well as sulfate reducers are involved in anaerobic methane oxidation has broadened the importance of these organisms in the global carbon and sulfur cycles (Knittel & Boetius, 2009).

Methanogens are strict anaerobes that require special measures for their growth in the lab. However, in the late 1970s the relatively tricky Hungate technique was replaced with the technique of Balch and Wolfe (Balch *et al.*, 1979), and the requirements for anaerobiosis are easily achieved with a modest expenditure on equipment and minimal training.

Key species of methanogens that have genetic systems

Species for which genetic tools have been developed belong to the genera *Methanococcus* and *Methanosarcina*. Thus, there are genetic systems for representatives of the two metabolic types, hydrogenotrophic and methylotrophic methanogens. Both genera have well-developed tools, but each has its intrinsic advantages.

Methanococcus species grow relatively fast (doubling times around 2 h) and liquid cultures grow to high densities overnight. For *Methanococcus maripaludis*, colonies of useful size often form in 2 days after inoculation on agar plates. In addition, for *M. maripaludis* a robust system for continuous culture in chemostats has been established and used effectively in studies of global regulation (Haydock *et al.*, 2004; Hendrickson *et al.*, 2008). The genomes of *Methanococcus* species are small (1.6–1.8 Mbp), streamlining annotation and transcriptomic and proteomic analyses. Genome sequences for four species [*M. maripaludis* (four strains), *Methanococcus voltae*, *Methanococcus vannielii*, and *Methanococcus aeolicus*] are available currently. The relatively restricted substrate range for methanogenesis in hydrogenotrophic species limits the utility of genetics in *Methanococcus* to study methanogenesis itself. Nevertheless, genetics demonstrated the role *in vivo* of an alternative pathway for the

Table 1. Genetic tools for methanogens

	Methanococcus	*Methanosarcina*
Negative enrichment	Enrichment of auxotrophic mutants (Ladapo & Whitman, 1990)	
DNA delivery	PEG-mediated transformation (Tumbula *et al*., 1994), conjugation (Dodsworth *et al*., 2010)	Liposome-mediated transformation (Metcalf *et al*., 1997)
Replicative shuttle vectors	Gardner & Whitman (1999)	Metcalf *et al*. (1997)
Positive selection	Puromycin (Gernhardt *et al*., 1990), neomycin (Argyle *et al*., 1996)	Puromycin (Gernhardt *et al*., 1990), pseudomonic acid (Boccazzi *et al*., 2000)
Counterselection	*hpt* (8-azahypoxanthine), *upt* (6-azauracil) (Moore & Leigh, 2005)	*hpt* (8-aza-2,6-diaminopurine) (Pritchett *et al*., 2004)
Markerless genetic exchange (pop-in/pop-out gene replacement)	Moore & Leigh (2005)	(Pritchett *et al*., 2004)
Ectopic integration	Into *hpt* or *upt* (Moore & Leigh, 2005)	Enhanced with ϕC31 site-specific recombination system (Guss *et al*., 2008)
Transposon insertion	*In vitro* (Porat & Whitman, 2009)	*In vivo* (Zhang *et al*., 2000)
Reporter genes	*lacZ* (β-galactosidase) (Lie & Leigh, 2002), *uidA* (β-glucuronidase) (Beneke *et al*., 1995)	*uidA* (β-glucuronidase) (Pritchett *et al*., 2004)
Regulated gene expression	*nif* promoter (Lie & Leigh, 2002; Chaban *et al*., 2007)	Tetracycline-responsive promoters (Guss *et al*., 2008)

reduction of the electron carrier coenzyme F_{420} (Hendrickson & Leigh, 2008), and the role of the energy-conserving hydrogenase Ehb in carbon fixation (Porat *et al*., 2006).

Methanosarcina species grow more slowly (doubling times around 8 h), and the formation of colonies requires about 14 days of incubation. The genomes of *Methanosarcina* species are relatively large, ranging from 4.1 to 5.8 Mbp. Genome sequences for three species (*Methanosarcina acetivorans*, *Methanosarcina barkeri*, and *Methanosarcina mazei*) are available currently. Despite their slower growth, the metabolic versatility of *Methanosarcina* allows more possibilities for the study of the methanogenic pathway. For example, mutants in oxidation/reduction steps between the formyl and methyl levels lost the ability to grow on H_2 and CO_2 or methanol alone, but grew well on H_2 and methanol (Welander & Metcalf, 2008).

Genetic tools for methanogens

The basic elements of the genetic toolbox consist of a means of DNA delivery, selection for that DNA, and a way for the DNA to replicate. Reliable plating of single cells, which grow into clonal colonies, is also needed for mutant screening. In methanogens a genetic manipulation of this kind was first achieved in 1987 when Bertani (of Luria–Bertani medium fame) and Baresi transformed auxotrophs of *M. voltae* to prototrophy (Bertani & Baresi, 1987). Selection by antibiotic resistance was initiated when A. Klein transformed *M. voltae* by expressing a puromycin resistance marker from *Streptomyces* (Gernhardt *et al*., 1990). In addition, W. Whitman devised a strategy for the enrichment of auxotrophic mutants in *M. maripaludis* (Ladapo & Whitman, 1990). Since then, the genetic tools for methanogens have been expanded and improved. Genetics became feasible in *Methanosarcina*, which normally grows in multicellular packets, when conditions for growth as single cells were found (Sowers *et al*., 1993), and W. Metcalf documented the high-efficiency transformation of *Methanosarcina* using liposomes (Metcalf *et al*., 1997). Table 1 outlines the genetic tools available for methanogens. Most of these techniques were worked out for *M. maripaludis* and *M. acetivorans*, but many of them have also been applied successfully in *M. voltae*, *M. barkeri*, and *M. mazei*. It has been possible to adapt many tools from standard bacterial genetics to the methanogens because they grow at moderate temperatures and salt concentrations.

DNA delivery, positive selection, shuttle vectors, and insertional gene disruption

DNA is introduced into *Methanococcus* and *Methanosarcina* species by transformation and plating under anaerobic conditions. In *M. maripaludis* the polyethylene glycol (PEG)-mediated transformation of spheroplasts results in frequencies near 10^5 transformants μg^{-1} DNA and 10^{-5} transformants CFU^{-1}. In *M. acetivorans* a liposome-mediated method achieves higher frequencies, as high as 10^8 transformants μg^{-1} DNA and 20% of the CFU. In both cases, one easily obtains thousands of colonies in a single experiment. Puromycin transacetylase from *Streptomyces* works well (Gernhardt *et al*., 1990), and selection in both genera is most commonly achieved using puromycin. In *M. maripaludis*, neomycin resistance is also achieved using aminoglycoside phosphotransferase genes (Argyle *et al*., 1996). Replicative shuttle vectors have been devised for both

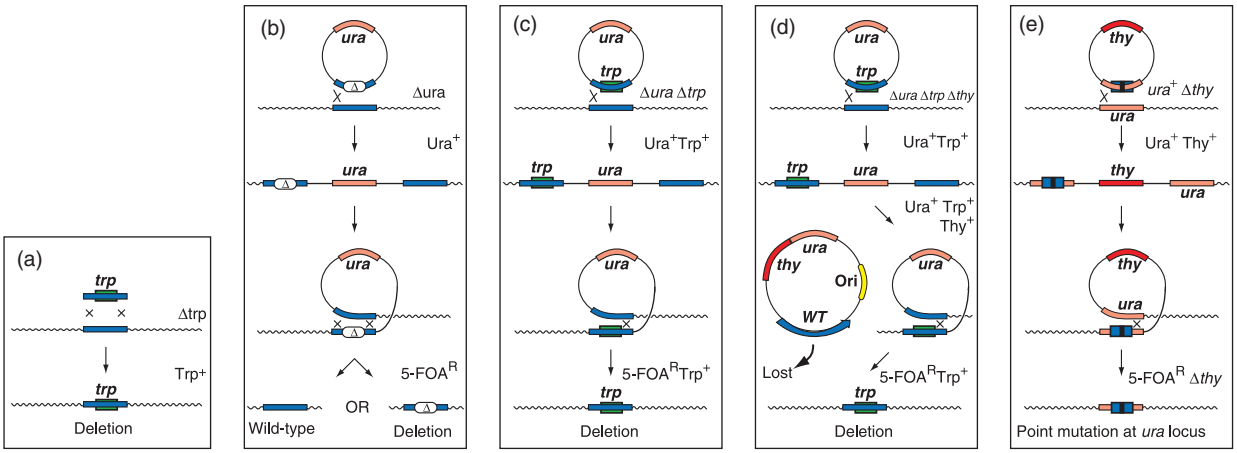

Fig. 2. Gene knockout methods used in archaeal genetics. Specific details (such as selectable markers) are illustrative and do not necessarily apply for all archaeal groups. See text for further details. (a) Gene replacement with selectable marker (in this case *trp*), by recombination between flanking regions of the gene and a chromosomal target, uses linear DNA. (b) Pop-in/pop-out deletion method uses circular DNA. Integration of the deletion construct (pop-in) is selected by transformation to uracil prototrophy. Intramolecular recombinants that have lost the plasmid (pop-out) are counterselected using 5-FOA. In methanogens, two different markers are used for selection and counterselection, respectively. (c) Variant of the pop-in/pop-out method for gene deletion, where the gene is replaced with a marker allowing direct selection (in this case *trp*). Used for deletion of genes that are important for cell viability. (d) Refinement of the pop-in/pop-out gene replacement method, where the gene function is complemented *in trans* from a shuttle vector. Loss of the shuttle vector (plasmid shuffling) and gene deletion is ensured by counterselection with 5-FOA. (e) Ectopic integration at the *ura* locus. Pop-in of a construct bearing the point mutation (of experimental gene, not *ura*) is selected by transformation to thymidine prototrophy. Counterselection with 5-FOA ensures that the *ura* gene is replaced with the point mutation.

genera, using replicative elements from naturally occurring plasmids of strains of each genus. DNA lacking autonomous replication can integrate into the chromosome via homologous recombination, which appears to occur at a particularly high frequency in *M. maripaludis* (Tumbula *et al.*, 1997). This allows for gene replacement and disruption by insertion of the selectable marker (Fig. 2a), and other genetic manipulations described below. In addition to transformation, a conjugation system from *E. coli* to *M. maripaludis* has been described recently (Dodsworth *et al.*, 2010). It is less laborious than transformation and may be useful for routine genetic manipulation of this methanogen.

Markerless genetic exchange

In both *Methanococcus* and *Methanosarcina*, systems have also been devised for markerless genetic exchange. Using these systems, mutations, including in-frame deletions, can be made in which the selectable marker is removed, allowing its re-use for subsequent manipulations. Vectors for this purpose contain both selectable and counterselectable markers. *hpt* and *upt*, encoding hypoxanthine and uracil phosphoribosyltransferase, respectively, allow for counterselection in the presence of nucleobase analogs in genetic backgrounds from which these genes have been deleted. In this approach, often termed pop-in/pop-out gene replacement, the construct to be exchanged into the genome is cloned with homologous flanking DNA on both sides (Fig. 2b). After transformation, positive selection results in a merodiploid in which integration of the entire plasmid has occurred in a single recombination event. Counterselection results in a second recombination event, removing the vector. Because the second recombination event can occur on the same or the opposite side from the first, the result is either a wild type or a mutant locus, which must be distinguished by screening. For *Methanosarcina*, vectors have been equipped with recognition sites for the Flp site-specific recombination system, allowing more expedient removal of the marker (Rother & Metcalf, 2005).

Ectopic integration

Integration of constructs into the genome is desirable not only for gene disruption or for modification but also in cases where artifacts due to multiple copies on a plasmid are to be avoided. In *M. maripaludis* this has been achieved by ectopic incorporation of constructs into the sites of the counterselectable genes *hpt* or *upt* (Fig. 2e) (Moore & Leigh, 2005). For *Methanosarcina* a system has been devised that uses the ϕC31 site-specific recombination system to considerably increase the efficiency of integration (Guss *et al.*, 2008).

Overexpression and controlled expression

Replicative vectors for *Methanococcus* and *Methanosarcina* are equipped with strong promoters that allow overexpression of genes. These vectors have been used to overexpress

His-tagged proteins in their native species for subsequent purification (Dodsworth & Leigh, 2006). A tetracycline-inducible promoter has been constructed for *Methanosarcina* by combining a strong promoter from *M. barkeri* with binding sites for the bacterial TetR protein, and used in a test for gene essentiality (Guss *et al.*, 2008). This system also has promise for the induction of gene expression for the purpose of protein production and purification. Attempts to adapt the tetracycline induction system for *Methanococcus* have not been successful. However, the *nif* (nitrogen fixation) promoter has been used in *M. maripaludis* for differential controlled expression (Lie & Leigh, 2002; Chaban *et al.*, 2007) and has potential application in tests for gene essentiality.

Transposon insertion

In vivo transposon insertion has been devised for *M. acetivorans*. The transposon system is derived from a mini-mariner element and inserts randomly into the genome at high frequency. The transposon contains selectable markers for *E. coli* as well as for *Methanosarcina*, and contains an *E. coli* origin of replication, facilitating cloning of the transposon insertion sites (Zhang *et al.*, 2000). This system works in *M. maripaludis* only at low frequency. However, transposition *in vitro* (Porat & Whitman, 2009) and into an *E. coli* λ lysate (Blank *et al.*, 1995) have been used successfully to generate insertions in a cloned *M. maripaludis* gene cluster, followed by transformation into *M. maripaludis*. These approaches have potential for development into an efficient transposon insertion system for *Methanococcus*.

Reporter genes

Genes used in reporter gene fusions are *uidA* (encoding β-glucuronidase) for *Methanosarcina* (Pritchett *et al.*, 2004) and *uidA* (Beneke *et al.*, 1995) and *lacZ* (β-galactosidase) (Lie & Leigh, 2002) for *Methanococcus*. Most applications measure reporter enzyme activity in cell extracts. The use of reporter genes for *in vivo* screening is more limited, because color development requires oxygen. However, *M. maripaludis* colonies have been exposed to air and sprayed with X-gal. Color development occurs before loss of viability, and colonies can be returned to anaerobic conditions and picked. This approach was used to identify super-repressor variants of the transcriptional repressor NrpR (Lie & Leigh, 2007).

Discoveries and recent progress

Genetic approaches have been particularly useful in *Methanosarcina* for filling gaps in our knowledge of the methanogenic pathway. For example, the energy-conserving hydrogenase Ech was shown to be required for methanogenesis and carbon fixation (Meuer *et al.*, 2002) in *Methanosarcina*. In another study, hydrogen cycling, a fundamental strategy in chemiosmotic energy conservation, was shown to occur (Kulkarni *et al.*, 2009). A striking feature in *Methanosarcina* is that the methylamine methyltransferases contain pyrrolysine, the 22nd genetically encoded amino acid. Genetic studies have helped determine the function of a dedicated tRNA and aminoacyl-tRNA synthetase in pyrrolysyl-tRNA synthesis (Mahapatra *et al.*, 2006, 2007).

Genetic studies in *Methanococcus* have addressed a wide range of questions. A number of regulatory mechanisms have been studied, and regulatory factors have been identified that govern the expression of genes for hydrogen metabolism (Sun & Klein, 2004) and nitrogen assimilation (Lie & Leigh, 2003; Lie *et al.*, 2005). A novel mechanism for the regulation of nitrogenase activity was discovered in *M. maripaludis*, and evidently exists in a variety of diazotrophic Archaea and Bacteria (Dodsworth *et al.*, 2005; Dodsworth & Leigh, 2006). Genetic studies in *M. maripaludis* have led to the identification of components of the archaeal flagellum system (Chaban *et al.*, 2007), tRNA-dependent cysteine biosynthesis (Stathopoulos *et al.*, 2001; Sauerwald *et al.*, 2005), and requirements for selenocysteine biosynthesis (Rother *et al.*, 2001; Yuan *et al.*, 2006; Stock *et al.*, 2010).

Numerous studies of global regulation at the transcriptomic and proteomic levels have been carried out with *Methanococcus* and *Methanosarcina* species, showing the global responses to alternative substrates, salt stress, and availabilities of nutrients including hydrogen and nitrogen (Hovey *et al.*, 2005; Li *et al.*, 2005a, b; Lessner *et al.*, 2006; Veit *et al.*, 2006; Xia *et al.*, 2006, 2009; Hendrickson *et al.*, 2007, 2008; Pfluger *et al.*, 2007; Jager *et al.*, 2009).

Halophiles

Introduction to haloarchaea, the heterotrophic, aerobic halophiles of the *Euryarchaeota*

Halophilic archaea inhabit the most saline environments on earth, including solar salterns and natural salt lakes. Like many other habitats where archaea are found, salt lakes were once thought devoid of life. In 1936, Benjamin Elazari-Volcani published the first report of microbial life in the Dead Sea (Elazari-Volcani, 1936). His work was commemorated by the naming of *Haloferax volcanii*, which was isolated from Dead Sea mud in 1977 (Mullakhanbhai & Larsen, 1975). In fact, the discovery of halophilic archaea predates the proposal of the domain Archaea by Carl Woese in the late 1970s (Woese & Fox, 1977; Woese *et al.*, 1990). For instance, *H. salinarum* was unwittingly discovered in 1922 as a red discoloration of salted fish (Harrison & Kennedy, 1922). All halophilic archaea are members of the *Euryarchaeota* and somewhat confusingly, the family *Halobacteriaceae* (Oren *et al.*, 2009). In this review, we will use

instead the term haloarchaea unless when referring to the genus *Halobacterium*.

Archaea are not alone in hypersaline environments; they share this habitat with bacteria, fungi and algae. In contrast to most halophilic bacteria and eukaryotes, haloarchaea maintain an osmotic balance with their medium by accumulating equimolar salt concentrations in the cytoplasm (Christian & Waltho, 1962; Oren, 2008). This 'salt-in' strategy predominantly uses potassium because it attracts less water than sodium. The converse 'salt-out' strategy favored by halotolerant bacteria excludes salt from the cytoplasm and uses organic solutes such as glycerol or glycine betaine to maintain an osmotic balance. The salt-out strategy is energetically costly and less suitable at saturating salt concentrations, which is probably why haloarchaea predominate under hypersaline conditions (Oren, 1999). There are notable exceptions: the bacterium *Salinibacter ruber* uses the archaeal salt-in strategy and coexists with haloarchaea at near-saturating salt concentrations (Oren *et al.*, 2002).

Because of the salt-in strategy, haloarchaeal proteins are adapted to function in molar salt concentrations and commonly denature in low-salt solutions. The adaptation to salt relies on several different strategies (Lanyi, 1974; Mevarech *et al.*, 2000).

- A reduction in overall hydrophobicity, by replacing large hydrophobic residues on the protein surface with small hydrophilic residues. This strategy is used by the dihydrofolate reductase of *Hfx. volcanii*, which requires higher salt concentrations for correct folding than the *E. coli* enzyme (Wright *et al.*, 2002).
- An increase in acidic residues. A high density of negative charges coordinates a network of hydrated cations, which maintain the protein in solution (Lanyi, 1974). For example, glucose dehydrogenase of *Haloferax mediterranei* is very acidic (Britton *et al.*, 2006), and the median pI of the *Hbt. salinarum* proteome is predicted to be 4.9 (Kennedy *et al.*, 2001). By altering the total charge, it is possible to interconvert halophilic and mesophilic forms of a protein (Tadeo *et al.*, 2009).
- An additional domain that is not found in mesohalic proteins, as seen in the ferredoxin of *Hbt. salinarum* (Marg *et al.*, 2005). The latter features a 30-amino acid insertion near its N-terminus, which is extremely rich in acidic amino acids and is essential for correct protein folding at high salt concentrations.

Besides their adaptation to salt, haloarchaea have other characteristic features. They are aerobic heterotrophs, some of which have the potential for anaerobic growth (Falb *et al.*, 2008). They are slightly thermophilic with an optimum temperature of 40–50 °C, can withstand up to 60 °C, and grow reasonably well at 37 °C (Robinson *et al.*, 2005). Even *Halorubrum lacusprofundi*, which was isolated in Antarctica, grows best at 36 °C. Haloarchaea are generally pigmented with C-50 bacterioruberins and some species contain retinal proteins such as bacteriorhodopsin. It is unlikely that these pigments play a significant role in protection against UV. While *Hbt. salinarum* can withstand very high doses of UV (McCready & Marcello, 2003), other pigmented species such as *Hfx. volcanii* are no more resistant than the model bacterium *E. coli* (Delmas *et al.*, 2009).

