Genetic analysis of homologous recombination in Archaea: *Haloferax volcanii* as a model organism

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**Abstract**

Homologous recombination is a fundamental cellular process that rearranges genes both within and between chromosomes, promotes repair of damaged DNA and underpins replication. Much of our understanding of recombination stems from pioneering studies of bacterial and eukaryotic systems such as *Escherichia coli* and *Saccharomyces cerevisiae*. Since most archaeal species are extremophilic and difficult to cultivate, current knowledge of recombination in the Archaea is confined largely to comparative genomics and biochemistry. A clear view of what we can learn will not emerge until genetic and molecular systems have been established. We are developing such systems using *Haloferax volcanii* as a model organism, as it can be cultivated in the laboratory with ease and offers great potential for establishing tractable and informative genetic systems.

**The importance of archaeal genetics**

Classic comedy duos rely on the partnership between a straight man and a funny man. This formula was used to great effect by Laurel and Hardy and also by Morecombe and Wise. Biochemistry has traditionally allied itself with genetics, and the enormous potential of this partnership is amply illustrated by the fascinating discoveries that have been made using bacterial and eukaryotic systems such as *Escherichia coli* and *Saccharomyces cerevisiae* respectively. Critically, these model organisms lend themselves to both biochemical and genetic approaches.

However, there is a third domain of life, the Archaea [1], which is characterized by organisms adapted to extremely harsh environments that pose enormous challenges for growth and DNA metabolism. The Archaea provide new opportunities to study how recombination has evolved to meet these challenges, and how it operates in a lineage fundamentally distinct from bacteria and eukaryotes. While this adaptation to extreme environments has helped bring the Archaea to prominence, it remains a major obstacle to the development of archaeal genetics [2]; organisms that grow at 105 °C under strictly anaerobic conditions are not conducive to routine genetic techniques.

*Haloferax volcanii* as a model organism

We have been developing a genetic system using *H. volcanii* as a model organism, in order to study the pathways and mechanics of DNA repair and homologous recombination in the Archaea. *H. volcanii* was isolated from the Dead Sea [3] and grows aerobically at 30–50 °C in medium containing 20% NaCl; it can be cultivated in the laboratory with ease. *H. volcanii* is one of only two archaean species in which a natural process of genetic exchange has been observed [4]. Several strains and a number of auxotrophic mutants are available [5], which in combination with antibiotic-resistance markers [6,7], an efficient transformation system [8], plasmid vectors for cloning and gene expression [9,10], reporter genes [11] and a genome sequencing project, provide a formidable arsenal of genetic tools. Foremost among these is the ability to generate precise gene modifications using a counter-selectable ‘pop-in/pop-out’ system that exploits uracil auxotrophy and resistance to 5-fluoro-oorotic acid (Figure 1) [12].

**Genetic analysis of homologous recombination**

The study of recombination mechanisms is well suited to a genetic approach. Unlike mutants in essential processes such as DNA replication, cells with deficiencies in DNA repair or recombination are seldom inviable. More commonly they exhibit genomic instability, which is due to an inadequate response to DNA damage such as double-strand breaks. In higher eukaryotes, the consequences of this genomic instability include chromosomal aberrations and a predisposition to cancer [13]. The accurate repair of double-strand breaks, whether they arise spontaneously by radiation or intentionally during meiosis, lies at the root of homologous recombination [14]. The mechanism by which cells perform this repair has been the subject of decades of research using bacterial [15] and eukaryotic systems [16]; a widely-accepted view of recombination is presented in the double-strand-break repair model of Szostak et al. [17,18] (Figure 2).

One of the most compelling reasons to study homologous recombination in the Archaea has come from genomics. Although they are prokaryotic and morphologically similar to bacteria, it is clear from DNA sequence data that the Archaea share greater similarity with eukaryotes [19], and that this kinship is most prominent amongst enzymes involved in nucleic acid metabolism. Recent work on DNA
replication has shown that both the core components and mode of action appear to have been conserved between Archaea and eukaryotes, but that archaeal systems are less complex and are therefore more amenable to analysis [20]. As with DNA replication, eukaryotic recombination and repair proteins often assemble in large complexes, complicating the analysis of their individual functions. Genetic studies of Archaea recombination proteins may therefore generate stripped-down models that facilitate the dissection of the more complex eukaryotic systems.

Notwithstanding the sequence similarity between Archaea and eukaryotes, the third domain of life holds many mysteries. Analyses of archaeal genomic sequences have revealed that only around 50% of the predicted genes can be assigned a putative role with confidence (e.g. [21]). A number of novel archaeal enzymes, such as the Holliday junction resolvase Hjc [22], have already been isolated. Furthermore, distant homology with a novel archael topoisomerase has allowed the identification of Spo11 as the eukaryotic enzyme responsible for double-strand DNA breaks formed during meiosis [23]. Genetics provides some of the most powerful tools to uncover the function of these novel archaeal genes, through traditional methods of generating mutants, screening, cloning and complementation.