To date, 12 haloarchaeal genomes have been sequenced [*Halalkalicoccus jeotgali* B3(T), *Haloarcula marismortui* ATCC 43049, *Halobacterium* sp. NRC-1, *Hfx. volcanii* DS2, *Halogeometricum borinquense* DSM 11551, *Halomicrobium mukohataei* DSM 12286, *Haloquadratum walsbyi* DSM 16790, *Halorhabdus utahensis* DSM 12940, *Halorubrum lacusprofundi* ATCC 49239, *Haloterrigena turkmenica* DSM 5511, *Natronomonas pharaonis* DSM 2160, *Natrialba magadii* ATCC 43099] and many more are underway. They usually consist of one main chromosome and a number of megaplasmids (Pfeiffer *et al.*, 2008a; Soppa *et al.*, 2008). Structural differences in their respective megaplasmids underlie the distinction between *Hbt. salinarum* and *Halobacterium* sp. NRC-1, but they are essentially the same species (we refer to both as *Hbt. salinarum*) (Ng *et al.*, 2000; Pfeiffer *et al.*, 2008b). Polyploidy is a signature of haloarchaea; there are 15–30 genome copies in *Hfx. volcanii* and *Hbt. salinarum* (Breuert *et al.*, 2006). Haloarchaeal genomes are characterized by a high G+C content (~65%) (Soppa *et al.*, 2008); the one known exception is *Haloquadratum walsbyi* (45% G+C) (Bolhuis *et al.*, 2006). It is often stated that the high G+C content is linked to the acidic proteome of haloarchaea (average pI of ~4.4), but it is more probably due to evasion of insertion sequence (IS) elements that target A+T-rich sequences (Pfeifer & Betlach, 1985; Cohen *et al.*, 1992; Hartman *et al.*, 2010). Interestingly, IS elements in *H. walsbyi* have a higher GC content than the rest of the genome (Bolhuis *et al.*, 2006). While all halophiles are infested with IS elements (Brugger *et al.*, 2002), their activity varies between species. *Halobacterium salinarum* exhibits genome instability due to frequent IS-mediated rearrangements (Sapienza *et al.*, 1982; Simsek *et al.*, 1982), but this is much less of a problem in *Hfx. volcanii*, where IS elements are confined to nonessential regions on the megaplasmids (Cohen *et al.*, 1992; Lopez-Garcia *et al.*, 1995).

In common with methanogens, the genomes of haloarchaea encode multiple isoforms of genes that are present as a single copy in other organisms. For instance, there are 16 *orc1/cdc6* genes for the DNA replication initiator in *Hfx. volcanii* and 10 in *Hbt. salinarum* (Berquist *et al.*, 2007; Norais *et al.*, 2007; Hartman *et al.*, 2010). This might be due to a requirement for regulatory and metabolic flexibility in haloarchaea (Facciotti *et al.*, 2007), but it is also possible that these redundant homologs have accumulated as a result of lateral gene transfer (LGT). There is ample evidence for large-scale LGT, often with bacteria, and it has been proposed that haloarchaea originally descended from methanogens that had acquired the genes for aerobic respiration

from bacteria (Boucher *et al.*, 2003). Underpinning this LGT is a system for mating and gene transfer in *Hfx. volcanii* (Rosenshine *et al.*, 1989) and a wide variety of haloarchaeal viruses (Dyall-Smith *et al.*, 2003).

Why study halophiles?

A significant motivation for working on haloarchaea is the sophistication of their genetic systems. They are easy to cultivate in the laboratory, have fast growth kinetics (Robinson *et al.*, 2005), and are resistant to contamination by nonhalophilic microorganisms (of course, cross-contamination of haloarchaeal strains within the laboratory remains a constant threat). Halophiles were the first archaeal group in which routine transformation with foreign DNA was possible (Charlebois *et al.*, 1987; Cline *et al.*, 1989). The ease, efficiency, and broad applicability of PEG-mediated transformation has ensured that haloarchaea have remained at the forefront of genetic tool development (Soppa, 2006). Perhaps the greatest testament to the popularity of haloarchaea is the Halohandbook, an invaluable compendium of methods for working with halophiles, which is diligently curated by Mike Dyall-Smith (Dyall-Smith, 2009).

Besides sophisticated genetics, there are many other reasons for working on halophiles. *Haloferax volcanii* and *Hbt. salinarum* can grow over a range of salinities and have been exploited to uncover genes involved in osmotic stress (Bidle *et al.*, 2007, 2008; Coker *et al.*, 2007). Because halophilic proteins function under conditions of low water availability, they offer distinct advantages for structural biology and biotechnology. For example, the structure of the ribosome was solved using the complex from *Har. marismortui*, leading to the Nobel Prize for Chemistry in 2009 (Ban *et al.*, 2000). In biotechnology, there are few success stories that can match bacteriorhodopsin. This purple membrane protein from *Hbt. salinarum* was identified by Walther Stoeckenius in 1971 (Oesterhelt & Stoeckenius, 1971) and has been used in countless photochemical applications (Margesin & Schinner, 2001; Oren, 2010).

Haloarchaea are an excellent choice to address the 'prokaryotic species question': do prokaryotic organisms form genomic and phenomic clusters that are sufficiently cohesive that we might legitimately call them species (Doolittle & Zhaxybayeva, 2009)? Haloarchaea are physiologically diverse and inhabit distinct ecological niches (Oren, 2008), they have dynamic genomes with systems for gene exchange and show evidence for LGT. Work on isolates of the genus *Halorubrum* from solar salterns and natural salt lakes has shown that haloarchaea exchange genetic information promiscuously, leading to the suggestion that there is no nonarbitrary way to define a prokaryotic species (Papke *et al.*, 2004, 2007). Genetics has been used to answer the question of whether genes acquired by LGT can supplant an endogenous function. The UvrABC complex, which is of bacterial origin, is functional in the repair of UV-induced DNA damage in both *Hbt. salinarum* and *Hfx. volcanii* (Crowley *et al.*, 2006; Lestini *et al.*, 2010).

Key species of halophiles that have genetic systems

There are two haloarchaeal model organisms: *Hbt. salinarum* and *Hfx. volcanii*. Both species have mature genetic systems, but each has intrinsic advantages. *Halobacterium salinarum* is the traditional choice for haloarchaeal cell biology; it was isolated many years ago and quickly became a popular choice owing to its purple membrane protein, bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971; Margesin & Schinner, 2001). The genome of *Hbt. salinarum* NRC-1 was published in 2000 (Ng *et al.*, 2000), a second annotation with a different assembly of the megaplasmids was published for *Hbt. salinarum* R1 in 2008 (Pfeiffer *et al.*, 2008b). The first archaeal method for gene knockout using a counterselectable marker was also published in 2000, using the *ura3* gene of *Hbt. salinarum* (Peck *et al.*, 2000) (Fig. 2). Availability of the genome sequence coincided with the growth of transcriptomics, and consequently *Hbt. salinarum* has been a favorite model for systems biology (DasSarma *et al.*, 2006). DNA repair has been a fruitful topic, because *Hbt. salinarum* is extremely resistant to UV and ionizing radiation (Baliga *et al.*, 2004; Whitehead *et al.*, 2006). However, for those wishing to carry out traditional genetics, *Hbt. salinarum* is perhaps not ideal. It grows slowly, its genome is unstable due to frequent IS-mediated rearrangements (Sapienza *et al.*, 1982; Simsek *et al.*, 1982), and the range of selectable markers is somewhat limited.

Haloferax volcanii is better suited to traditional genetics. It has a generation time of $\sim$2 h (Robinson *et al.*, 2005), its genome is stable (Lopez-Garcia *et al.*, 1995), and it grows on synthetic media (Mevarech & Werczberger, 1985). PEG-mediated transformation protocols were originally developed for *Hfx. volcanii* (Charlebois *et al.*, 1987; Cline *et al.*, 1989), and methods for gene knockout have incorporated additional selectable markers (Bitan-Banin *et al.*, 2003; Allers *et al.*, 2004), thus facilitating the construction of multiply-mutated cells (Fig. 2). A Gateway system for deletion construction (El Yacoubi *et al.*, 2009), several reporter genes (Holmes & Dyall-Smith, 2000; Reuter & Maupin-Furlow, 2004), an inducible promoter (Large *et al.*, 2007), and a system for protein overexpression (Allers *et al.*, 2010) have been developed in the last decade. *Haloferax volcanii* has a long history of genome research going back to 1991, when a physical map of overlapping genomic clones was published (Charlebois *et al.*, 1991). Publication of the annotated genome sequence in 2010 (Hartman *et al.*, 2010) has spurred the development of whole-genome microarrays (S. Chimileski & T. Papke, pers. commun.), which will allow faster

data analysis than the shotgun DNA microarrays available previously (Zaigler *et al.*, 2003).

Rudimentary genetic systems are available for other haloarchaea. *Haloarcula marismortui* can be transformed with shuttle plasmids from *Hbt. salinarum* and *Hfx. volcanii* (Cline & Doolittle, 1992) and gene replacement studies have been used to investigate the *Har. marismortui* ribosome (Tu *et al.*, 2005). However, this species harbors restriction/modification systems that reduce the efficiency of transformation by 10^4-fold (Cline & Doolittle, 1992). Systems for *Halorubrum* are currently under development (S. Chimileski & T. Papke, pers. commun.). *Haloferax mediterranei* is closely related to *Hfx. volcanii*; they differ in that *Hfx. mediterranei* produces gas vesicles. These gas-filled proteinaceous particles are used by cells to increase their buoyancy and float to the surface of the brine; they have been studied intensively by the laboratory of Felicitas Pfeifer, often using *Hfx. volcanii* as a heterologous host (Pfeifer *et al.*, 2002; Hechler & Pfeifer, 2009). This is one of the great strengths of haloarchaeal genetics: because there are several organisms with mature genetic systems, complementation by genes from a related species can be used to identify random mutations and thereby isolate novel enzymes. This approach was used to find a novel thymidylate synthase and an alternative pathway for reduced folate biosynthesis in *Hbt. salinarum*, using genes from *Hfx. volcanii* (Giladi *et al.*, 2002; Levin *et al.*, 2004). More recently, the essential *pitA* gene of *Hfx. volcanii* was replaced by an ortholog from the haloalkaliphile *Natronomonas pharaonis*; the latter lacks the histidine-rich linker region found in *Hfx. volcanii* PitA and does not copurify with His-tagged recombinant proteins (Allers *et al.*, 2010).

Genetic tools for halophiles

Transformation

Modern genetics takes for granted a method for introducing DNA into cells, and a means to select for cells that have taken up the DNA. The development of transformation protocols is intimately linked with selectable markers. This was an acute problem in the early days of archaeal genetics, because bacterial antibiotics are largely ineffective against archaea (Hilpert *et al.*, 1981). Cline and Doolittle overcame this hurdle by assaying for transfection of *Hbt. salinarum* with naked DNA from halovirus ΦH, and scoring for plaques on a lawn of cells (Cline & Doolittle, 1987). This allowed them to develop the PEG transformation protocol that is used today (Cline *et al.*, 1989): the glycoprotein cell surface layer, which depends on Mg^{2+}, is removed by treatment with EDTA and DNA is introduced into spheroplasts using PEG 600, after which cells recover in rich broth before plating on selective medium. This protocol yields up to 10^7 transformants μg^{-1} DNA, depending on restriction/modification systems. *Haloferax volcanii* has two such systems: one that targets unmethylated 5′-CTAG-3′ sites (Charlebois *et al.*, 1987) (T. Allers, unpublished data) and another that restricts methylated 5′-G^{me}ATC-3′ DNA (Holmes & Dyall-Smith, 1991). The latter results in a 10^2-fold drop in transformation efficiency and has been circumvented by passaging DNA through an *E. coli dam* mutant, which lacks the methylase that modifies 5′-GATC-3′ sites. This is no longer necessary, because an*Δmrr* mutant of *Hfx. volcanii* was recently shown to lack the restriction enzyme that targets 5′-G^{me}ATC-3′ DNA (Allers *et al.*, 2010).

Shuttle vectors

Many replicative shuttle vectors have been developed, using origins of DNA replication taken from indigenous haloarchaeal plasmids (Table 2). For *Hbt. salinarum* there are plasmids based on pGRB1, pHH1, and pNRC100 origins (Blaseio & Pfeifer, 1990; Krebs *et al.*, 1991; DasSarma, 1995), while for *Hfx. volcanii* there are vectors based on pHK2, pHV2, and pHV1/4 origins (Lam & Doolittle, 1989; Holmes *et al.*, 1994; Allers *et al.*, 2004; Norais *et al.*, 2007). Some of these origins are broad range: for example, pHV2-based vectors replicate in both species (Blaseio & Pfeifer, 1990). Interestingly, pHV2-based plasmids do not function in *Hfx. volcanii* mutants deficient in the RadA recombinase, where pHK2-based vectors are used instead (Woods & Dyall-Smith, 1997). For a comprehensive list of plasmid vectors, see Allers & Mevarech (2005) or Berquist *et al.* (2006).

Selectable markers

Bacterial antibiotics that are safe to use in eukaryotes are largely ineffective against archaea, because the targets of these drugs (e.g. peptidoglycan cell walls) are not encountered in archaeal or eukaryotic cells. There are some exceptions (Hilpert *et al.*, 1981); they have been exploited to develop selectable markers for haloarchaeal genetics. Novobiocin is an inhibitor of DNA gyrase (*gyrB*), an essential enzyme found in both bacteria and archaea, and a resistant form of the *gyrB* gene was isolated from *Haloferax* strain Aa2.2 (Holmes & Dyall-Smith, 1991). Novobiocin has since become the most widely used haloarchaeal antibiotic. An alternative is mevinolin (simvastatin), which inhibits HMG-CoA reductase. In humans, it is prescribed as a cholesterol-lowering drug, while in archaea it inhibits membrane synthesis. A mutant allele of the *Hfx. volcanii hmgA* gene that leads to overexpression of the enzyme has been harnessed as a mevinolin-resistant marker for both *Hfx. volcanii* and *Hbt. salinarum* (Blaseio & Pfeifer, 1990; Lam & Doolittle, 1992).

The last decade has seen a shift away from antibiotics and towards auxotrophic selectable markers, where genes involved in amino acid or nucleotide biosynthesis are used to complement chromosomal mutations. Deletion of the gene

Table 2. Genetic tools for haloarchaea

	Hbt. salinarum	*Hfx. volcanii*
Synthetic media	No, lacks biosynthetic capability for five amino acids (Falb *et al*., 2008)	Yes (Mevarech & Werczberger, 1985)
DNA delivery	PEG-mediated transformation (Cline *et al*., 1989)	PEG-mediated transformation (Cline *et al*., 1989)
Restriction barrier	Some restriction of methylated CTTCCT DNA*, lacking in some strains (Schinzel & Burger, 1986; Blaseio & Pfeifer, 1990)	Severe restriction of $G^{me}ATC$ DNA, eliminated in*mrr* mutant (Holmes & Dyall-Smith, 1991; Allers *et al*., 2010)
Replicative shuttle vectors	Based on pGRB1, pHH1 and pNRC100 origins (Blaseio & Pfeifer, 1990; Krebs *et al*., 1991; DasSarma, 1995; Berquist *et al*., 2006)	Based on pHK2, pHV2 and pHV1/4 origins (Lam & Doolittle, 1989; Holmes *et al*., 1994; Allers *et al*., 2004; Allers & Mevarech, 2005; Norais *et al*., 2007)
Positive selection	Mevinolin, novobiocin, uracil (*ura3*) (Blaseio & Pfeifer, 1990; Holmes & Dyall-Smith, 1991; Peck *et al*., 2000)	Mevinolin, novobiocin, uracil (*pyrE2*), leucine (*leuB*), thymidine (*hdrB*), tryptophan (*trpA*) (Lam & Doolittle, 1989; Holmes & Dyall-Smith, 1991; Bitan-Banin *et al*., 2003; Allers *et al*., 2004). Also histidine (*hisC*) and methionine (*metX*) (M. Mevarech, pers. commun.)
Counterselection	5-FOA (*ura3*) (Peck *et al*., 2000)	5-FOA (*pyrE2*) (Bitan-Banin *et al*., 2003; Allers *et al*., 2004)
Random mutagenesis	Using UV and X-rays (Soppa & Oesterhelt, 1989)	Using ethyl methanesulphonate (EMS) (Mevarech & Werczberger, 1985)
Negative enrichment	Using 5-bromo-2′-deoxyuridine (BrdU) (Soppa & Oesterhelt, 1989)	Using 5-bromo-2′-deoxyuridine (BrdU) (Soppa & Oesterhelt, 1989; Wanner & Soppa, 1999)
Markerless gene knockout or replacement	Using *ura3* (Peck *et al*., 2000)	Using *pyrE2* (Bitan-Banin *et al*., 2003; Allers *et al*., 2004). Gateway system available (El Yacoubi *et al*., 2009)
Ectopic integration	At *ura3* (Peck *et al*., 2000)	At *pyrE2* (T. Allers, unpublished data)
Natural genetic exchange	Not observed (Tchelet & Mevarech, 1994)	Involves cell-cell contact (Rosenshine *et al*., 1989)
Reporter genes	β-Galactosidase (*bgaH*) and GFP (Nomura & Harada, 1998; Patenge *et al*., 2000)	β-Galactosidase (*bgaH*) and GFP (Holmes & Dyall-Smith, 2000; Reuter & Maupin-Furlow, 2004)
Regulated gene expression	No	Tryptophan-inducible p.*tnaA* promoter (Large *et al*., 2007)
Protein overexpression	pBBEV1 expression plasmid with constitutive *bop* promoter (Berquist *et al*., 2006), pNBPA expression plasmid with constitutive *fdx* promoter (Facciotti *et al*., 2007)	$pitA_{Nph}$ gene replacement strain H1209 and tryptophan-inducible expression plasmid pTA963, for His-tagged proteins (Allers *et al*., 2010)

*M. Dyall-Smith, pers. commun.

circumvents the instability that results from homologous recombination of the marker with a chromosomal allele; this is a problem for antibiotics, because both novobiocin- and mevinolin-resistance markers have homology to essential chromosomal genes. The first auxotrophic marker to be developed was the *ura3* gene for uracil biosynthesis in *Hfx. salinarum* (Peck *et al*., 2000); it was followed by a similar system based on the *pyrE2* gene of *Hfx. volcanii* (Bitan-Banin *et al*., 2003). These markers are particularly useful for gene knockout or replacement (Fig. 2b), because they can be counterselected using 5-fluoroorotic acid (5-FOA). Integration of a deletion construct is selected by transformation to uracil prototrophy and loss of the construct (resulting in gene deletion) is counterselected with 5-FOA, which is converted to toxic 5-fluorouracil in ura^+ (but not ura^-) cells. Selection for 5-FOA-resistance is also used to bring about ectopic integration, where a gene construct is used to replace the *ura3* or *pyrE2* gene (Fig. 2e).

In *Hfx. volcanii* there are five additional auxotrophic markers. The *trpA* gene for tryptophan and *leuB* gene for leucine biosynthesis are often used in conjunction with *pyrE2* (Allers *et al*., 2004), for gene replacement with a selectable marker (Fig. 2c). This has proved essential for the construction of some mutants, where the gene is important for cell viability and therefore difficult to delete. The *hdrB* marker for thymidine biosynthesis is well suited for shuttle plasmids, because it allows them to be maintained in rich media based on yeast extract (which lacks thymidine) (Allers *et al*., 2004). For example, a shuttle vector with the *hdrB* marker was used for *in trans* complementation of *radA* (Fig. 2d) to generate a *radA mre11 rad50* mutant (Delmas *et al*., 2009). This approach, known as plasmid shuffling, was also used to demonstrate that *Hfx. volcanii* mutants lacking both the Holliday junction resolvase Hjc and the Xpf homolog Hef are synthetically lethal (Lestini *et al*., 2010). Additional selectable markers based on the *hisC* and *metX* genes of *Hfx. volcanii* (involved in histidine and methionine biosynthesis, respectively) have recently been developed (M. Mevarech, pers. commun.). This approach has been less fruitful for *Hbt. salinarum*, because it lacks the biosynthetic capability for five amino acids and cannot grow on synthetic media (Falb *et al*., 2008).

Regulated gene expression

The ability to regulate gene expression via a tightly repressed promoter is taken for granted in bacterial and eukaryotic systems. Heat-inducible chaperonin promoters have been available for some time in *Hfx. volcanii* (Kuo *et al.*, 1997), but given the pleiotropic effects of heat-shock, they are far from ideal. In 2007 the p.*tnaA* promoter from the tryptophanase gene of *Hfx. volcanii* was characterized; it is tightly repressed in the absence of tryptophan and shows rapid, strong induction upon addition of ≥ 1 mM tryptophan (Large *et al.*, 2007). It has been used to construct a depletion mutant of the essential *cct1* gene (Large *et al.*, 2007) and to generate an overexpression system for hexahistidine-tagged halophilic proteins (Allers *et al.*, 2010). This overexpression system features a *Hfx. volcanii* host strain with deletion of the *mrr* gene, allowing high-efficiency transformation with DNA isolated from *E. coli*, and replacement of the *pitA* gene by its ortholog from *N. pharaonis*, preventing copurification with His-tagged recombinant proteins (Allers *et al.*, 2010).

Reporter genes

A number of biosynthetic genes have been used as reporters in haloarchaea: for example the *dhfr* gene has been used to study transcription and translation in *Hfx. volcanii* (Danner & Soppa, 1996; Hering *et al.*, 2009). Two colorimetric reporter genes are available for monitoring gene expression in haloarchaea: β-galactosidase and green fluorescent protein (GFP). They succinctly illustrate the 'halophilic adaptation' of existing genetic tools, which takes into account the high intracellular salt concentrations of haloarchaea. Because the *lacZ* gene product of *E. coli* is not active at high salt concentrations, the laboratory of Mike Dyall-Smith isolated a β-galactosidase gene *bgaH* from *Haloferax alicantei* that develops a blue color from X-gal (Holmes & Dyall-Smith, 2000). It has been used extensively as a transcriptional reporter in *Hfx. volcanii* and *Hbt. salinarum*, both of which lack detectable β-galactosidase activity (Patenge *et al.*, 2000). A different approach was used to generate a halophilic version of GFP. Because GFP is a stable protein that is resistant to many denaturants, it was possible, by introducing just four mutations, to generate a soluble modified derivative that exhibits fluorescence in *Hfx. volcanii* cells (Reuter & Maupin-Furlow, 2004).

Discoveries and recent progress

The last 5 years have seen considerable progress in the area of 'whole genome' studies of *Hbt. salinarum*. Systems biology has taken advantage of the genome sequence since its publication in 2000 (Ng *et al.*, 2000), leading to insights into the cellular response to anaerobic growth, phototrophy, X-ray and UV irradiation, salinity and temperature shifts, heavy metal resistance, and phosphate limitation (Baliga *et al.*, 2004; McCready *et al.*, 2005; Muller & DasSarma, 2005; Kaur *et al.*, 2006; Whitehead *et al.*, 2006; Coker *et al.*, 2007; Twellmeyer *et al.*, 2007; Wende *et al.*, 2009). Now that the *Hfx. volcanii* genome sequence is published (Hartman *et al.*, 2010), we hope to see similar progress in the other model haloarchaeon. This species holds considerable potential for combining mutant strain construction by reverse genetics with '-omics' technology, for example proteomics and metabolomics (Kirkland *et al.*, 2008; Sisignano *et al.*, 2010).

One of the most fascinating aspects of halophiles is how their proteins can function in molar salt concentrations. The stability of halophilic proteins is partly due to their unusually hydrophilic surfaces, which leads to a requirement for efficient protein folding. This poses problems for secreted proteins, owing to a lack of ATP-dependent chaperones in the extracytoplasmic space. In halophiles, an unusually large number of proteins are rerouted via the twin-arginine transport (Tat) pathway, which allows cytoplasmic folding of proteins before their secretion (Rose *et al.*, 2002; Hutcheon & Bolhuis, 2003). Haloarchaea have also yielded surprising insights into the evolutionary origin of cellular processes that were once thought to be exclusive to eukaryotes or bacteria. Ubiquitin-like proteins have been found in *Hfx. volcanii* and shown to act in protein conjugation, probably targeting these proteins for proteasome-mediated proteolysis (Humbard *et al.*, 2010). A caspase-like activity has also been found; its expression is induced by salt stress and may play a role in programmed cell death (Bidle *et al.*, 2010). N-linked glycosylation has been shown to be widespread in archaea, with a wider variety of sugar subunits than seen in eukaryal or bacterial glycoproteins it is commonly encountered in S-layer proteins and flagellins (Calo *et al.*, 2010; Jarrell *et al.*, 2010). Archaeal flagellins assemble to form a structure with superficial similarity to bacterial flagella, but on a molecular level they are unrelated (Ng *et al.*, 2006). Studies on *Hbt. salinarum* have shown how a chemotaxis signal transduction system consisting of bacterial-like proteins, and proteins unique to archaea, is used to modulate rotation of the flagellum (Schlesner *et al.*, 2009).

Thermococcales

Introduction to *Thermococcales*, the heterotrophic, sulfur-reducing hyperthermophiles of the *Euryarchaeota*

The *Euryarchaeota* include an abundant number of hyperthermophiles that exhibit considerable diversity in terms of metabolism. Hyperthermophiles are found in the orders *Archaeoglobales* (sulfate reducers), *Methanococcales*/*Methanopyrales* (methanogens), and *Thermococcales* (sulfur reducers). The *Thermococcales* consist of three genera: *Pyrococcus* (Fiala & Stetter, 1986), *Thermococcus* (Zillig *et al.*, 1983), and

Palaeococcus (Takai *et al.*, 2000). Although several exceptions are present such as the freshwater *Thermococcus waiotapuensis* (Gonzalez *et al.*, 1999), the majority of the *Thermococcales* have been isolated from shallow marine thermal springs or deep-sea hydrothermal vents. They are all considered to be obligate anaerobes and heterotrophs that assimilate amino acids, peptides, pyruvate, and oligosaccharides, coupled with sulfur reduction or hydrogen fermentation (Amend & Shock, 2001). Among the three genera, *Thermococcus* contains the highest number of characterized isolates, and environmental studies have indicated that the members of *Thermococcus* are ubiquitously present in deep-sea hydrothermal vent systems (Orphan *et al.*, 2000; Holden *et al.*, 2001).

Most members of the *Thermococcales* exhibit optimal growth temperatures $> 80\,^{\circ}$C. Members of the genus *Thermococcus* exhibit growth temperature ranges that fall between 50 and 100 °C, with optimal temperatures between 80 and 90 °C. Members of *Pyrococcus* exhibit slightly higher growth temperatures, with growth observed between 65 and 105 °C, with optimums between 95 and 105 °C. *Palaeococcus ferrophilus*, the only member of the genus studied in detail, grows between 60 and 88 °C, with an optimum of 83 °C (Takai *et al.*, 2000). As the members of the *Thermococcales* grow at these high temperature ranges, all of their cell components, including membrane lipids, nucleic acids, and proteins, must display extreme thermostability in order to function at these high temperatures. Due to both fundamental and application-based interests, an abundant number of studies have focused on the structures of the thermostable proteins from hyperthermophiles.

In most cases, enzymes from hyperthermophiles are highly similar to their counterparts from mesophilic organisms in terms of both primary and tertiary structure, and share common catalytic mechanisms. Studies on enzymes from (hyper)thermophilic organisms have helped us understand the effects of temperature on enzyme activity (Daniel *et al.*, 2010), and the structural characteristics that lead to protein thermostability. Enhancement of protein thermostability is brought about by several general strategies and their degrees of contribution vary according to the protein. One major strategy is the presence of extensive ion pair networks formed by acidic and basic amino acid residues. Glutamate dehydrogenases from a variety of *Thermococcales* species have been studied as model proteins, confirming the importance of ion pair networks in protein thermostability (Yip *et al.*, 1995, 1998; Rice *et al.*, 1996; Rahman *et al.*, 1998; Vetriani *et al.*, 1998). Another factor is increased packing and loop shortening. An increase in the number of buried atoms and a decrease in internal cavity volume is observed in the citrate synthase from *Pyrococcus furiosus* compared with its counterparts from mesophiles. The enzyme also has six loops that are shorter than those found in the pig citrate synthase (Russell *et al.*, 1997; Arnott *et al.*, 2000). These studies on citrate synthases have also revealed the importance of subunit interaction towards thermostability. The third major strategy is an increase in hydrophobic interactions in the protein core. O^6-methylguanine DNA methyltransferase from *Thermococcus kodakarensis* harbors increased aromatic amino acids in the enzyme core compared with its counterpart from *E. coli* (Hashimoto *et al.*, 1999).

Why study *Thermococcales*?

Other than the fact that they are obligate anaerobes, the *Thermococcales* can be grown on simple, organic media and exhibit cell yields sufficient for biochemical analyses. In addition, as complete genome sequences of three *Pyrococcus* species became available at a relatively early stage, members of the *Thermococcales* have been utilized to study a wide range of archaeal biology. These include DNA replication and repair (Hopkins & Paull, 2008; Williams *et al.*, 2008; Yoshimochi *et al.*, 2008; Kiyonari *et al.*, 2009; Mayanagi *et al.*, 2009; Nishida *et al.*, 2009), transcription and its regulation (Vierke *et al.*, 2003; Lee *et al.*, 2005, 2007; Goede *et al.*, 2006; Kanai *et al.*, 2007; Santangelo *et al.*, 2007; Hirata *et al.*, 2008a), carbon and energy metabolism (Sapra *et al.*, 2003; Verhees *et al.*, 2003; Siebers & Schönheit, 2005), CRISPR systems (Hale *et al.*, 2009), and cellular responses to stress, such as oxidative (Jenney *et al.*, 1999; Clay *et al.*, 2003), osmotic (Neves *et al.*, 2005; Rodrigues *et al.*, 2007), and temperature stress (Laksanalamai & Robb, 2004; Danno *et al.*, 2008; Fujiwara *et al.*, 2008; Kanzaki *et al.*, 2008; Kida *et al.*, 2008). The abundant genome sequences have also promoted a wealth of '-omics' research including transcriptomics (Schut *et al.*, 2003; Lee *et al.*, 2006; Trauger *et al.*, 2008), proteomics (Menon *et al.*, 2009), structural genomics (Hura *et al.*, 2009), and other genome-based high-throughput strategies (Keese *et al.*, 2010).

In terms of metabolism, the *Thermococcales* assimilate a wide range of organic compounds, in many cases via novel or modified metabolic pathways that have not been identified in other organisms. They have thus attracted much attention to those interested in carbon/energy metabolism. The *Thermococcales* are also known to efficiently utilize polymeric substrates, namely poly- and oligosaccharides and peptides, and are armed with a vast array of stable, polymer-degrading hydrolases, which are expected to be applicable in various fields of biotechnology (Atomi, 2005; Egorova & Antranikian, 2005).

A variety of thermostable poly(oligo)maltosaccharide-modifying enzymes have been identified and characterized, which include α-amylases (Laderman *et al.*, 1993; Dong *et al.*, 1997a), amylopullulanases (Dong *et al.*, 1997b), cyclodextrin glucanotransferases (Tachibana *et al.*, 1999; Rashid *et al.*, 2002a), 4-α-glucanotransferases (Jeon *et al.*, 1997), maltodextrin phosphorylases (Mizanur *et al.*, 2008), cyclodextrinases (Hashimoto *et al.*, 2001), and branching enzymes (Murakami

et al., 2006). In addition, many enzymes that cleave β-1,3- or β-1,4-glycosidic bonds have been studied such as β-glucosidases, β-galactosidases, β-mannosidases, endo-β-1,3-glucanases, chitinases, and β-glucosaminidases (Kengen *et al.*, 1993; Voorhorst *et al.*, 1995; Bauer *et al.*, 1996; Gueguen *et al.*, 1997; Driskill *et al.*, 1999; Matsui *et al.*, 2000). Based on genome sequence predictions, members of the *Thermococcales* each harbor over 30 protease/peptidase-related genes (Ward *et al.*, 2002), and many of their protein products have been examined (Halio *et al.*, 1996; Voorhorst *et al.*, 1996; Story *et al.*, 2005). In terms of the active site nucleophile, hyperthermophilic proteases include serine proteases, cysteine proteases, the threonine-dependent proteasomes, metal-dependent proteases, and those of which the catalytic mechanisms have not been elucidated. It is worthy to note that a remarkable number of crystal structures have been reported for the proteases/peptidases from the *Thermococcales* (Du *et al.*, 2000; Arndt *et al.*, 2002; Maher *et al.*, 2004; Yokoyama *et al.*, 2006; Delfosse *et al.*, 2009; Dura *et al.*, 2009).

Intracellular sugar metabolism has been another major topic of interest in the *Thermococcales*. Glycolysis is carried out through a modified Embden–Meyerhof (EM) pathway. Studies with *P. furiosus* have revealed that sugar phosphorylation is carried out by novel ADP-dependent glucokinases and ADP-dependent phosphofructokinases that are structurally unrelated to the ATP-dependent enzymes of the classical EM pathway (Kengen *et al.*, 1994, 1995; Tuininga *et al.*, 1999). Fructose-1,6-bisphosphate aldolases are structurally distinct to the previously known enzymes from bacteria/eukaryotes (Galperin *et al.*, 2000; Siebers *et al.*, 2001; Imanaka *et al.*, 2002), as is the case with the gluconeogenic enzyme fructose-1,6-bisphosphatase (Rashid *et al.*, 2002b; Sato *et al.*, 2004). Metabolism of glyceraldehyde 3-phosphate (GAP) involves a novel GAP:ferredoxin oxidoreductase (Mukund & Adams, 1995; van der Oost *et al.*, 1998), in addition to the phosphorylating GAP dehydrogenase and phosphoglycerate kinase. In the final step from phosphoenolpyruvate to pyruvate, phosphoenolpyruvate synthase is the major enzyme rather than the well-known pyruvate kinase (Imanaka *et al.*, 2006). Studies on this single glycolytic pathway have revealed the presence of enzymes with novel structures, novel activities, and novel metabolic roles.

An encouraging fact for those interested in studying the *Thermococcales* is the wealth of sequence information that has accumulated in recent years. Complete genome sequences have been reported for *P. furiosus* JCM8422 (Robb *et al.*, 2001), *Pyrococcus abyssi* GE5 (Cohen *et al.*, 2003), *Pyrococcus horikoshii* OT3 (Kawarabayasi *et al.*, 1998), *T. kodakarensis* KOD1 (Fukui *et al.*, 2005), *Thermococcus gammatolerans* EJ3 (Zivanovic *et al.*, 2009), *Thermococcus onnurineus* NA1 (Lee *et al.*, 2008), and *Thermococcus sibiricus* MM739 (Mardanov *et al.*, 2009), and are publicly available for *Thermococcus barophilus* MP and *Thermococcus* sp. AM4. This will provide an advantage not only in structure–function studies of individual proteins, but also in predicting functional relationships of genes in various biological systems of the *Thermococcales*.

Genetic tools for *Thermococcales*

Genetic manipulation techniques have been developed for *T. kodakarensis* (Morikawa *et al.*, 1994; Atomi *et al.*, 2004a) by the groups of Tadayuki Imanaka and John Reeve. As will be described below, gene disruption, insertion, and replacement on the chromosome occur through homologous recombination. *Thermococcus kodakarensis–E. coli* shuttle vectors have also been developed. Strong constitutive promoters have been identified and can be used for gene expression. A β-glycosidase gene and a chitinase gene have been utilized as reporter genes. As for members of *Pyrococcus*, shuttle vector-based transformation systems are available for *P. abyssi* (Lucas *et al.*, 2002), and have recently been developed in *P. furiosus* (Waege *et al.*, 2010). The current range of genetic tools for *Thermococcales* is shown in Table 3.

Genetic systems using auxotrophic host strains in defined media

Uracil auxotrophs with mutations in the *pyrE* or *pyrF* genes of *T. kodakarensis* were positively selected in a medium containing uracil and 5-FOA. Homologous recombination was tested using a *pyrF*$^{-}$ strain as the host strain and the wild-type *pyrF* gene as the marker. Specific gene disruption was achieved with plasmids designed for double-crossover recombination (Fig. 2a). Double crossover plasmids were used to delete the *pyrF* gene from the wild-type KOD1 strain, resulting in strain KU216 (Δ*pyrF*), and the *pyrF* marker gene was subsequently used to disrupt the *trpE* gene, leading to strain KW128 (Δ*pyrF*, Δ*trpE*::*pyrF*), a tryptophan auxotroph. The *pyrF* and *trpE* genes can be used as markers for gene manipulation in strains KU216 and KW128, respectively (Sato *et al.*, 2003, 2005).

Thermococcus kodakarensis displays natural competency and cells collected from a routine culture can be used directly for transformation. However, frequencies are low ($\sim 10^2$ mg^{-1} DNA with homologous regions of 1000 bp), and the use of this methodology is limited to specific gene modifications, and is not applicable for experiments such as random mutagenesis/gene complementation. Frequencies become even lower when the length of the homologous region becomes shorter. No prototrophs are obtained with flanking regions of 100 bp (Sato *et al.*, 2003, 2005).

The pop-in/pop-out strategy using the counterselectable *pyrF* gene is applicable in *T. kodakarensis*. Plasmids are designed so that two regions on the chromosome are directly fused on the plasmid, with the *pyrF* marker gene flanking this

Table 3. Genetic tools for *Thermococcales*

	T. kodakarensis	*P. abyssi*	*P. furiosus*
Defined media	Yes (Sato *et al*., 2003)	Yes (Lucas *et al*., 2002)	Yes (Blumentals *et al*., 1990)
DNA delivery	Transformation (Sato *et al*., 2003)	PEG-mediated transformation (Lucas *et al*., 2002)	Transformation (Waege *et al*., 2010)
Restriction barrier	No	No	No
Replicative shuttle vectors	pLC70 (Santangelo *et al*., 2008a, b)	pYS2 (Lucas *et al*., 2002)	pYS3 (Waege *et al*., 2010)
Positive selection	Simvastatin (Matsumi *et al*., 2007)		Simvastatin (Waege *et al*., 2010)
Counterselection	5-FOA (*pyrF*) (Sato *et al*., 2005; Yokooji *et al*., 2009) 6-Methyl purine (TK0664) (Santangelo *et al*., 2010)		
Markerless gene knockout or replacement	Using *pyrF* (Sato *et al*., 2005; Yokooji *et al*., 2009) Using TK0664 (Santangelo *et al*., 2010)		
Ectopic integration	At chitinase gene (TK1765) locus (Mueller *et al*., 2009)		
Reporter genes	TK1761 (β-galactosidase) (Santangelo *et al*., 2008a, b, 2010)		
Regulated gene expression	*fbp* (TK2164) promoter (Hirata *et al*., 2008a, b)		
Protein overexpression	*gdh* (TK1431) and *csg* (TK0895) promoters (Matsumi *et al*., 2007; Mueller *et al*., 2009; Yokooji *et al*., 2009)		*gdh* (PF1602) promoter (Waege *et al*., 2010)

construct (Fig. 2b). Host cells (Δ*pyrF*) are transformed with the plasmid, and transformants that have undergone single-crossover recombination ($pyrF^+$) at either one of the homologous regions are enriched in liquid medium depleted of pyrimidines. Cells are then spread on Gelrite solid medium containing uracil and 5-FOA, which allows only growth of cells that have undergone a second recombination event that removes the *pyrF* marker gene. If the second recombination event occurs at the same homologous region that was used in the first recombination, the genotype returns to that of the host strain, whereas recombination at the opposite region results in gene modification and marker removal. This strategy and other similar strategies utilizing counterselection with the *pyrF* gene have been used to construct double and triple auxotrophic host strains such as KUW1 (Δ*pyrF*, Δ*trpE*) and KUWH1 (Δ*pyrF*, Δ*trpE*, Δ*hisD*) (Sato *et al*., 2005), and various markerless gene knockout strains (Yokooji *et al*., 2009).

Counterselection is also possible using the TK0664 gene, annotated as hypoxanthine–guanine phosphoribosyltransferase. Deletion of this gene results in a strain that is resistant to 6-methyl purine. Therefore, by utilizing a host strain deleted of the TK0664 and *trpE* genes (*T. kodakarensis* TS517), a gene cassette consisting of the TK0664 and *trpE* gene under the control of strong promoters (trp-$6MP^S$ cassette) can be used for selection/counterselection (Fig. 2c) (Santangelo *et al*., 2010).