Recombination in *H. volcanii*

We are currently generating a number of recombination mutants by reverse genetics, using sequence data from the *H. volcanii* genome-sequencing project, in combination with sequence data from related archaeal species, to identify genes of interest (Figure 2). Mutations to delete or modify these recombination genes are introduced by the pop-in/pop-out method shown in Figure 1. We are developing further selectable markers, which will be of widespread utility to archaeal genetics.

Our initial efforts have focused on RecA-like strand-exchange proteins (RadA and B). Strand exchange is the key step in recombination as it registers homology and provides a means to abort illegitimate events. The basic reaction has been conserved throughout evolution [24] and is catalysed by a family of related proteins (RecA, Rad51 and RadA) that form helical nucleoprotein filaments in which the DNA is extended and underwound to facilitate pairing and base-pair switching. A radA mutant of *H. volcanii* has been constructed elsewhere, and displays recombination defects and sensitivity to DNA damage [25]. The Archaea possess a second RecA-like protein called RadB, which has diverged significantly from its RadA counterpart [26]. Although RadB expression is not induced by DNA damage [27], a specific interaction between RadB and the Hjc Holliday junction resolvase of *Pyrococcus furiosus* has been observed [28]. A number of RecA homologues exist in eukaryotes, and play distinct roles at different developmental stages. RadB proteins may perform a specialized function in Archaea, which will best be elucidated by a genetic study of *H. volcanii* radB and hjc mutants. Initial tests of these mutants for sensitivity to UV and γ-radiation have yielded promising results (T.A. and H.-P.N., unpublished work).

A more detailed study of recombination mutants calls for the development of an analytical system. We are using a plasmid×chromosome recombination assay that monitors exchanges between a mutant allele of the β-galactosidase gene *bgaH* on the *H. volcanii* chromosome, and a *bgaH* heteroallele on a selectable plasmid. Recombinants can be scored by a blue/white colour assay after spraying colonies with X-gal [11]. We have also developed a variation of this assay using the leucine biosynthesis gene *leuB* (Figure 3). These systems will be used to determine the effect of plasmid substrate (e.g. linear versus circular DNA) and DNA mismatches on homologous recombination. Both autonomously replicating and non-replicating plasmids will be used, so that products of both gene conversion (by gap repair) and crossing-over (by integration) may be recovered.
Figure 2 | The double-strand-break repair model
In the double-strand break repair model for recombination [17,18], DNA ends are processed to generate 3′-ended single-strand tails, which invade an intact homologue to generate a heteroduplex intermediate in which parental duplexes are linked via Holliday junctions. Resolution of these junctions by cutting the outside strands (filled arrowheads) or crossed strands (open arrowheads) of each junction generates recombinant duplex products, which may be further processed by repair of DNA base-pair mismatches in heteroduplex regions. Key enzymes involved in each of these steps in bacteria (B), eukaryotes (E) and Archaea (A) are shown.

Figure 3 | Recombination assay system
Exchanges between a mutant allele of the leucine biosynthesis gene *leuB* on the *H. volcanii* chromosome, and a *leuB* heteroallele on a plasmid are scored by the ability to grow in the absence of leucine. The plasmid additionally contains the *MevR* gene for resistance to the archaeal antibiotic mevinolin [7], allowing non-crossover (gene conversion) events to be distinguished from crossovers, the latter result in integration of the *MevR* gene on the *H. volcanii* chromosome.
Such an approach has proved fundamental to the elucidation of homologous recombination in S. cerevisiae [16]. We are also exploiting the analytical recombination system to screen for novel archaeal genes. Mutagenized cells that fail to recombine the bgaH heteroalleles, and therefore fail to stain blue with X-gal, will be tested for sensitivity to DNA-damaging agents such as γ-radiation; there is considerable overlap between the processes needed for repair of double-stranded DNA breaks and those involved in recombination. Recombination genes identified in this screen will also be analysed using the archaeal species Methanothermobacter thermautotrophicus, in collaboration with Edward Bolt at the University of Nottingham. For biochemical work, M. thermautotrophicus is preferable to H. volcanii, as the halophilic proteins of the latter are difficult to purify and characterize. Using these complementary strategies of reverse genetics, we expect to shed light on novel aspects of recombination in the Archaea, and how these organisms meet the physiological challenges posed by the harsh environments they occupy.

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References

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