Genetic systems applicable in nutrient-rich media

Conventional antibiotic resistance marker genes cannot be used in hyperthermophiles due to the lack of thermostability in their protein products. However, a strategy based on inhibition of a particular endogenous protein by an antibiotic and relieving the inhibition by overexpressing the protein or by introducing a mutant protein insensitive to the antibiotic is feasible. As demonstrated in the halophiles, simvastatin, a specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, displays severe effects on the growth of *T. kodakarensis*. Growth of the wild-type *T. kodakarensis* KOD1 was completely inhibited for 5 days in the presence of 4 μM simvastatin. Overexpression cassettes for the endogenous HMG-CoA reductase gene from *T. kodakarensis* (hmg_{Tk}) and the heterologous gene from *P. furiosus* (hmg_{Pf}) have been shown to be applicable as selection markers (Matsumi *et al*., 2007). The promoter applied for overexpression was the 5′-upstream region of the glutamate dehydrogenase gene (P_{gdh}) from *T. kodakarensis*. Transformants harboring the overexpression cassette display resistance and can be selected in the presence of 10 μM simvastatin. Although both hmg_{Tk} and hmg_{Pf} can be used, the hmg_{Pf} gene is recommended as it will prevent unintended recombination at the native *hmg* locus that can occur with the endogenous hmg_{Tk}. This system allows gene disruption in nutrient-rich media, and can be directly applied on the wild-type *T. kodakarensis* KOD1. Moreover, this system has proved applicable in several other *Thermococcus* species such as *T. onnurineus* NA1 (Kim *et al*., 2010).

Another system that has recently been reported utilizes a *T. kodakarensis* strain deleted of its arginine decarboxylase gene (*pda*). Arginine decarboxylase converts arginine to agmatine, a vital precursor for polyamine biosynthesis. Even in a nutrient-rich medium, the *pda* disruption strain can only grow when agmatine is supplemented to the medium (Fukuda *et al*., 2008). The *pda* gene can thus be used as a

selectable marker in nutrient-rich medium (without agmatine) when a *pda* gene disruption strain (*T. kodakarensis* TS559) is used as a host (Santangelo *et al.*, 2010). This system allows selection in nutrient-rich medium without the addition of antibiotics.

Shuttle vectors in *Thermococcus* and *Pyrococcus*

Shuttle vectors have been developed that replicate stably and express selectable phenotypes in both *T. kodakarensis* and *E. coli* (Santangelo *et al.*, 2008b). A plasmid from *Thermococcus nautilis* (pTN1) was ligated to a commercial vector for *E. coli*, and the selectable markers *trpE* and the P_{gdh}-hmg_{Pf} overexpression cassette were added so that *T. kodakarensis* transformants could be selected by Δ*trpE* complementation and/or mevinolin resistance. The plasmids are maintained in *T. kodakarensis* at a copy number of approximately three copies per chromosome. The use of these plasmids for gene expression in *T. kodakarensis* has also been shown (Santangelo *et al.*, 2008b).

Shuttle vector-based transformation is also possible in *P. abyssi* (Lucas *et al.*, 2002) and *P. furiosus* (Waege *et al.*, 2010). In the *P. abyssi* system, strains with mutations in the *pyrE* gene were used as host cells. The shuttle vector pYS2, which harbors the *pyrE* gene from *S. acidocaldarius* as a selectable marker, can be introduced into *P. abyssi* cells by a PEG-spheroplast method. pYS2 is stably maintained in *P. abyssi* with a high copy number of 20–30 copies per chromosome (Lucas *et al.*, 2002). In the *P. furiosus* system, pYS2 was modified so that the *pyrE* gene was replaced by an hmg_{Pf} overexpression cassette (pYS3). The promoter used was the *gdh* promoter from *P. furiosus*. Transformants can be selected based on their resistance towards 10 μM simvastatin. pYS3 is stable in *P. furiosus*, and the copy number of the vector was 1–2 copies per chromosome. Induced expression of the RNA polymerase subunit D has been achieved using this vector. It has also been mentioned that the introduction of mutations on the chromosome of *P. furiosus* is now possible using hmg_{Pf} as a selectable marker (Waege *et al.*, 2010).

Reporter genes

Thermococcus kodakarensis harbors two nonessential genes that encode β-glycosidases (TK1761 and TK1827). As intracellular activity deriving from TK1827 was very low and remained constant; an *in vivo* gene reporter system was established based on TK1761 (Santangelo *et al.*, 2008a). This initial system has been utilized to display the occurrence of polarity in archaea (Santangelo *et al.*, 2008a) and to elucidate the transcription termination signal [oligo(T) sequence] recognized by archaeal RNA polymerases (Santangelo *et al.*, 2009). Recently, a *T. kodakarensis* strain deleted of both TK1761 and TK1827 has been constructed as an improved host for the *in vivo* β-glycosidase gene reporter system (Santangelo *et al.*, 2010).

Gene expression

Several strong promoters have been identified for use in gene expression in *T. kodakarensis*. The glutamate dehydrogenase gene promoter (P_{gdh}) mentioned above is a 551-bp 5′-flanking region of TK1431 (Matsumi *et al.*, 2007). A 200-bp 5′-flanking region of the TK0895 gene (P_{csg}), which encodes a cell surface glycoprotein, has also been utilized for gene expression (Yokooji *et al.*, 2009). In addition, a heterologous promoter of the histone-encoding *hmtB* gene from *Methanothermobacter thermautotrophicus* (P_{hmtB}) supports high expression levels in *T. kodakarensis* (Santangelo *et al.*, 2010). Heterologous expression of the α-1,4-glucan phosphorylase gene from *Sulfolobus solfataricus*, which could not be functionally expressed in mesophilic host cells, was achieved in *T. kodakarensis* using P_{csg} (Mueller *et al.*, 2009). Heterologous gene constructs can be integrated into the chitinase gene locus without any apparent decrease in growth rate and cell yield (Fig. 2e) (Mueller *et al.*, 2009).

Discoveries and recent progress

Until recently, the use of genetics in the *Thermococcales* has been carried out mainly in *T. kodakarensis*. In addition to the studies described above, disruption of the reverse gyrase gene in *T. kodakarensis* has demonstrated that the enzyme provides a significant advantage for hyperthermophiles to grow at high temperatures, but is not essential for life at 90 °C (Atomi *et al.*, 2004b). The use of genetics has also contributed in elucidating metabolic pathways unique to the archaea, such as pentose synthesis via the reverse flux of the ribulose monophosphate pathway (Orita *et al.*, 2006), AMP degradation via a novel route involving Type III Rubiscos (Sato *et al.*, 2007), and coenzyme A biosynthesis involving two novel enzymes pantoate kinase and phosphopantothenate synthetase (Yokooji *et al.*, 2009). Further genetic studies have provided insight into how *T. kodakarensis* responds to various carbon sources and environmental stress (Kanai *et al.*, 2007, 2010). Application of the simvastatin-based gene disruption system to *T. onnurineus* NA1 has contributed to the identification of genes involved in growth of this strain on formate (Kim *et al.*, 2010).

Sulfolobales

Introduction to the *Sulfolobales*, the aerobic thermoacidophiles of the *Crenarchaeota*

The first member of the *Sulfolobales*, *S. acidocaldarius*, was described by T. Brock in 1972 and isolated from a hot spring in Yellowstone National Park (Brock *et al.*, 1972). Later on, different members of *Sulfolobales* were characterized from

acidic hotsprings and mudholes all over the world, with *S. solfataricus* (Pozzuoli, Italy; Zillig *et al.*, 1980) and *Sulfolobus tokodaii* (Japan; Suzuki *et al.*, 2002) being the most commonly used strains in laboratories. Recently, seven genomes of *Sulfolobus islandicus* were sequenced from a variety of acidic hot springs in the United States, Iceland, and Russia (Whitaker *et al.*, 2005), and have been used to describe the mechanism of archaeal genome evolution (Reno *et al.*, 2009).

Sulfolobus solfataricus and *S. islandicus* are metabolically the most diverse species and are able to grow on a wide variety of peptide sources, amino acids mixtures, and minimal media containing only sugars such as arabinose, glucose, sucrose, trehalose, cellobiose, and others by aerobic respiration (Grogan, 1989). In contrast, although *S. acidocaldarius* and *S. tokodaii* contain the genes for the nonphosphorylated Entner–Doudoroff pathway and the partially overlapping alternative pathway that generates ATP (Siebers & Schönheit, 2005), they cannot grow in these media because they lack the wide variety of sugar and peptide uptake systems present in the previously mentioned strains (Elferink *et al.*, 2001; Albers *et al.*, 2004). Very recently, a systems biology approach was undertaken to understand the temperature adaptation of glucose metabolism in *S. solfataricus* (Albers *et al.*, 2009). These studies initiated the development of standard operating procedures for a wide variety of techniques for *Sulfolobales* including fermentation, transcriptomics, proteomics, and metabolomics, which should facilitate the comparability of results once researchers use these standardized protocols (Zaparty *et al.*, 2010).

The cell cycle in *Sulfolobales* is characterized by a short prereplication period and an extensive postreplication stage that accounts for up to 70% of the generation time (Lundgren *et al.*, 2008). In stationary-phase *Sulfolobus* cultures, all cells contain two genome copies resulting in an increase in the average cellular DNA content relative to an exponentially growing culture (Bernander, 2007). In contrast to *Euryarchaeota*, *Crenarchaeota* do not exhibit FtsZ for septum formation during cell division. Recently, it was found that in *S. solfataricus* and *S. acidocaldarius*, homologs of the eukaryotic endosomal sorting (ESCRT) pathway are located at midcell before cell division (Lindas *et al.*, 2008; Samson *et al.*, 2008). However, the exact role of these proteins during division is unknown.

As the archaeal enzymes involved in DNA replication are more similar to their eukaryal counterpart than to their bacterial one, a common origin of the eukaryal and archaeal replication apparatus was further strengthened by the demonstration of three origins of replication in the genomes of *S. solfataricus* and *S. acidocaldarius* (Duggin *et al.*, 2008). However, it is not yet clear whether all of these origins are used at the same time or are differentially induced.

As an important adaptation to their extreme habitat, membranes of *Sulfolobus* species contain a large amount of tetraether lipids (up to 98% of all membrane lipids) resulting in a monolayer membrane (Elferink *et al.*, 1992). These lipids are highly proton-impermeable and therefore enable *Sulfolobus* to maintain an internal pH of 6.5 in a highly acidic surrounding (Moll & Schäfer, 1988; van de Vossenberg *et al.*, 1995).

Full genome sequences have been determined for all commonly used *Sulfolobus* strains, and are characterized by a high A+T content (between 63% and 67%). The genome size ranges from 2 to 3 Mbp, with *S. solfataricus* having the largest genome (She *et al.*, 2001). This is mainly due to the fact that 11% of the *S. solfataricus* genome consists of mobile elements including over 200 different IS elements whereas other *Sulfolobus* strains only contain very few mobile elements. Large genome arrangements are reported for *S. solfataricus* species (Redder & Garrett, 2006).

Sulfolobus species have been a source for the isolation and characterization of a large number of genetic elements such as viruses, plasmids, and conjugative plasmids (Zillig *et al.*, 1996; Prangishvili *et al.*, 1998, 2006; Peng *et al.*, 2000; Greve *et al.*, 2004). These genetic elements are intensively being studied and some of them have been used for the development of genetic tools (Berkner & Lipps, 2008).

Why study *Sulfolobales*?

Sulfolobales are the only representatives of the *Crenarchaeota* that are amenable for genetic manipulation so far. All of the above-mentioned strains have growth optima between 70 and 85 °C and grow at pH values of 2–3. A few *Sulfolobus* species are reported to grow chemolithoautotrophically (Huber & Stetter, 1991), but are easily cultivated aerobically under heterotrophic conditions in the laboratory and exhibit doubling times of 3–6 h. *Sulfolobus* spp. have developed into model organisms for studies on DNA translation, transcription, and replication, DNA repair, cell division, RNA processing, metabolism, and many other cellular aspects.

One reason for this popularity is that the proteins of hyperthermophiles such as *Sulfolobus* are very amenable for obtaining 3D structures, and the PDB database contains 501 crystal structures obtained from different *Sulfolobus* species (December 2010). Among these is the structure of its complete RNA polymerase (Hirata *et al.*, 2008b; Korkhin *et al.*, 2009), and understanding in eukaryotic DNA repair mechanisms has been achieved interpreting structures obtained from *Sulfolobus* (Liu *et al.*, 2008).

As mentioned before, a systems biology project (SulfoSYS) was initiated leading to a wealth of metabolomic, transcriptomic, and proteomic data available and a set of standardized methods that will enable researchers to compare results from different laboratories better with each other (Albers *et al.*, 2009; Zaparty *et al.*, 2010). The first archaeal deep sequencing project was carried out with *S. solfataricus* providing detailed information of transcription start sites of all genes present in the genome, and identified

100 previously unknown expressed genes and antisense RNAs (Wurtzel *et al.*, 2010).

Key species of *Sulfolobales* that have genetic systems

A major problem for the development of genetic tools in the *Sulfolobales* has been and still is that only two selectable markers, uracil auxotrophy and growth on lactose, are available. Moreover, the main species researchers worked on, namely *S. solfataricus* P1 and P2, until now have not yet shown to recombine foreign DNA into their genome. The breakthrough came when Worthington *et al.* (2003) used a natural mutant of the *S. solfataricus* strain 98/2 PBL2002 for the construction of the first directed deletion mutant. The strain had an insertion in the β-galactosidase gene (*lac*S) that is necessary for *S. solfataricus* to grow in minimal medium containing lactose. Selection for growth on lactose often resulted in revertants; therefore, a naturally occurring mutant of PBL2002, PBL2025, is now being used that exhibits a large deletion of 50 genes (Schelert *et al.*, 2004). A disadvantage of working with the selection for growth on lactose is that this procedure is rather time consuming (7–14 days in liquid medium before plating; Albers & Driessen, 2008). PBL2025 has a different morphology to wild-type *S. solfataricus* cells, regularly showing very large cells in growing cultures. As the genome sequence of PBL2025 is not publicly available, it is unclear what causes the morphological changes and care should be taken when using this strain. For example, it has been demonstrated that PBL2025 shows a profound difference in the production of extracellular matrix once cells are attached to a variety of surfaces (Zolghadr *et al.*, 2010).

The first strain that was used for the expression of proteins in *Sulfolobus* was the uracil auxotrophic *S. solfataricus* P1 strain PH1-16, which also contains an IS element in the *lac*S gene (Martusewitsch *et al.*, 2000; Albers *et al.*, 2006). However, so far it has not been possible to introduce any foreign DNA into the genomic DNA of this strain using analogous methods as for *S. islandicus* and *S. acidocaldarius*.

Very recently, MR31, a uracil auxotrophic *S. acidocaldarius* mutant (Reilly & Grogan, 2001), was used for the construction of deletion mutants (Wagner *et al.*, 2009; Ellen *et al.*, 2010). An advantage of this strain is the possibility of direct plating after electroporation and only 4–6 days growth until colonies appear on gelrite plates. A markerless deletion mutant can be obtained in < 3 weeks, which makes this strain a prime candidate for deletion mutant studies (Wagner *et al.*, 2009). A plasmid-based system has been developed for maltose-induced protein expression in MR31 (Berkner *et al.*, 2010).

Another recently developed strain is the uracil auxotrophic *S. islandicus* E233S1 that has been used to obtain a markerless *lacS* mutant (Deng *et al.*, 2009; She *et al.*, 2009). A shuttle vector is available for this strain, which was used in a detailed study of the arabinose-binding protein promoter *araS* (Peng *et al.*, 2009). Both the *S. acidocaldarius* and the *S. islandicus* strains have the advantage that 5-FOA can be used for counterselection (Fig. 2b).

Genetic tools for *Sulfolobales*

The transformation of *Sulfolobus* strains was already established in 1992 when Christa Schleper demonstrated that *S. solfataricus* could efficiently be transfected with SSV1 virus DNA by electroporation (Schleper *et al.*, 1992). Because the lack of positive selection pressure made the use of plasmids for transformation impractical in early trials, *E. coli–Sulfolobus* shuttle vectors were constructed based on conjugative plasmids and viruses that would spread through a transfected culture (for a detailed review, see Berkner & Lipps, 2008). The most successfully used vector of this generation is pMJ0503, a shuttle vector based on SSV1 (Jonuscheit *et al.*, 2003). This vector was used for promoter studies and adapted for the homologous and heterologous expression of tagged proteins in *S. solfataricus* (Jonuscheit *et al.*, 2003; Albers *et al.*, 2006). The vector has also been used for the complementation of deletion mutants in PBL2025 (Zolghadr *et al.*, 2007; Frols *et al.*, 2008). The *in vivo* overexpression with the virus vector of the translation elongation factor a/eIF2-γ demonstrated that this factor stabilizes mRNA in *S. solfataricus* (Hasenohrl *et al.*, 2008).

Since the first deletion mutant was constructed in PBL2025 (Worthington *et al.*, 2003), the development of genetic tools for *Sulfolobales* has gone into warp speed and the most recent methods will be discussed and summarized in Table 4.

Insertional gene disruption and markerless genetic exchange

Marked gene disruptions in *S. solfataricus* PBL2025 were first obtained by single-crossover events using plasmid DNA and later by double-crossover events using linearized plasmids or PCR products (Fig. 2a and c) (Worthington *et al.*, 2003; Schelert *et al.*, 2004; Albers & Driessen, 2008; Wagner *et al.*, 2009). Integration of plasmid DNA via single crossover leads to tandem integration (Wagner *et al.*, 2009). Selection for positive transformants by growth on lactose is rather time consuming in this strain, and unfortunately direct plating of cells after electroporation does not yield colonies.

Very recently, in both *S. islandicus* and *S. acidocaldarius*, deletion mutants were obtained via homologous recombination via double-crossover and unmarked deletions via single-crossover events using uracil autotrophy for enrichment (Fig. 2b) (Deng *et al.*, 2009; Wagner *et al.*, 2009; Ellen *et al.*, 2010).

Ectopic integration

To avoid complication by multiple copies during expression of genes or promoter fusion constructs in cells, ectopic

Table 4. Genetic tools for *Sulfolobales*

	S. solfataricus PBL2025	*S. islandicus* E322S	*S. acidocaldarius*
Defined media	Yes (Grogan, 1989)	Yes	Yes (Grogan, 1989)
DNA delivery	Electroporation (Schleper *et al*., 1992; Worthington *et al*., 2003; Albers & Driessen, 2008)	Electroporation (Deng *et al*., 2009)	Electroporation (Kurosawa & Grogan, 2005; Wagner *et al*., 2009)
Restriction barrier	No	SuiI cuts at GCwGC (Sollner *et al*., 2006)	SuaI, restricts unmethylated DNA at CCGG (Prangishvili *et al*., 1985)
Replicative shuttle vectors	pEXSs (Cannio *et al*., 1998), pKMSD48 (Stedman *et al*., 1999), pMSS derivatives (Aucelli *et al*., 2006), pJlacS (Berkner *et al*., 2007), pMJ0503 derivatives (Jonuscheit *et al*., 2003)	pRN2 based vectors (Deng *et al*., 2009)	pCSV1 and pAG based vectors (Aagaard *et al*., 1996; Aravalli & Garrett, 1997), pCmalLacS (Berkner *et al*., 2010)
Positive selection	Lactose (Worthington *et al*., 2003), uracil (Jonuscheit *et al*., 2003), hygromycin (Cannio *et al*., 1998)	Uracil (Deng *et al*., 2009)	Uracil (Wagner *et al*., 2009), alcohols (Aravalli & Garrett, 1997)
Counterselection	No	5-FOA (*pyrEF*) (Deng *et al*., 2009)	5-FOA (*pyrEF*) (Wagner *et al*., 2009)
Markerless gene knockout or replacement	Using *lacS* (Schelert *et al*., 2004; Albers & Driessen, 2008)	Using *pyrE* (Deng *et al*., 2009)	Using *pyrE* (Wagner *et al*., 2009; Ellen *et al*., 2010)
Ectopic integration			At *pyrEF* and *amyA* (M. Wagner & S.-V. Albers, unpublished data)
Reporter genes	*lacS* (β-galactosidase)(Jonuscheit *et al*., 2003)	*lacS* (β-galactosidase) (Deng *et al*., 2009)	*lacS* (β-galactosidase)
Regulated gene expression	*araS* promoter (Albers *et al*., 2006)	*araS* promoter (Peng *et al*., 2009)	*malE* promoter (Berkner *et al*., 2010)
Protein overexpression	pMJ0503 virus vectors with arabinose inducible induction with His and Strep tags, pRN1-based vectors (Albers *et al*., 2006; Berkner *et al*., 2007)	Based on pRN2 pHZ2lacS (Peng *et al*., 2009)	Maltose inducible expression using pCmal (Berkner *et al*., 2010)

integration was successfully used by inserting promoter fusion constructs into the *amyA* (α-amylase) locus of *S. acidocaldarius* (Fig. 2e) (M. Wagner & S.-V. Albers, pers. commun.).

Reporter genes, overexpression and controlled expression

lacS, the gene encoding the β-galactosidase, has been used in all plasmids to demonstrate expression, as its activity can easily be determined by an X-gal assay. In the virus-based plasmid pMJ0503 (Jonuscheit *et al*., 2003), the pRN1-based pA-pK (Berkner *et al*., 2007) and the pRN2-based pHZ2lacS (Deng *et al*., 2009) *lac*S was used as a reporter gene for promoter studies. In PBL2025 and *S. islandicus* the arabinose-inducible arabinose-binding protein promoter *araS* has been implemented for the expression of proteins and complementation of deletion mutants (Albers *et al*., 2006; Zolghadr *et al*., 2007). Because *S. acidocaldarius* does not contain an arabinose uptake system, the promoter of the maltose-binding protein was used for homologous and heterologous expression (Berkner *et al*., 2010). Unfortunately, the *malE* promoter is quite leaky even in the absence of maltose.

Homologous expression of proteins in *Sulfolobus* leads to the correct assembly of cofactors in recombinant proteins, and Histidine and Strep-tags are stably expressed on fusion proteins, which have been successfully isolated by affinity chromatography (Albers *et al*., 2006). The overexpression of a mutant protein was used to demonstrate a dominant negative effect *in vivo* (Samson *et al*., 2008) and expression vectors were also used to complement deletion mutants (Fig. 2d) (Zolghadr *et al*., 2007; Frols *et al*., 2008).

Discoveries and recent progress

Sulfolobus species are the only genetically tractable members of the *Crenarchaeota*. Various genetic tools are now available and have led to important new discoveries including a eukaryotic-like cell division apparatus (Samson *et al*., 2008), identification of a general stress response (Maaty *et al*., 2009), the identification of several transcriptional regulators (Schelert *et al*., 2006; Peeters *et al*., 2009), and new cell surface structures (Szabo *et al*., 2007; Zolghadr *et al*., 2007; Frols *et al*., 2008). A detailed mapping of the *araS* promoter was obtained, showing unexpected parts of the promoter to be involved in regulation (Peng *et al*., 2009), and *in vivo* studies showed that a/ELF2g counteracts 5′–3′ mRNA decay in *Sulfolobus* (Hasenohrl *et al*., 2008).

Conclusion and outlook

To write a review encompassing an entire domain of life might seem overly ambitious, and archaea are indeed exceedingly diverse. On the other hand, the development of

genetic technologies for each branch of archaea has involved common challenges. Characteristics that make archaea so interesting, such as thermophily, halophily, and strict anaerobiosis, pose challenges to routine laboratory culture. Furthermore, the immunity of the archaea to most conventional antibiotics has meant that other means of selection have had to be devised. This article describes how these challenges have now been overcome for various archaeal species. These developments allow us to more effectively characterize and exploit the very features that make archaea so fascinating as well as challenging. For instance, the *Sulfolobales* and *Thermococcales* host thermostable proteins, which, in addition to being of inherent interest, are very often used for biochemical studies, because they crystallize easily and are stable under laboratory conditions. The availability of genetic tools for *Sulfolobus* and *Thermococcus* species will now enable us to study these proteins not only *in vitro* but also *in vivo*. Among the methanogens, genetic approaches are starting to prove useful in elucidating the relatively constrained world of hydrogenotrophic methanogenesis as well as the more versatile methylotrophic methanogenesis (Costa *et al.*, 2010). The halophiles have shown us how with a few cunning modifications, proteins can function in salt concentrations that were once thought to be incompatible with life.

Opportunities for studies in archaea have never been better. In addition to genetic tools, the number of genome sequences continues to expand. A glance at NCBI Microbial Genomes reveals a current repertoire of seven strains of *Methanococcus*, three of *Methanosarcina*, six of *Thermococcus*, three of *Pyrococcus*, 11 of *Sulfolobus*, two of *Halobacterium*, and one of *Haloferax*, to say nothing of taxa for which genetic tools are not yet available. Clearly, comparative studies are now feasible – they are important as well. The considerable diversity of archaea offers countless opportunities for interesting discoveries, but no single archaeal species can be representative of the domain as a whole, or even its own specific grouping. For example, *Hbt. salinarum* is known to be extremely resistant to UV irradiation, while *Hfx. volcanii*, a closely related species that grows in the same habitat, is not (Baliga *et al.*, 2004; Delmas *et al.*, 2009). Rather than extrapolating results from one archaeal model organism to another, it is better to study each organism in its own right. Encouragingly, genetic tools continue to expand into new taxa. For example, shuttle vector-based technology is available in *P. abyssi* and *P. furiosus*, and chromosome modification in the latter species is now possible. As the application of these tools to other members of this order has just begun, we will probably be able to witness the development of genetic systems in a wide range of *Thermococcales* in the near future. Systems biology is also making inroads. Numerous global regulation studies have been carried out in *Hbt. salinarum* and a predictive model for transcriptional control of physiology has been generated – the first in any domain of life (Bonneau *et al.*, 2007). Transcriptomic and proteomic studies have been carried out with members of all the archaeal groups described here. As systems biology matures in these organisms, genetics will play an increasing role in testing the hypotheses that are generated.

To be sure, the genetic toolbox for archaea needs to expand and the number of selectable markers is still limited. A driving force has been reverse genetics, where a gene is precisely deleted or mutated. Unfortunately, there is only so much that we can learn from deletion mutants. Regulated promoters, already in place for some archaea and under development in others, will facilitate the study of essential genes that cannot otherwise be mutated. In addition, if we are to take advantage of the explosion in genomic data, we must carry out mutagenesis on a whole-genome level. Traditional protocols for mutagenesis with chemicals, UV or X-rays have been used in *Hbt. salinarum* and *Hfx. volcanii*, and negative enrichment for nondividing cells is possible using 5-bromo-2′-deoxyuridine (BrdU) (Soppa & Oesterhelt, 1989; Wanner & Soppa, 1999). Similarly, in *M. maripaludis*, after mutagenesis with ethyl methanesulfonate, growing cells were selectively killed upon exposure to the base analogs 6-azauracil and 8-azahypoxanthine (Ladapo & Whitman, 1990). However, with these methods it is difficult to isolate the resulting mutations. Insertion mutagenesis using recombinant transposons is a solution that allows for facile identification of the mutant allele. Transposon mutagenesis has been worked out for *Methanosarcina*, but is still lacking in other archaea; in halophiles, early attempts met with limited success (Dyall-Smith & Doolittle, 1994; Woods *et al.*, 1999). An alternative is to use next generation sequencing to identify mutations; this is currently too expensive for routine usage, but costs will inevitably fall to within the reach of all researchers.

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Note added in the proof

During the final stages of the reviewing process, Lipscomb *et al.* (2011) reported the development of a gene disruption system in *Pyrococcus furiosus* (REF). A *pyrF* deletion strain was used as the host strain, and markerless gene disruption was demonstrated via selection and counterselection with the *pyrF* marker based on uracil prototrophy and resistance towards 5-FOA.

References

Aagaard C, Leviev I, Aravalli RN, Forterre P, Prieur D & Garrett RA (1996) General vectors for archaeal hyperthermophiles: strategies based on a mobile intron and a plasmid. *FEMS Microbiol Rev* **18**: 93–104.

Albers SV & Driessen AJM (2008) Conditions for gene disruption by homologous recombination of exogenous DNA into the *Sulfolobus solfataricus* genome. *Archaea* **2**: 145–149.

Albers SV, Koning SM, Konings WN & Driessen AJ (2004) Insights into ABC transport in archaea. *J Bioenerg Biomembr* **36**: 5–15.

Albers SV, Jonuscheit M, Dinkelaker S *et al*. (2006) Production of recombinant and tagged proteins in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *Appl Environ Microb* **72**: 102–111.

Albers SV, Birkeland NK, Driessen AJ *et al*. (2009) SulfoSYS (Sulfolobus Systems Biology): towards a silicon cell model for the central carbohydrate metabolism of the archaeon *Sulfolobus solfataricus* under temperature variation. *Biochem Soc T* **37**: 58–64.

Allers T & Mevarech M (2005) Archaeal genetics – the third way. *Nat Rev Genet* **6**: 58–73.

Allers T, Ngo HP, Mevarech M & Lloyd RG (2004) Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. *Appl Environ Microb* **70**: 943–953.

Allers T, Barak S, Liddell S, Wardell K & Mevarech M (2010) Improved strains and plasmid vectors for conditional overexpression of His-tagged proteins in *Haloferax volcanii*. *Appl Environ Microb* **76**: 1759–1769.

Amend JP & Shock EL (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and bacteria. *FEMS Microbiol Rev* **25**: 175–243.

Aravalli RN & Garrett RA (1997) Shuttle vectors for hyperthermophilic archaea. *Extremophiles* **1**: 183–191.

Argyle JL, Tumbula DL & Leigh JA (1996) Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. *Appl Environ Microb* **62**: 4233–4237.

Arndt JW, Hao B, Ramakrishnan V, Cheng T, Chan SI & Chan MK (2002) Crystal structure of a novel carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Structure* **10**: 215–224.

Arnott MA, Michael RA, Thompson CR, Hough DW & Danson MJ (2000) Thermostability and thermoactivity of citrate synthases from the thermophilic and hyperthermophilic archaea, *Thermoplasma acidophilum* and *Pyrococcus furiosus*. *J Mol Biol* **304**: 657–668.

Atomi H (2005) Recent progress towards the application of hyperthermophiles and their enzymes. *Curr Opin Chem Biol* **9**: 166–173.

Atomi H, Fukui T, Kanai T, Morikawa M & Imanaka T (2004a) Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. *Archaea* **1**: 263–267.

Atomi H, Matsumi R & Imanaka T (2004b) Reverse gyrase is not a prerequisite for hyperthermophilic life. *J Bacteriol* **186**: 4829–4833.

Aucelli T, Contursi P, Girfoglio M, Rossi M & Cannio R (2006) A spreadable, non-integrative and high copy number shuttle vector for *Sulfolobus solfataricus* based on the genetic element pSSVx from *Sulfolobus islandicus*. *Nucleic Acids Res* **34**: e114.

Balch WE, Fox GE, Magrum LJ, Woese CR & Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**: 260–296.

Baliga NS, Bjork SJ, Bonneau R *et al*. (2004) Systems level insights into the stress response to UV radiation in the halophilic archaeon *Halobacterium* NRC-1. *Genome Res* **14**: 1025–1035.

Ban N, Nissen P, Hansen J, Moore PB & Steitz TA (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289**: 905–920.

Bauer MW, Bylina EJ, Swanson RV & Kelly RM (1996) Comparison of a β-glucosidase and a β-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Purification, characterization, gene cloning, and sequence analysis. *J Biol Chem* **271**: 23749–23755.

Beneke S, Bestgen H & Klein A (1995) Use of the *Escherichia coli uidA* gene as a reporter in *Methanococcus voltae* for the analysis of the regulatory function of the intergenic region between the operons encoding selenium-free hydrogenases. *Mol Gen Genet* **248**: 225–228.

Berkner S & Lipps G (2008) Genetic tools for *Sulfolobus* spp.: vectors and first applications. *Arch Microbiol* **190**: 217–230.

Berkner S, Grogan D, Albers SV & Lipps G (2007) Small multicopy, non-integrative shuttle vectors based on the plasmid pRN1 for *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*, model organisms of the (cren-)archaea. *Nucleic Acids Res* **35**: e88.

Berkner S, Wlodkowski A, Albers SV & Lipps G (2010) Inducible and constitutive promoters for genetic systems in *Sulfolobus acidocaldarius*. *Extremophiles* **14**: 249–259.

Bernander R (2007) The cell cycle of *Sulfolobus*. *Mol Microbiol* **66**: 557–562.

Berquist BR, Muller JA & Dassarma S (2006) Genetic systems for halophilic archaea. *Methods in Microbiology: Extremophiles* (Rainey F & Oren A, eds), pp. 649–680. Elsevier/Academic Press, Amsterdam.

Berquist BR, DasSarma P & DasSarma S (2007) Essential and non-essential DNA replication genes in the model halophilic Archaeon, *Halobacterium* sp. NRC-1. *BMC Genet* **8**: 31.

Bertani G & Baresi L (1987) Genetic transformation in the methanogen *Methanococcus voltae* PS. *J Bacteriol* **169**: 2730–2738.

Bidle KA, Hanson TE, Howell K & Nannen J (2007) HMG-CoA reductase is regulated by salinity at the level of transcription in *Haloferax volcanii*. *Extremophiles* **11**: 49–55.

Bidle KA, Kirkland PA, Nannen JL & Maupin-Furlow JA (2008) Proteomic analysis of *Haloferax volcanii* reveals salinity-mediated regulation of the stress response protein PspA. *Microbiology* **154**: 1436–1443.

Bidle KA, Haramaty L, Baggett N, Nannen J & Bidle KD (2010) Tantalizing evidence for caspase-like protein expression and activity in the cellular stress response of Archaea. *Environ Microbiol* **12**: 1161–1172.

Bitan-Banin G, Ortenberg R & Mevarech M (2003) Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene. *J Bacteriol* **185**: 772–778.

Blank CE, Kessler PS & Leigh JA (1995) Genetics in methanogens: transposon insertion mutagenesis of a *Methanococcus maripaludis nifH* gene. *J Bacteriol* **177**: 5773–5777.

Blaseio U & Pfeifer F (1990) Transformation of *Halobacterium halobium*: development of vectors and investigation of gas vesicle synthesis. *P Natl Acad Sci USA* **87**: 6772–6776.

Blumentals II, Brown SH, Schicho RN, Skaja AK, Costantino HR & Kelly RM (1990) The hyperthermophilic archaebacterium, *Pyrococcus furiosus*. Development of culturing protocols, perspectives on scaleup, and potential applications. *Ann NY Acad Sci* **589**: 301–314.

Boccazzi P, Zhang JK & Metcalf WW (2000) Generation of dominant selectable markers for resistance to pseudomonic acid by cloning and mutagenesis of the *ileS* gene from the archaeon *Methanosarcina barkeri* fusaro. *J Bacteriol* **182**: 2611–2618.

Bolhuis H, Palm P, Wende A *et al.* (2006) The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. *BMC Genomics* **7**: 169.

Bonneau R, Facciotti MT, Reiss DJ *et al.* (2007) A predictive model for transcriptional control of physiology in a free living cell. *Cell* **131**: 1354–1365.

Boucher Y, Douady CJ, Papke RT *et al.* (2003) Lateral gene transfer and the origins of prokaryotic groups. *Annu Rev Genet* **37**: 283–328.

Breuert S, Allers T, Spohn G & Soppa J (2006) Regulated polyploidy in halophilic archaea. *PLoS ONE* **1**: e92.

Britton KL, Baker PJ, Fisher M *et al.* (2006) Analysis of protein solvent interactions in glucose dehydrogenase from the extreme halophile *Haloferax mediterranei*. *P Natl Acad Sci USA* **103**: 4846–4851.

Brock TD, Brock KM, Belly RT & Weiss RL (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol* **84**: 54–68.

Brugger K, Redder P, She Q, Confalonieri F, Zivanovic Y & Garrett RA (2002) Mobile elements in archaeal genomes. *FEMS Microbiol Lett* **206**: 131–141.

Bult CJ, White O, Olsen GJ *et al.* (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**: 1058–1073.

Calo D, Kaminski L & Eichler J (2010) Protein glycosylation in Archaea: sweet and extreme. *Glycobiology* **20**: 1065–1076.

Cannio R, Contursi P, Rossi M & Bartolucci S (1998) An autonomously replicating transforming vector for *Sulfolobus solfataricus*. *J Bacteriol* **180**: 3237–3240.

Chaban B, Ng SY, Kanbe M, Saltzman I, Nimmo G, Aizawa S & Jarrell KF (2007) Systematic deletion analyses of the *fla* genes in the flagella operon identify several genes essential for proper assembly and function of flagella in the archaeon, *Methanococcus maripaludis*. *Mol Microbiol* **66**: 596–609.

Charlebois RL, Lam WL, Cline SW & Doolittle WF (1987) Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. *P Natl Acad Sci USA* **84**: 8530–8534.

Charlebois RL, Schalkwyk LC, Hofman JD & Doolittle WF (1991) Detailed physical map and set of overlapping clones covering the genome of the archaebacterium *Haloferax volcanii* DS2. *J Mol Biol* **222**: 509–524.

Christian JH & Waltho JA (1962) Solute concentrations within cells of halophilic and non-halophilic bacteria. *Biochim Biophys Acta* **65**: 506–508.

Clay MD, Cosper CA, Jenney FE Jr, Adams MW & Johnson MK (2003) Nitric oxide binding at the mononuclear active site of reduced *Pyrococcus furiosus* superoxide reductase. *P Natl Acad Sci USA* **100**: 3796–3801.

Cline SW & Doolittle WF (1987) Efficient transfection of the archaebacterium *Halobacterium halobium*. *J Bacteriol* **169**: 1341–1344.

Cline SW & Doolittle WF (1992) Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. *J Bacteriol* **174**: 1076–1080.

Cline SW, Lam WL, Charlebois RL, Schalkwyk LC & Doolittle WF (1989) Transformation methods for halophilic archaebacteria. *Can J Microbiol* **35**: 148–152.

Cohen A, Lam WL, Charlebois RL, Doolittle WF & Schalkwyk LC (1992) Localizing genes on the map of the genome of *Haloferax volcanii*, one of the Archaea. *P Natl Acad Sci USA* **89**: 1602–1606.

Cohen GN, Barbe V, Flament D *et al.* (2003) An integrated analysis of the genome of the hyperthermophilic archaeon *Pyrococcus abyssi*. *Mol Microbiol* **47**: 1495–1512.

Coker JA, DasSarma P, Kumar J, Muller JA & DasSarma S (2007) Transcriptional profiling of the model Archaeon *Halobacterium* sp. NRC-1: responses to changes in salinity and temperature. *Saline Systems* **3**: 6.

Costa KC, Wong PM, Wang T *et al.* (2010) Protein complexing in a methanogen suggests electron bifurcation and electron delivery from formate to heterodisulfide reductase. *P Natl Acad Sci USA* **107**: 11050–11055.

Crowley DJ, Boubriak I, Berquist BR *et al.* (2006) The *uvrA*, *uvrB* and *uvrC* genes are required for repair of ultraviolet light induced DNA photoproducts in *Halobacterium* sp. NRC-1. *Saline Systems* **2**: 11.

Daniel RM, Peterson ME, Danson MJ *et al.* (2010) The molecular basis of the effect of temperature on enzyme activity. *Biochem J* **425**: 353–360.

Danner S & Soppa J (1996) Characterization of the distal promoter element of halobacteria *in vivo* using saturation mutagenesis and selection. *Mol Microbiol* **19**: 1265–1276.

Danno A, Fukuda W, Yoshida M *et al.* (2008) Expression profiles and physiological roles of two types of prefoldins from the

hyperthermophilic archaeon *Thermococcus kodakaraensis*. *J Mol Biol* **382**: 298–311.

DasSarma S (1995) Natural plasmids and plasmid vectors of halophiles. *Archaea: A Laboratory Manual, Vol. Halophiles* (DasSarma S & Fleischmann EM, eds), pp. 241–252. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

DasSarma S, Berquist BR, Coker JA, DasSarma P & Muller JA (2006) Post-genomics of the model haloarchaeon *Halobacterium* sp. NRC-1. *Saline Systems* **2**: 3.

Delfosse V, Girard E, Birck C *et al.* (2009) Structure of the archaeal pab87 peptidase reveals a novel self-compartmentalizing protease family. *PLoS One* **4**: e4712.

Delmas S, Shunburne L, Ngo HP & Allers T (2009) Mre11-Rad50 promotes rapid repair of DNA damage in the polyploid archaeon *Haloferax volcanii* by restraining homologous recombination. *PLoS Genet* **5**: e1000552.

DeLong EF (1998) Everything in moderation: archaea as 'non-extremophiles'. *Curr Opin Genet Dev* **8**: 649–654.

Deng L, Zhu H, Chen Z, Liang YX & She Q (2009) Unmarked gene deletion and host–vector system for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*. *Extremophiles* **13**: 735–746.

Dodsworth JA & Leigh JA (2006) Regulation of nitrogenase by 2-oxoglutarate-reversible, direct binding of a PII-like nitrogen sensor protein to dinitrogenase. *P Natl Acad Sci USA* **103**: 9779–9784.

Dodsworth JA, Cady NC & Leigh JA (2005) 2-Oxoglutarate and the PII homologues NifI1 and NifI2 regulate nitrogenase activity in cell extracts of *Methanococcus maripaludis*. *Mol Microbiol* **56**: 1527–1538.

Dodsworth JA, Li L, Wei S, Hedlund BP, Leigh JA & de Figueiredo P (2010) Interdomain conjugal transfer of DNA from bacteria to archaea. *Appl Environ Microb* **76**: 5644–5647.

Dong G, Vieille C, Savchenko A & Zeikus JG (1997a) Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Appl Environ Microb* **63**: 3569–3576.

Dong G, Vieille C & Zeikus JG (1997b) Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Appl Environ Microb* **63**: 3577–3584.

Doolittle WF & Zhaxybayeva O (2009) On the origin of prokaryotic species. *Genome Res* **19**: 744–756.

Driskill LE, Bauer MW & Kelly RM (1999) Synergistic interactions among β-laminarinase, β-1,4-glucanase, and β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* during hydrolysis of β-1,4-, β-1,3-, and mixed-linked polysaccharides. *Biotechnol Bioeng* **66**: 51–60.

Du X, Choi IG, Kim R, Wang W, Jancarik J, Yokota H & Kim SH (2000) Crystal structure of an intracellular protease from *Pyrococcus horikoshii* at 2Å resolution. *P Natl Acad Sci USA* **97**: 14079–14084.

Duggin IG, McCallum SA & Bell SD (2008) Chromosome replication dynamics in the archaeon *Sulfolobus acidocaldarius*. *P Natl Acad Sci USA* **105**: 16737–16742.

Dura MA, Rosenbaum E, Larabi A, Gabel F, Vellieux FM & Franzetti B (2009) The structural and biochemical characterizations of a novel TET peptidase complex from *Pyrococcus horikoshii* reveal an integrated peptide degradation system in hyperthermophilic Archaea. *Mol Microbiol* **72**: 26–40.

Dyall-Smith M (2009) *The Halohandbook – Protocols for haloarchaeal genetics*. Available at http://www.haloarchaea.com/resources/halohandbook/index.html

Dyall-Smith M, Tang SL & Bath C (2003) Haloarchaeal viruses: how diverse are they? *Res Microbiol* **154**: 309–313.

Dyall-Smith ML & Doolittle WF (1994) Construction of composite transposons for halophilic Archaea. *Can J Microbiol* **40**: 922–929.

Egorova K & Antranikian G (2005) Industrial relevance of thermophilic Archaea. *Curr Opin Microbiol* **8**: 649–655.

Elazari-Volcani B (1936) Life in the Dead Sea. *Nature* **138**: 467.

Elferink MG, de Wit JG, Demel R, Driessen AJ & Konings WN (1992) Functional reconstitution of membrane proteins in monolayer liposomes from bipolar lipids of *Sulfolobus acidocaldarius*. *J Biol Chem* **267**: 1375–1381.

Elferink MG, Albers SV, Konings WN & Driessen AJ (2001) Sugar transport in *Sulfolobus solfataricus* is mediated by two families of binding protein-dependent ABC transporters. *Mol Microbiol* **39**: 1494–1503.

Ellen AF, Albers SV & Driessen AJ (2010) Comparative study of the extracellular proteome of *Sulfolobus* species reveals limited secretion. *Extremophiles* **14**: 87–98.

El Yacoubi B, Phillips G, Blaby IK, Haas CE, Cruz Y, Greenberg J & de Crecy-Lagard V (2009) A Gateway platform for functional genomics in *Haloferax volcanii*: deletion of three tRNA modification genes. *Archaea* **2**: 211–219.

Facciotti MT, Reiss DJ, Pan M *et al.* (2007) General transcription factor specified global gene regulation in archaea. *P Natl Acad Sci USA* **104**: 4630–4635.

Falb M, Muller K, Konigsmaier L *et al.* (2008) Metabolism of halophilic archaea. *Extremophiles* **12**: 177–196.

Fiala G & Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100 °C. *Arch Microbiol* **145**: 56–61.

Fields S & Johnston M (2005) Cell biology. Whither model organism research? *Science* **307**: 1885–1886.

Fox GE, Magrum LJ, Balch WE, Wolfe RS & Woese CR (1977) Classification of methanogenic bacteria by 16S ribosomal RNA characterization. *P Natl Acad Sci USA* **74**: 4537–4541.

Frols S, Ajon M, Wagner M *et al.* (2008) UV-inducible cellular aggregation of the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by pili formation. *Mol Microbiol* **70**: 938–952.

Fujiwara S, Aki R, Yoshida M, Higashibata H, Imanaka T & Fukuda W (2008) Expression profiles and physiological roles of two types of molecular chaperonins from the

hyperthermophilic archaeon *Thermococcus kodakarensis*. *Appl Environ Microb* **74**: 7306–7312.

Fukuda W, Morimoto N, Imanaka T & Fujiwara S (2008) Agmatine is essential for the cell growth of *Thermococcus kodakaraensis*. *FEMS Microbiol Lett* **287**: 113–120.

Fukui T, Atomi H, Kanai T, Matsumi R, Fujiwara S & Imanaka T (2005) Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. *Genome Res* **15**: 352–363.

Galperin MY, Aravind L & Koonin EV (2000) Aldolases of the DhnA family: a possible solution to the problem of pentose and hexose biosynthesis in archaea. *FEMS Microbiol Lett* **183**: 259–264.

Gardner WL & Whitman WB (1999) Expression vectors for *Methanococcus maripaludis*: overexpression of acetohydroxyacid synthase and beta-galactosidase. *Genetics* **152**: 1439–1447.

Garrett RA & Klenk HP (2007) *Archaea: Evolution, Physiology and Molecular Biology*. Blackwell Publishing, Oxford.

Geiduschek EP & Ouhammouch M (2005) Archaeal transcription and its regulators. *Mol Microbiol* **56**: 1397–1407.

Gernhardt P, Possot O, Foglino M, Sibold L & Klein A (1990) Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. *Mol Gen Genet* **221**: 273–279.

Giladi M, Bitan-Banin G, Mevarech M & Ortenberg R (2002) Genetic evidence for a novel thymidylate synthase in the halophilic archaeon *Halobacterium salinarum* and in *Campylobacter jejuni*. *FEMS Microbiol Lett* **216**: 105–109.

Goede B, Naji S, von Kampen O, Ilg K & Thomm M (2006) Protein–protein interactions in the archaeal transcriptional machinery: binding studies of isolated RNA polymerase subunits and transcription factors. *J Biol Chem* **281**: 30581–30592.

Gonzalez JM, Sheckells D, Viebahn M, Krupatkina D, Borges KM & Robb FT (1999) *Thermococcus waiotapuensis* sp. nov., an extremely thermophilic archaeon isolated from a freshwater hot spring. *Arch Microbiol* **172**: 95–101.

Greve B, Jensen S, Brugger K, Zillig W & Garrett RA (2004) Genomic comparison of archaeal conjugative plasmids from *Sulfolobus*. *Archaea* **1**: 231–239.

Grogan DW (1989) Phenotypic characterization of the archaebacterial genus *Sulfolobus*: comparison of five wild-type strains. *J Bacteriol* **171**: 6710–6719.

Gueguen Y, Voorhorst WG, van der Oost J & de Vos WM (1997) Molecular and biochemical characterization of an endo-β-1,3-glucanase of the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **272**: 31258–31264.

Guss AM, Rother M, Zhang JK, Kulkarni G & Metcalf WW (2008) New methods for tightly regulated gene expression and highly efficient chromosomal integration of cloned genes for *Methanosarcina* species. *Archaea* **2**: 193–203.

Hale CR, Zhao P, Olson S *et al.* (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139**: 945–956.

Halio SB, Blumentals II, Short SA, Merrill BM & Kelly RM (1996) Sequence, expression in *Escherichia coli*, and analysis of the gene encoding a novel intracellular protease (PfpI) from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* **178**: 2605–2612.

Harrison FC & Kennedy ME (1922) The red discoloration of cured cod fish. *T Roy Soc Can Sect V* **16**: 101–152.

Hartman AL, Norais C, Badger JH *et al.* (2010) The complete genome sequence of *Haloferax volcanii* DS2, a model archaeon. *PLoS ONE* **5**: e9605.

Hasenohrl D, Lombo T, Kaberdin V, Londei P & Blasi U (2008) Translation initiation factor a/eIF2(-gamma) counteracts 5′ to 3′ mRNA decay in the archaeon *Sulfolobus solfataricus*. *P Natl Acad Sci USA* **105**: 2146–2150.

Hashimoto H, Inoue T, Nishioka M, Fujiwara S, Takagi M, Imanaka T & Kai Y (1999) Hyperthermostable protein structure maintained by intra-and inter-helix ion-pairs in archaeal O6-methylguanine-DNA methyltransferase. *J Mol Biol* **292**: 707–716.

Hashimoto Y, Yamamoto T, Fujiwara S, Takagi M & Imanaka T (2001) Extracellular synthesis, specific recognition, and intracellular degradation of cyclomaltodextrins by the hyperthermophilic archaeon *Thermococcus* sp. strain B1001. *J Bacteriol* **183**: 5050–5057.

Haydock AK, Porat I, Whitman WB & Leigh JA (2004) Continuous culture of *Methanococcus maripaludis* under defined nutrient conditions. *FEMS Microbiol Lett* **238**: 85–91.

Hechler T & Pfeifer F (2009) Anaerobiosis inhibits gas vesicle formation in halophilic Archaea. *Mol Microbiol* **71**: 132–145.

Hendrickson EL & Leigh JA (2008) Roles of coenzyme F420-reducing hydrogenases and hydrogen- and F420-dependent methylenetetrahydromethanopterin dehydrogenases in reduction of F420 and production of hydrogen during methanogenesis. *J Bacteriol* **190**: 4818–4821.

Hendrickson EL, Haydock AK, Moore BC, Whitman WB & Leigh JA (2007) Functionally distinct genes regulated by hydrogen limitation and growth rate in methanogenic Archaea. *P Natl Acad Sci USA* **104**: 8930–8934.

Hendrickson EL, Liu Y, Rosas-Sandoval G, Porat I, Soll D, Whitman WB & Leigh JA (2008) Global responses of *Methanococcus maripaludis* to specific nutrient limitations and growth rate. *J Bacteriol* **190**: 2198–2205.

Hering O, Brenneis M, Beer J, Suess B & Soppa J (2009) A novel mechanism for translation initiation operates in haloarchaea. *Mol Microbiol* **71**: 1451–1463.

Hilpert R, Winter J, Hammes W & Kandler O (1981) The sensitivity of archaebacteria to antibiotics. *Zbl Bakt Hyg I C* **2**: 11–20.

Hirata A, Kanai T, Santangelo TJ *et al.* (2008a) Archaeal RNA polymerase subunits E and F are not required for transcription *in vitro*, but a *Thermococcus kodakarensis* mutant lacking subunit F is temperature-sensitive. *Mol Microbiol* **70**: 623–633.

Hirata A, Klein BJ & Murakami KS (2008b) The X-ray crystal structure of RNA polymerase from Archaea. *Nature* **451**: 851–854.

Holden JF, Takai K, Summit M, Bolton S, Zyskowski J & Baross JA (2001) Diversity among three novel groups of hyperthermophilic deep-sea *Thermococcus* species from three sites in the northeastern Pacific Ocean. *FEMS Microbiol Ecol* **36**: 51–60.

Holmes M, Pfeifer F & Dyall-Smith M (1994) Improved shuttle vectors for *Haloferax volcanii* including a dual-resistance plasmid. *Gene* **146**: 117–121.

Holmes ML & Dyall-Smith ML (1991) Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria. *J Bacteriol* **173**: 642–648.

Holmes ML & Dyall-Smith ML (2000) Sequence and expression of a halobacterial beta-galactosidase gene. *Mol Microbiol* **36**: 114–122.

Hopkins BB & Paull TT (2008) The *P. furiosus* mre11/rad50 complex promotes 5′ strand resection at a DNA double-strand break. *Cell* **135**: 250–260.

Hovey R, Lentes S, Ehrenreich A *et al.* (2005) DNA microarray analysis of *Methanosarcina mazei* Go1 reveals adaptation to different methanogenic substrates. *Mol Genet Genomics* **273**: 225–239.

Huber G & Stetter KO (1991) *Sulfolobus metallicus*, sp. nov., a novel strictly chemolithotrophic thermophilic archaeal species of metal-mobilizers. *Syst Appl Microbiol* **14**: 372–378.

Huet J, Schnabel R, Sentenac A & Zillig W (1983) Archaebacteria and eukaryotes possess DNA-dependent RNA polymerases of a common type. *EMBO J* **2**: 1291–1294.

Hugenholtz P, Goebel BM & Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**: 4765–4774.

Humbard MA, Miranda HV, Lim JM *et al.* (2010) Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii*. *Nature* **463**: 54–60.

Hura GL, Menon AL, Hammel M *et al.* (2009) Robust, high-throughput solution structural analyses by small angle X-ray scattering (SAXS). *Nat Methods* **6**: 606–612.

Hutcheon GW & Bolhuis A (2003) The archaeal twin-arginine translocation pathway. *Biochem Soc T* **31**: 686–689.

Imanaka H, Fukui T, Atomi H & Imanaka T (2002) Gene cloning and characterization of fructose-1,6-bisphosphate aldolase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J Biosci Bioeng* **94**: 237–243.

Imanaka H, Yamatsu A, Fukui T, Atomi H & Imanaka T (2006) Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden–Meyerhof pathway in *Thermococcus kodakarensis*. *Mol Microbiol* **61**: 898–909.

Jager D, Sharma CM, Thomsen J, Ehlers C, Vogel J & Schmitz RA (2009) Deep sequencing analysis of the *Methanosarcina mazei* Go1 transcriptome in response to nitrogen availability. *P Natl Acad Sci USA* **106**: 21878–21882.

Jarrell KF, Jones GM, Kandiba L, Nair DB & Eichler J (2010) S-layer glycoproteins and flagellins: reporters of archaeal posttranslational modifications. *Archaea* **2010** pii: 612948.

Jenney FE Jr, Verhagen MF, Cui X & Adams MW (1999) Anaerobic microbes: oxygen detoxification without superoxide dismutase. *Science* **286**: 306–309.

Jeon BS, Taguchi H, Sakai H, Ohshima T, Wakagi T & Matsuzawa H (1997) 4-α-glucanotransferase from the hyperthermophilic archaeon *Thermococcus litoralis* – enzyme purification and characterization, and gene cloning, sequencing and expression in *Escherichia coli*. *Eur J Biochem* **248**: 171–178.

Jonuscheit M, Martusewitsch E, Stedman KM & Schleper C (2003) A reporter gene system for the hyperthermophilic archaeon *Sulfolobus solfataricus* based on a selectable and integrative shuttle vector. *Mol Microbiol* **48**: 1241–1252.

Kanai T, Akerboom J, Takedomi S *et al.* (2007) A global transcriptional regulator in *Thermococcus kodakaraensis* controls the expression levels of both glycolytic and gluconeogenic enzyme-encoding genes. *J Biol Chem* **282**: 33659–33670.

Kanai T, Takedomi S, Fujiwara S, Atomi H & Imanaka T (2010) Identification of the Phr-dependent heat shock regulon in the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. *J Biochem* **147**: 361–370.

Kanzaki T, Iizuka R, Takahashi K *et al.* (2008) Sequential action of ATP-dependent subunit conformational change and interaction between helical protrusions in the closure of the built-in lid of group II chaperonins. *J Biol Chem* **283**: 34773–34784.

Karner MB, DeLong EF & Karl DM (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409**: 507–510.

Kasting JF & Siefert JL (2002) Life and the evolution of Earth's atmosphere. *Science* **296**: 1066–1068.

Kaur A, Pan M, Meislin M, Facciotti MT, El-Gewely R & Baliga NS (2006) A systems view of haloarchaeal strategies to withstand stress from transition metals. *Genome Res* **16**: 841–854.

Kawarabayasi Y, Sawada M, Horikawa H *et al.* (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. *DNA Res* **5**: 55–76.

Keese AM, Schut GJ, Ouhammouch M, Adams MW & Thomm M (2010) Genome-wide identification of targets for the archaeal heat shock regulator phr by cell-free transcription of genomic DNA. *J Bacteriol* **192**: 1292–1298.

Kengen SW, Luesink EJ, Stams AJ & Zehnder AJ (1993) Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur J Biochem* **213**: 305–312.

Kengen SW, de Bok FA, van Loo ND, Dijkema C, Stams AJ & de Vos WM (1994) Evidence for the operation of a novel Embden–Meyerhof pathway that involves ADP-dependent kinases during sugar fermentation by *Pyrococcus furiosus*. *J Biol Chem* **269**: 17537–17541.

Kengen SW, Tuininga JE, de Bok FA, Stams AJ & de Vos WM (1995) Purification and characterization of a novel ADP-

dependent glucokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **270**: 30453–30457.

Kennedy SP, Ng WV, Salzberg SL, Hood L & DasSarma S (2001) Understanding the adaptation of *Halobacterium* species NRC-1 to its extreme environment through computational analysis of its genome sequence. *Genome Res* **11**: 1641–1650.

Kida H, Sugano Y, Iizuka R, Fujihashi M, Yohda M & Miki K (2008) Structural and molecular characterization of the prefoldin beta subunit from *Thermococcus* strain KS-1. *J Mol Biol* **383**: 465–474.

Kim YJ, Lee HS, Kim ES *et al.* (2010) Formate-driven growth coupled with H(2) production. *Nature* **467**: 352–355.

Kirkland PA, Gil MA, Karadzic IM & Maupin-Furlow JA (2008) Genetic and proteomic analyses of a proteasome-activating nucleotidase A mutant of the haloarchaeon *Haloferax volcanii*. *J Bacteriol* **190**: 193–205.

Kiyonari S, Tahara S, Uchimura M, Shirai T, Ishino S & Ishino Y (2009) Studies on the base excision repair (BER) complex in *Pyrococcus furiosus*. *Biochem Soc T* **37**: 79–82.

Knittel K & Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* **63**: 311–334.

Konneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB & Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.

Korkhin Y, Unligil UM, Littlefield O *et al.* (2009) Evolution of complex RNA polymerases: the complete Archaeal RNA polymerase structure. *PLoS Biol* **7**: e102.

Krebs MP, Hauss T, Heyn MP, RajBhandary UL & Khorana HG (1991) Expression of the bacterioopsin gene in *Halobacterium halobium* using a multicopy plasmid. *P Natl Acad Sci USA* **88**: 859–863.

Kulkarni G, Kridelbaugh DM, Guss AM & Metcalf WW (2009) Hydrogen is a preferred intermediate in the energy-conserving electron transport chain of *Methanosarcina barkeri*. *P Natl Acad Sci USA* **106**: 15915–15920.

Kuo YP, Thompson DK, St Jean A, Charlebois RL & Daniels CJ (1997) Characterization of two heat shock genes from *Haloferax volcanii*: a model system for transcription regulation in the Archaea. *J Bacteriol* **179**: 6318–6324.

Kurosawa N & Grogan DW (2005) Homologous recombination of exogenous DNA with the *Sulfolobus acidocaldarius* genome: properties and uses. *FEMS Microbiol Lett* **253**: 141–149.

Ladapo J & Whitman WB (1990) Method for isolation of auxotrophs in the methanogenic archaebacteria: role of the acetyl-CoA pathway of autotrophic CO_2 fixation in *Methanococcus maripaludis*. *P Natl Acad Sci USA* **87**: 5598–5602.

Laderman KA, Asada K, Uemori T, Mukai H, Taguchi Y, Kato I & Anfinsen CB (1993) α-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Cloning and sequencing of the gene and expression in *Escherichia coli*. *J Biol Chem* **268**: 24402–24407.

Laksanalamai P & Robb FT (2004) Small heat shock proteins from extremophiles: a review. *Extremophiles* **8**: 1–11.

Lam WL & Doolittle WF (1989) Shuttle vectors for the archaebacterium *Halobacterium volcanii*. *P Natl Acad Sci USA* **86**: 5478–5482.

Lam WL & Doolittle WF (1992) Mevinolin-resistant mutations identify a promoter and the gene for a eukaryote-like 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the archaebacterium *Haloferax volcanii*. *J Biol Chem* **267**: 5829–5834.

Lanyi JK (1974) Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriol Rev* **38**: 272–290.

Large A, Stamme C, Lange C, Duan Z, Allers T, Soppa J & Lund PA (2007) Characterization of a tightly controlled promoter of the halophilic archaeon *Haloferax volcanii* and its use in the analysis of the essential *cct1* gene. *Mol Microbiol* **66**: 1092–1106.

Lee HS, Shockley KR, Schut GJ *et al.* (2006) Transcriptional and biochemical analysis of starch metabolism in the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* **188**: 2115–2125.

Lee HS, Kang SG, Bae SS *et al.* (2008) The complete genome sequence of *Thermococcus onnurineus* NA1 reveals a mixed heterotrophic and carboxydotrophic metabolism. *J Bacteriol* **190**: 7491–7499.

Lee SJ, Moulakakis C, Koning SM, Hausner W, Thomm M & Boos W (2005) TrmB, a sugar sensing regulator of ABC transporter genes in *Pyrococcus furiosus* exhibits dual promoter specificity and is controlled by different inducers. *Mol Microbiol* **57**: 1797–1807.

Lee SJ, Surma M, Seitz S, Hausner W, Thomm M & Boos W (2007) Characterization of the TrmB-like protein, PF0124, a TGM-recognizing global transcriptional regulator of the hyperthermophilic archaeon *Pyrococcus furiosus*. *Mol Microbiol* **65**: 305–318.

Lessner DJ, Li L, Li Q *et al.* (2006) An unconventional pathway for reduction of CO_2 to methane in CO-grown *Methanosarcina acetivorans* revealed by proteomics. *P Natl Acad Sci USA* **103**: 17921–17926.

Lestini R, Duan Z & Allers T (2010) The archaeal Xpf/Mus81/FANCM homolog Hef and the Holliday junction resolvase Hjc define alternative pathways that are essential for cell viability in *Haloferax volcanii*. *DNA Repair (Amst)* **9**: 994–1002.

Levin I, Giladi M, Altman-Price N, Ortenberg R & Mevarech M (2004) An alternative pathway for reduced folate biosynthesis in bacteria and halophilic archaea. *Mol Microbiol* **54**: 1307–1318.

Li Q, Li L, Rejtar T, Karger BL & Ferry JG (2005a) Proteome of *Methanosarcina acetivorans* Part I: an expanded view of the biology of the cell. *J Proteome Res* **4**: 112–128.

Li Q, Li L, Rejtar T, Karger BL & Ferry JG (2005b) Proteome of *Methanosarcina acetivorans* Part II: comparison of protein levels in acetate- and methanol-grown cells. *J Proteome Res* **4**: 129–135.

Lie TJ & Leigh JA (2002) Regulatory response of *Methanococcus maripaludis* to alanine, an intermediate nitrogen source. *J Bacteriol* **184**: 5301–5306.

Lie TJ & Leigh JA (2003) A novel repressor of *nif* and *glnA* expression in the methanogenic archaeon *Methanococcus maripaludis*. *Mol Microbiol* **47**: 235–246.

Lie TJ & Leigh JA (2007) Genetic screen for regulatory mutations in *Methanococcus maripaludis* and its use in identification of induction-deficient mutants of the euryarchaeal repressor NrpR. *Appl Environ Microb* **73**: 6595–6600.

Lie TJ, Wood GE & Leigh JA (2005) Regulation of *nif* expression in *Methanococcus maripaludis*: roles of the euryarchaeal repressor NrpR, 2-oxoglutarate, and two operators. *J Biol Chem* **280**: 5236–5241.

Lindas AC, Karlsson EA, Lindgren MT, Ettema TJ & Bernander R (2008) A unique cell division machinery in the Archaea. *P Natl Acad Sci USA* **105**: 18942–18946.

Lipscomb GL, Stirrett K, Schut GJ, Yang F, Jenney FE Jr, Scott RA, Michael WW & Westpheling J (2011) Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: construction of multiple markerless deletions of genes encoding the two cytoplasmic hydrogenases. *Appl Environ Microbiol* (in press).

Liu H, Rudolf J, Johnson KA *et al.* (2008) Structure of the DNA repair helicase XPD. *Cell* **133**: 801–812.

Liu Y & Whitman WB (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann NY Acad Sci* **1125**: 171–189.

Lopez-Garcia P, St Jean A, Amils R & Charlebois RL (1995) Genomic stability in the archaea *Haloferax volcanii* and *Haloferax mediterranei*. *J Bacteriol* **177**: 1405–1408.

Lucas S, Toffin L, Zivanovic Y *et al.* (2002) Construction of a shuttle vector for, and spheroplast transformation of, the hyperthermophilic archaeon *Pyrococcus abyssi*. *Appl Environ Microb* **68**: 5528–5536.

Lundgren M, Malandrin L, Eriksson S, Huber H & Bernander R (2008) Cell cycle characteristics of crenarchaeota: unity among diversity. *J Bacteriol* **190**: 5362–5367.

Maaty WS, Wiedenheft B, Tarlykov P *et al.* (2009) Something old, something new, something borrowed; how the thermoacidophilic archaeon *Sulfolobus solfataricus* responds to oxidative stress. *PLoS One* **4**: e6964.

Mahapatra A, Patel A, Soares JA, Larue RC, Zhang JK, Metcalf WW & Krzycki JA (2006) Characterization of a *Methanosarcina acetivorans* mutant unable to translate UAG as pyrrolysine. *Mol Microbiol* **59**: 56–66.

Mahapatra A, Srinivasan G, Richter KB *et al.* (2007) Class I and class II lysyl-tRNA synthetase mutants and the genetic encoding of pyrrolysine in *Methanosarcina* spp. *Mol Microbiol* **64**: 1306–1318.

Maher MJ, Ghosh M, Grunden AM, Menon AL, Adams MW, Freeman HC & Guss JM (2004) Structure of the prolidase from *Pyrococcus furiosus*. *Biochemistry* **43**: 2771–2783.

Mardanov AV, Ravin NV, Svetlitchnyi VA, Beletsky AV, Miroshnichenko ML, Bonch-Osmolovskaya EA & Skryabin KG (2009) Metabolic versatility and indigenous origin of the archaeon *Thermococcus sibiricus*, isolated from a siberian oil reservoir, as revealed by genome analysis. *Appl Environ Microb* **75**: 4580–4588.

Marg BL, Schweimer K, Sticht H & Oesterhelt D (2005) A two-alpha-helix extra domain mediates the halophilic character of a plant-type ferredoxin from halophilic archaea. *Biochemistry* **44**: 29–39.

Margesin R & Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* **5**: 73–83.

Martusewitsch E, Sensen CW & Schleper C (2000) High spontaneous mutation rate in the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by transposable elements. *J Bacteriol* **182**: 2574–2581.

Matsui I, Sakai Y, Matsui E, Kikuchi H, Kawarabayasi Y & Honda K (2000) Novel substrate specificity of a membrane-bound β-glycosidase from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *FEBS Lett* **467**: 195–200.

Matsumi R, Manabe K, Fukui T, Atomi H & Imanaka T (2007) Disruption of a sugar transporter gene cluster in a hyperthermophilic archaeon using a host-marker system based on antibiotic resistance. *J Bacteriol* **189**: 2683–2691.

Mayanagi K, Kiyonari S, Saito M, Shirai T, Ishino Y & Morikawa K (2009) Mechanism of replication machinery assembly as revealed by the DNA ligase-PCNA-DNA complex architecture. *P Natl Acad Sci USA* **106**: 4647–4652.

McCready S & Marcello L (2003) Repair of UV damage in *Halobacterium salinarum*. *Biochem Soc T* **31**: 694–698.

McCready S, Muller JA, Boubriak I, Berquist BR, Ng WL & Dassarma S (2005) UV irradiation induces homologous recombination genes in the model archaeon, *Halobacterium* sp. NRC-1. *Saline Systems* **1**: 3.

Menon AL, Poole FL II, Cvetkovic A *et al.* (2009) Novel multiprotein complexes identified in the hyperthermophilic archaeon *Pyrococcus furiosus* by non-denaturing fractionation of the native proteome. *Mol Cell Proteomics* **8**: 735–751.

Metcalf WW, Zhang JK, Apolinario E, Sowers KR & Wolfe RS (1997) A genetic system for Archaea of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. *P Natl Acad Sci USA* **94**: 2626–2631.

Meuer J, Kuettner HC, Zhang JK, Hedderich R & Metcalf WW (2002) Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation. *P Natl Acad Sci USA* **99**: 5632–5637.

Mevarech M & Werczberger R (1985) Genetic transfer in *Halobacterium volcanii*. *J Bacteriol* **162**: 461–462.

Mevarech M, Frolow F & Gloss LM (2000) Halophilic enzymes: proteins with a grain of salt. *Biophys Chem* **86**: 155–164.

Mizanur RM, Griffin AK & Pohl NL (2008) Recombinant production and biochemical characterization of a hyperthermostable α-glucan/maltodextrin phosphorylase from *Pyrococcus furiosus*. *Archaea* **2**: 169–176.

Moll R & Schäfer G (1988) Chemiosmotic H+ cycling across the plasma membrane of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *FEBS Lett* **232**: 359–363.

Moore BC & Leigh JA (2005) Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J Bacteriol* **187**: 972–979.

Morikawa M, Izawa Y, Rashid N, Hoaki T & Imanaka T (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. *Appl Environ Microb* **60**: 4559–4566.

Mueller M, Takemasa R, Schwarz A, Atomi H & Nidetzky B (2009) "Short-chain" alpha-1,4-glucan phosphorylase having a truncated N-terminal domain: functional expression and characterization of the enzyme from *Sulfolobus solfataricus*. *Biochim Biophys Acta* **1794**: 1709–1714.

Mukund S & Adams MW (1995) Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **270**: 8389–8392.

Mullakhanbhai MF & Larsen H (1975) *Halobacterium volcanii* spec. nov., a Dead Sea halobacterium with a moderate salt requirement. *Arch Microbiol* **104**: 207–214.

Muller JA & DasSarma S (2005) Genomic analysis of anaerobic respiration in the archaeon *Halobacterium* sp. strain NRC-1: dimethyl sulfoxide and trimethylamine N-oxide as terminal electron acceptors. *J Bacteriol* **187**: 1659–1667.

Murakami T, Kanai T, Takata H, Kuriki T & Imanaka T (2006) A novel branching enzyme of the GH-57 family in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J Bacteriol* **188**: 5915–5924.

Neves C, da Costa MS & Santos H (2005) Compatible solutes of the hyperthermophile *Palaeococcus ferrophilus*: osmoadaptation and thermoadaptation in the order thermococcales. *Appl Environ Microb* **71**: 8091–8098.

Ng SY, Chaban B & Jarrell KF (2006) Archaeal flagella, bacterial flagella and type IV pili: a comparison of genes and post-translational modifications. *J Mol Microb Biotech* **11**: 167–191.

Ng WV, Kennedy SP, Mahairas GG *et al.* (2000) Genome sequence of *Halobacterium* species NRC-1. *P Natl Acad Sci USA* **97**: 12176–12181.

Nishida H, Mayanagi K, Kiyonari S, Sato Y, Oyama T, Ishino Y & Morikawa K (2009) Structural determinant for switching between the polymerase and exonuclease modes in the PCNA-replicative DNA polymerase complex. *P Natl Acad Sci USA* **106**: 20693–20698.

Nomura S & Harada Y (1998) Functional expression of green fluorescent protein derivatives in *Halobacterium salinarum*. *FEMS Microbiol Lett* **167**: 287–293.

Norais C, Hawkins M, Hartman AL, Eisen JA, Myllykallio H & Allers T (2007) Genetic and physical mapping of DNA replication origins in *Haloferax volcanii*. *PLoS Genet* **3**: e77.

Oesterhelt D & Stoeckenius W (1971) Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat New Biol* **233**: 149–152.

Olsen GJ & Woese CR (1996) Lessons from an Archaeal genome: what are we learning from *Methanococcus jannaschii*? *Trends Genet* **12**: 377–379.

Oren A (1999) Bioenergetic aspects of halophilism. *Microbiol Mol Biol R* **63**: 334–348.

Oren A (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems* **4**: 2.

Oren A (2010) Industrial and environmental applications of halophilic microorganisms. *Environ Technol* **31**: 825–834.

Oren A, Heldal M, Norland S & Galinski EA (2002) Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*. *Extremophiles* **6**: 491–498.

Oren A, Arahal DR & Ventosa A (2009) Emended descriptions of genera of the family Halobacteriaceae. *Int J Syst Evol Micr* **59**: 637–642.

Orita I, Sato T, Yurimoto H, Kato N, Atomi H, Imanaka T & Sakai Y (2006) The ribulose monophosphate pathway substitutes for the missing pentose phosphate pathway in the archaeon *Thermococcus kodakaraensis*. *J Bacteriol* **188**: 4698–4704.

Orphan VJ, Taylor LT, Hafenbradl D & Delong EF (2000) Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl Environ Microb* **66**: 700–711.

Papke RT, Koenig JE, Rodriguez-Valera F & Doolittle WF (2004) Frequent recombination in a saltern population of *Halorubrum*. *Science* **306**: 1928–1929.

Papke RT, Zhaxybayeva O, Feil EJ, Sommerfeld K, Muise D & Doolittle WF (2007) Searching for species in haloarchaea. *P Natl Acad Sci USA* **104**: 14092–14097.

Patenge N, Haase A, Bolhuis H & Oesterhelt D (2000) The gene for a halophilic beta-galactosidase (*bgaH*) of *Haloferax alicantei* as a reporter gene for promoter analyses in *Halobacterium salinarum*. *Mol Microbiol* **36**: 105–113.

Peck RF, Dassarma S & Krebs MP (2000) Homologous gene knockout in the archaeon *Halobacterium salinarum* with *ura3* as a counterselectable marker. *Mol Microbiol* **35**: 667–676.

Peeters E, Albers SV, Vassart A, Driessen AJ & Charlier D (2009) Ss-LrpB, a transcriptional regulator from *Sulfolobus solfataricus*, regulates a gene cluster with a pyruvate ferredoxin oxidoreductase-encoding operon and permease genes. *Mol Microbiol* **71**: 972–988.

Peng N, Xia Q, Chen Z, Liang YX & She Q (2009) An upstream activation element exerting differential transcriptional activation on an archaeal promoter. *Mol Microbiol* **74**: 928–939.

Peng X, Holz I, Zillig W, Garrett RA & She Q (2000) Evolution of the family of pRN plasmids and their integrase-mediated insertion into the chromosome of the crenarchaeon *Sulfolobus solfataricus*. *J Mol Biol* **303**: 449–454.

Pfeifer F & Betlach M (1985) Genome organization in *Halobacterium halobium*: a 70 kb island of more (AT) rich DNA in the chromosome. *Mol Gen Genet* **198**: 449–455.

Pfeifer F, Gregor D, Hofacker A, Plosser P & Zimmermann P (2002) Regulation of gas vesicle formation in halophilic archaea. *J Mol Microb Biotech* **4**: 175–181.

Pfeiffer F, Broicher A, Gillich T, Klee K, Mejia J, Rampp M & Oesterhelt D (2008a) Genome information management and integrated data analysis with HaloLex. *Arch Microbiol* **190**: 281–299.

Pfeiffer F, Schuster SC, Broicher A *et al.* (2008b) Evolution in the laboratory: the genome of *Halobacterium salinarum* strain R1 compared to that of strain NRC-1. *Genomics* **91**: 335–346.

Pfluger K, Ehrenreich A, Salmon K, Gunsalus RP, Deppenmeier U, Gottschalk G & Muller V (2007) Identification of genes involved in salt adaptation in the archaeon *Methanosarcina mazei* Go1 using genome-wide gene expression profiling. *FEMS Microbiol Lett* **277**: 79–89.

Porat I & Whitman WB (2009) Tryptophan auxotrophs were obtained by random transposon insertions in the *Methanococcus maripaludis* tryptophan operon. *FEMS Microbiol Lett* **297**: 250–254.

Porat I, Kim W, Hendrickson EL *et al.* (2006) Disruption of the operon encoding Ehb hydrogenase limits anabolic CO2 assimilation in the archaeon *Methanococcus maripaludis*. *J Bacteriol* **188**: 1373–1380.

Prangishvili D, Albers SV, Holz I *et al.* (1998) Conjugation in archaea: frequent occurrence of conjugative plasmids in *Sulfolobus*. *Plasmid* **40**: 190–202.

Prangishvili D, Forterre P & Garrett RA (2006) Viruses of the Archaea: a unifying view. *Nat Rev Microbiol* **4**: 837–848.

Prangishvili DA, Vashakidze RP, Chelidze MG & Gabriadze I (1985) A restriction endonuclease *Sua*I from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *FEBS Lett* **192**: 57–60.

Pritchett MA, Zhang JK & Metcalf WW (2004) Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. *Appl Environ Microb* **70**: 1425–1433.

Rahman RN, Fujiwara S, Nakamura H, Takagi M & Imanaka T (1998) Ion pairs involved in maintaining a thermostable structure of glutamate dehydrogenase from a hyperthermophilic archaeon. *Biochem Bioph Res Co* **248**: 920–926.

Rashid N, Cornista J, Ezaki S, Fukui T, Atomi H & Imanaka T (2002a) Characterization of an archaeal cyclodextrin glucanotransferase with a novel C-terminal domain. *J Bacteriol* **184**: 777–784.

Rashid N, Imanaka H, Kanai T, Fukui T, Atomi H & Imanaka T (2002b) A novel candidate for the true fructose-1,6-bisphosphatase in archaea. *J Biol Chem* **277**: 30649–30655.

Redder P & Garrett RA (2006) Mutations and rearrangements in the genome of *Sulfolobus solfataricus* P2. *J Bacteriol* **188**: 4198–4206.

Reilly MS & Grogan DW (2001) Characterization of intragenic recombination in a hyperthermophilic archaeon via conjugational DNA exchange. *J Bacteriol* **183**: 2943–2946.

Reno ML, Held NL, Fields CJ, Burke PV & Whitaker RJ (2009) Biogeography of the *Sulfolobus islandicus* pan-genome. *P Natl Acad Sci USA* **106**: 8605–8610.

Reuter CJ & Maupin-Furlow J (2004) Analysis of proteasome-dependent proteolysis in *Haloferax volcanii* cells, using short-lived green fluorescent proteins. *Appl Environ Microb* **70**: 7530–7538.

Rice DW, Yip KS, Stillman TJ *et al.* (1996) Insights into the molecular basis of thermal stability from the structure determination of *Pyrococcus furiosus* glutamate dehydrogenase. *FEMS Microbiol Rev* **18**: 105–117.

Rivera MC, Jain R, Moore JE & Lake JA (1998) Genomic evidence for two functionally distinct gene classes. *P Natl Acad Sci USA* **95**: 6239–6244.

Robb FT, Maeder DL, Brown JR *et al.* (2001) Genomic sequence of hyperthermophile, *Pyrococcus furiosus*: implications for physiology and enzymology. *Method Enzymol* **330**: 134–157.

Robertson CE, Harris JK, Spear JR & Pace NR (2005) Phylogenetic diversity and ecology of environmental Archaea. *Curr Opin Microbiol* **8**: 638–642.

Robinson JL, Pyzyna B, Atrasz RG *et al.* (2005) Growth kinetics of extremely halophilic archaea (family halobacteriaceae) as revealed by arrhenius plots. *J Bacteriol* **187**: 923–929.

Rodrigues MV, Borges N, Henriques M *et al.* (2007) Bifunctional CTP:inositol-1-phosphate cytidylyltransferase/CDP-inositol:inositol-1-phosphate transferase, the key enzyme for di-myo-inositol-phosphate synthesis in several (hyper)thermophiles. *J Bacteriol* **189**: 5405–5412.

Rose RW, Bruser T, Kissinger JC & Pohlschroder M (2002) Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol Microbiol* **45**: 943–950.

Rosenshine I, Tchelet R & Mevarech M (1989) The mechanism of DNA transfer in the mating system of an archaebacterium. *Science* **245**: 1387–1389.

Rother M & Metcalf WW (2005) Genetic technologies for Archaea. *Curr Opin Microbiol* **8**: 745–751.

Rother M, Resch A, Gardner WL, Whitman WB & Bock A (2001) Heterologous expression of archaeal selenoprotein genes directed by the SECIS element located in the 3′ non-translated region. *Mol Microbiol* **40**: 900–908.

Russell RJ, Ferguson JM, Hough DW, Danson MJ & Taylor GL (1997) The crystal structure of citrate synthase from the hyperthermophilic archaeon *Pyrococcus furiosus* at 1.9 A resolution. *Biochemistry* **36**: 9983–9994.

Samson RY, Obita T, Freund SM, Williams RL & Bell SD (2008) A role for the ESCRT system in cell division in archaea. *Science* **322**: 1710–1713.

Santangelo TJ, Cubonova L, James CL & Reeve JN (2007) TFB1 or TFB2 is sufficient for *Thermococcus kodakaraensis* viability and for basal transcription *in vitro*. *J Mol Biol* **367**: 344–357.

Santangelo TJ, Cubonova L, Matsumi R, Atomi H, Imanaka T & Reeve JN (2008a) Polarity in archaeal operon transcription in *Thermococcus kodakaraensis*. *J Bacteriol* **190**: 2244–2248.

Santangelo TJ, Cubonova L & Reeve JN (2008b) Shuttle vector expression in *Thermococcus kodakaraensis*: contributions of cis elements to protein synthesis in a hyperthermophilic archaeon. *Appl Environ Microb* **74**: 3099–3104.

Santangelo TJ, Cubonova L, Skinner KM & Reeve JN (2009) Archaeal intrinsic transcription termination *in vivo*. *J Bacteriol* **191**: 7102–7108.

Santangelo TJ, Cubonova L & Reeve JN (2010) *Thermococcus kodakarensis* genetics: TK1827-encoded β-glycosidase, new positive-selection protocol, and targeted and repetitive deletion technology. *Appl Environ Microb* **76**: 1044–1052.

Sapienza C, Rose MR & Doolittle WF (1982) High-frequency genomic rearrangements involving archaebacterial repeat sequence elements. *Nature* **299**: 182–185.

Sapra R, Bagramyan K & Adams MW (2003) A simple energy-conserving system: proton reduction coupled to proton translocation. *P Natl Acad Sci USA* **100**: 7545–7550.

Sato T, Fukui T, Atomi H & Imanaka T (2003) Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J Bacteriol* **185**: 210–220.

Sato T, Imanaka H, Rashid N, Fukui T, Atomi H & Imanaka T (2004) Genetic evidence identifying the true gluconeogenic fructose-1,6-bisphosphatase in *Thermococcus kodakaraensis* and other hyperthermophiles. *J Bacteriol* **186**: 5799–5807.

Sato T, Fukui T, Atomi H & Imanaka T (2005) Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Appl Environ Microb* **71**: 3889–3899.

Sato T, Atomi H & Imanaka T (2007) Archaeal type III RuBisCOs function in a pathway for AMP metabolism. *Science* **315**: 1003–1006.

Sauerwald A, Zhu W, Major TA *et al.* (2005) RNA-dependent cysteine biosynthesis in archaea. *Science* **307**: 1969–1972.

Schelert J, Dixit V, Hoang V, Simbahan J, Drozda M & Blum P (2004) Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon *Sulfolobus solfataricus* by use of gene disruption. *J Bacteriol* **186**: 427–437.

Schelert J, Drozda M, Dixit V, Dillman A & Blum P (2006) Regulation of mercury resistance in the crenarchaeote *Sulfolobus solfataricus*. *J Bacteriol* **188**: 7141–7150.

Schinzel R & Burger KJ (1986) A site-specific endonuclease activity in *Halobacterium halobium*. *FEMS Microbiol Lett* **37**: 325–329.

Schleper C, Kubo K & Zillig W (1992) The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and of transfection with viral DNA. *P Natl Acad Sci USA* **89**: 7645–7649.

Schleper C, Jurgens G & Jonuscheit M (2005) Genomic studies of uncultivated archaea. *Nat Rev Microbiol* **3**: 479–488.

Schlesner M, Miller A, Streif S *et al.* (2009) Identification of Archaea-specific chemotaxis proteins which interact with the flagellar apparatus. *BMC Microbiol* **9**: 56.

Schut GJ, Brehm SD, Datta S & Adams MW (2003) Whole-genome DNA microarray analysis of a hyperthermophile and an archaeon: *Pyrococcus furiosus* grown on carbohydrates or peptides. *J Bacteriol* **185**: 3935–3947.

She Q, Singh RK, Confalonieri F *et al.* (2001) The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. *P Natl Acad Sci USA* **98**: 7835–7840.

She Q, Zhang C, Deng L, Peng N, Chen Z & Liang YX (2009) Genetic analyses in the hyperthermophilic archaeon *Sulfolobus islandicus*. *Biochem Soc T* **37**: 92–96.

Shimoyama T, Kato S, Ishii S & Watanabe K (2009) Flagellum mediates symbiosis. *Science* **323**: 1574.

Siebers B & Schönheit P (2005) Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Curr Opin Microbiol* **8**: 695–705.

Siebers B, Brinkmann H, Dorr C, Tjaden B, Lilie H, van der Oost J & Verhees CH (2001) Archaeal fructose-1,6-bisphosphate aldolases constitute a new family of archaeal type class I aldolase. *J Biol Chem* **276**: 28710–28718.

Simsek M, DasSarma S, RajBhandary UL & Khorana HG (1982) A transposable element from *Halobacterium halobium* which inactivates the bacteriorhodopsin gene. *P Natl Acad Sci USA* **79**: 7268–7272.

Sisignano M, Morbitzer D, Gatgens J, Oldiges M & Soppa J (2010) A 2-oxoacid dehydrogenase complex of *Haloferax volcanii* is essential for growth on isoleucine but not on other branched-chain amino acids. *Microbiology* **156**: 521–529.

Sollner S, Berkner S & Lipps G (2006) Characterisation of the novel restriction endonuclease *Sui*I from *Sulfolobus islandicus*. *Extremophiles* **10**: 629–634.

Soppa J (2006) From genomes to function: haloarchaea as model organisms. *Microbiology* **152**: 585–590.

Soppa J & Oesterhelt D (1989) Bacteriorhodopsin mutants of *Halobacterium* sp. GRB. I. The 5-bromo-2′-deoxyuridine selection as a method to isolate point mutants in halobacteria. *J Biol Chem* **264**: 13043–13048.

Soppa J, Baumann A, Brenneis M, Dambeck M, Hering O & Lange C (2008) Genomics and functional genomics with haloarchaea. *Arch Microbiol* **190**: 197–215.

Sowers KR, Boone JE & Gunsalus RP (1993) Disaggregation of *Methanosarcina* spp. and growth as single cells at elevated osmolarity. *Appl Environ Microb* **59**: 3832–3839.

Spanheimer R & Muller V (2008) The molecular basis of salt adaptation in *Methanosarcina mazei* Go1. *Arch Microbiol* **190**: 271–279.

Stathopoulos C, Kim W, Li T *et al.* (2001) Cysteinyl-tRNA synthetase is not essential for viability of the archaeon *Methanococcus maripaludis*. *P Natl Acad Sci USA* **98**: 14292–14297.

Stedman KM, Schleper C, Rumpf E & Zillig W (1999) Genetic requirements for the function of the archaeal virus SSV1 in *Sulfolobus solfataricus*: construction and testing of viral shuttle vectors. *Genetics* **152**: 1397–1405.

Stock T, Selzer M & Rother M (2010) *In vivo* requirement of selenophosphate for selenoprotein synthesis in archaea. *Mol Microbiol* **75**: 149–160.

Story SV, Shah C, Jenney FE Jr & Adams MW (2005) Characterization of a novel zinc-containing, lysine-specific aminopeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* **187**: 2077–2083.

Sun J & Klein A (2004) A lysR-type regulator is involved in the negative regulation of genes encoding selenium-free hydrogenases in the archaeon *Methanococcus voltae*. *Mol Microbiol* **52**: 563–571.

Suzuki T, Iwasaki T, Uzawa T *et al.* (2002) *Sulfolobus tokodaii* sp. nov. (f. *Sulfolobus* sp. strain 7), a new member of the genus *Sulfolobus* isolated from Beppu Hot Springs, Japan. *Extremophiles* **6**: 39–44.

Szabo Z, Sani M, Groeneveld M *et al.* (2007) Flagellar motility and structure in the hyperthermoacidophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol* **189**: 4305–4309.

Tachibana Y, Kuramura A, Shirasaka N *et al.* (1999) Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. *Appl Environ Microb* **65**: 1991–1997.

Tadeo X, Lopez-Mendez B, Trigueros T, Lain A, Castano D & Millet O (2009) Structural basis for the aminoacid composition of proteins from halophilic archea. *PLoS Biol* **7**: e1000257.

Takai K, Sugai A, Itoh T & Horikoshi K (2000) *Palaeococcus ferrophilus* gen. nov., sp. nov., a barophilic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney. *Int J Syst Evol Micr* **50**: 489–500.

Tchelet R & Mevarech M (1994) Interspecies genetic transfer in halophilic archaebacteria. *Syst Appl Microbiol* **15**: 578–581.

Thauer RK, Kaster AK, Seedorf H, Buckel W & Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* **6**: 579–591.

Trauger SA, Kalisak E, Kalisiak J *et al.* (2008) Correlating the transcriptome, proteome, and metabolome in the environmental adaptation of a hyperthermophile. *J Proteome Res* **7**: 1027–1035.

Tu D, Blaha G, Moore PB & Steitz TA (2005) Gene replacement in *Haloarcula marismortui*: construction of a strain with two of its three chromosomal rRNA operons deleted. *Extremophiles* **9**: 427–435.

Tuininga JE, Verhees CH, van der Oost J, Kengen SW, Stams AJ & de Vos WM (1999) Molecular and biochemical characterization of the ADP-dependent phosphofructokinase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **274**: 21023–21028.

Tumbula DL & Whitman WB (1999) Genetics of *Methanococcus*: possibilities for functional genomics in Archaea. *Mol Microbiol* **33**: 1–7.

Tumbula DL, Makula RA & Whitman WB (1994) Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. *FEMS Microbiol Lett* **121**: 309–314.

Tumbula DL, Bowen TL & Whitman WB (1997) Characterization of pURB500 from the archaeon *Methanococcus maripaludis* and construction of a shuttle vector. *J Bacteriol* **179**: 2976–2986.

Twellmeyer J, Wende A, Wolfertz J *et al.* (2007) Microarray analysis in the archaeon *Halobacterium salinarum* strain R1. *PLoS ONE* **2**: e1064.

van der Oost J, Schut G, Kengen SW, Hagen WR, Thomm M & de Vos WM (1998) The ferredoxin-dependent conversion of glyceraldehyde-3-phosphate in the hyperthermophilic archaeon *Pyrococcus furiosus* represents a novel site of glycolytic regulation. *J Biol Chem* **273**: 28149–28154.

van de Vossenberg JL, Ubbink-Kok T, Elferink MG, Driessen AJ & Konings WN (1995) Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of bacteria and archaea. *Mol Microbiol* **18**: 925–932.

Veit K, Ehlers C, Ehrenreich A *et al.* (2006) Global transcriptional analysis of *Methanosarcina mazei* strain Go1 under different nitrogen availabilities. *Mol Genet Genomics* **276**: 41–55.

Verhees CH, Kengen SW, Tuininga JE, Schut GJ, Adams MW, De Vos WM & Van Der Oost J (2003) The unique features of glycolytic pathways in Archaea. *Biochem J* **375**: 231–246.

Vetriani C, Maeder DL, Tolliday N *et al.* (1998) Protein thermostability above 100 °C: a key role for ionic interactions. *P Natl Acad Sci USA* **95**: 12300–12305.

Vierke G, Engelmann A, Hebbeln C & Thomm M (2003) A novel archaeal transcriptional regulator of heat shock response. *J Biol Chem* **278**: 18–26.

Voorhorst WG, Eggen RI, Luesink EJ & de Vos WM (1995) Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutation in *Escherichia coli*. *J Bacteriol* **177**: 7105–7111.

Voorhorst WG, Eggen RI, Geerling AC, Platteeuw C, Siezen RJ & Vos WM (1996) Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Biol Chem* **271**: 20426–20431.

Waege I, Schmid G, Thumann S, Thomm M & Hausner W (2010) Shuttle vector-based transformation system for *Pyrococcus furiosus*. *Appl Environ Microb* **76**: 3308–3313.

Wagner M, Berkner S, Ajon M, Driessen AJ, Lipps G & Albers SV (2009) Expanding and understanding the genetic toolbox of the hyperthermophilic genus *Sulfolobus*. *Biochem Soc T* **37**: 97–101.

Walters AD & Chong JP (2009) *Methanococcus maripaludis*: an archaeon with multiple functional MCM proteins? *Biochem Soc T* **37**: 1–6.

Wanner C & Soppa J (1999) Genetic identification of three ABC transporters as essential elements for nitrate respiration in *Haloferax volcanii*. *Genetics* **152**: 1417–1428.

Ward DE, Shockley KR, Chang LS, Levy RD, Michel JK, Conners SB & Kelly RM (2002) Proteolysis in hyperthermophilic microorganisms. *Archaea* **1**: 63–74.

Welander PV & Metcalf WW (2008) Mutagenesis of the C1 oxidation pathway in *Methanosarcina barkeri*: new insights into the Mtr/Mer bypass pathway. *J Bacteriol* **190**: 1928–1936.

Wende A, Furtwangler K & Oesterhelt D (2009) Phosphate-dependent behavior of the archaeon *Halobacterium salinarum* strain R1. *J Bacteriol* **191**: 3852–3860.

Whitaker RJ, Grogan DW & Taylor JW (2005) Recombination shapes the natural population structure of the hyperthermophilic archaeon *Sulfolobus islandicus*. *Mol Biol Evol* **22**: 2354–2361.

Whitehead K, Kish A, Pan M *et al.* (2006) An integrated systems approach for understanding cellular responses to gamma radiation. *Mol Syst Biol* **2**: 47.

Whitman WB, Boone DR, Koga Y & Keswani J (2001) Taxonomy of methanogenic Archaea. *Bergey's Manual of Systematic Bacteriology, Vol. 1* (Boone DR & Castenholz RW, eds), pp. 211–294. Springer-Verlag, New York.

Williams RS, Moncalian G, Williams JS *et al.* (2008) Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell* **135**: 97–109.

Woese CR & Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *P Natl Acad Sci USA* **74**: 5088–5090.

Woese CR, Kandler O & Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *P Natl Acad Sci USA* **87**: 4576–4579.

Wolfe RS (1996) 1776-1996: alessandro volta's combustible air. *ASM News* **62**: 529–534.

Woods WG & Dyall-Smith ML (1997) Construction and analysis of a recombination-deficient *(radA)* mutant of *Haloferax volcanii*. *Mol Microbiol* **23**: 791–797.

Woods WG, Ngui K & Dyall-Smith ML (1999) An improved transposon for the halophilic archaeon *Haloarcula hispanica*. *J Bacteriol* **181**: 7140–7142.

Worthington P, Hoang V, Perez-Pomares F & Blum P (2003) Targeted disruption of the alpha-amylase gene in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol* **185**: 482–488.

Wright DB, Banks DD, Lohman JR, Hilsenbeck JL & Gloss LM (2002) The effect of salts on the activity and stability of *Escherichia coli* and *Haloferax volcanii* dihydrofolate reductases. *J Mol Biol* **323**: 327–344.

Wurtzel O, Sapra R, Chen F, Zhu Y, Simmons BA & Sorek R (2010) A single-base resolution map of an archaeal transcriptome. *Genome Res* **20**: 133–141.

Xia Q, Hendrickson EL, Zhang Y *et al.* (2006) Quantitative proteomics of the archaeon *Methanococcus maripaludis* validated by microarray analysis and real time PCR. *Mol Cell Proteomics* **5**: 868–881.

Xia Q, Wang T, Hendrickson EL, Lie TJ, Hackett M & Leigh JA (2009) Quantitative proteomics of nutrient limitation in the hydrogenotrophic methanogen *Methanococcus maripaludis*. *BMC Microbiol* **9**: 149.

Yip KS, Stillman TJ, Britton KL *et al.* (1995) The structure of *Pyrococcus furiosus* glutamate dehydrogenase reveals a key role for ion-pair networks in maintaining enzyme stability at extreme temperatures. *Structure* **3**: 1147–1158.

Yip KS, Britton KL, Stillman TJ *et al.* (1998) Insights into the molecular basis of thermal stability from the analysis of ion-pair networks in the glutamate dehydrogenase family. *Eur J Biochem* **255**: 336–346.

Yokooji Y, Tomita H, Atomi H & Imanaka T (2009) Pantoate kinase and phosphopantothenate synthetase, two novel enzymes necessary for CoA biosynthesis in the Archaea. *J Biol Chem* **284**: 28137–28145.

Yokoyama H, Matsui E, Akiba T, Harata K & Matsui I (2006) Molecular structure of a novel membrane protease specific for a stomatin homolog from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *J Mol Biol* **358**: 1152–1164.

Yoshimochi T, Fujikane R, Kawanami M, Matsunaga F & Ishino Y (2008) The GINS complex from *Pyrococcus furiosus* stimulates the MCM helicase activity. *J Biol Chem* **283**: 1601–1609.

Yuan J, Palioura S, Salazar JC *et al.* (2006) RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *P Natl Acad Sci USA* **103**: 18923–18927.

Yutin N, Makarova KS, Mekhedov SL, Wolf YI & Koonin EV (2008) The deep archaeal roots of eukaryotes. *Mol Biol Evol* **25**: 1619–1630.

Zaigler A, Schuster SC & Soppa J (2003) Construction and usage of a onefold coverage shotgun DNA microarray to characterize the metabolism of the archaeon *Haloferax volcanii*. *Mol Microbiol* **48**: 1089–1105.

Zaparty M, Esser D, Gertig S *et al.* (2010) "Hot standards" for the thermoacidophilic archaeon *Sulfolobus solfataricus*. *Extremophiles* **14**: 119–142.

Zhang JK, Pritchett MA, Lampe DJ, Robertson HM & Metcalf WW (2000) *In vivo* transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner-family transposable element Himar1. *P Natl Acad Sci USA* **97**: 9665–9670.

Zillig W, Stetter KO, Wunderl S, Schulz W, Priess H & Scholz I (1980) The *Sulfolobus*–"Caldariella" group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. *Arch Microbiol* **125**: 259–269.

Zillig W, Holz I, Janekovic D, Schäfer W & Reiter WD (1983) The archaebacterium *Thermococcus celer* represents, a novel genus within the thermophilic branch of the archaebacteria. *Syst Appl Microbiol* **4**: 88–94.

Zillig W, Prangishvilli D, Schleper C *et al.* (1996) Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic Archaea. *FEMS Microbiol Rev* **18**: 225–236.

Zivanovic Y, Armengaud J, Lagorce A *et al.* (2009) Genome analysis and genome-wide proteomics of *Thermococcus gammatolerans*, the most radioresistant organism known amongst the Archaea. *Genome Biol* **10**: R70.

Zolghadr B, Weber S, Szabo Z, Driessen AJ & Albers SV (2007) Identification of a system required for the functional surface localization of sugar binding proteins with class III signal peptides in *Sulfolobus solfataricus*. *Mol Microbiol* **64**: 795–806.

Zolghadr B, Klingl A, Koerdt A, Driessen AJ, Rachel R & Albers SV (2010) Appendage-mediated surface adherence of *Sulfolobus solfataricus*. *J Bacteriol* **192**: 104–110.