Homologous recombination in *Sulfolobus acidocaldarius*: genetic assays and functional properties

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Abstract

HR (homologous recombination) is expected to play important roles in the molecular biology and genetics of archaea, but, so far, few functional properties of archaeal HR have been measured *in vivo*. In the extreme thermoacidophile *Sulfolobus acidocaldarius*, a conjugational mechanism of DNA transfer enables quantitative analysis of HR between chromosomal markers. Early studies of this system indicated that HR occurred frequently between closely spaced mutations within the *pyrE* gene, and this result was later supported by various analyses involving defined point mutations and deletions. These properties of intragenic HR suggested a non-reciprocal mechanism in which donor sequences become incorporated into the recipient genome as short segments. Because fragmentation of donor DNA during cell-to-cell transfer could not be excluded from contributing to this result, subsequent analyses have focused on electroporation of selectable donor DNA directly into recipient strains. For example, *S. acidocaldarius* was found to incorporate synthetic ssDNA (single-stranded DNA) of more than \(\sim 20\) nt readily into its genome. With respect to various molecular properties of the ssDNA substrates, the process resembled bacteriophage \(\lambda\) Red-mediated ‘recombineering’ in *Escherichia coli*. Another approach used electroporation of a multiply marked *pyrE* gene to measure donor sequence tracts transferred to the recipient genome in individual recombination events. Initial results indicate multiple discontinuous tracts in the majority of recombinants, representing a relatively broad distribution of tract lengths. This pattern suggests that properties of the HR process could, in principle, account for many of the apparent peculiarities of intragenic recombination initiated by *S. acidocaldarius* conjugation.

Evolutionary conservation and divergence of DNA strand-exchange proteins

Micro-organisms offer practical advantages for experimental analysis of HR (homologous recombination), and the underlying DNA transactions have broad significance for genome replication and cell division in all organisms. In particular, certain processes that enable DNA replication forks to proceed past unrepaired damage or reassemble after breaking depend on formation and resolution of the same type of four-way Holliday junctions responsible for classical genetic recombination in micro-organisms [1–4]. The most intensively studied examples of HR require a specialized protein to initiate the strand exchanges leading to these Holliday junctions. These synapsis-promoting proteins are not essential for cellular viability, yet they occur in all cellular organisms and share basic functional properties, including ssDNA (single-stranded DNA) binding, ATP hydrolysis and the ability to promote strand invasion [5]. Although broadly conserved, these proteins have nevertheless diversified significantly among and within the three domains of cellular life. Thus the Rad51, RecA and RadA families represent orthologous proteins of Eukarya, Bacteria and Archaea respectively, which differ with respect to structural motifs [6,7]. In addition, numerous paralogues of these proteins have been identified which have less obvious connections to HR. Among archaea, for example, paralogues of RadA proteins, designated RadB, have been identified in divergent euryarcheotes. The RadB protein of *Thermococcus kodakarensis* lacks strand-exchange activity and exhibits affinity for a Holliday junction resolvase [8,9]. Similarly, genetic and biochemical analyses indicate that the RadB protein of *Haloferax volcanii* interacts with DNA and ATP and contributes to the survival of cells after UV irradiation [10].

HR in archaea

Although used routinely for genetic manipulations, archaeal HR has not been analysed extensively as a genetic process. In methanogens and the hyperthermophilic non-methanogenic anaerobe *T. kodakarensis*, HR has been used extensively to disrupt chromosomal genes [11–13]. In the extreme halophile *H. volcanii*, genetic constructs provide assays of reciprocal and non-reciprocal exchanges, as well as genetic screens for novel mutants defective in these processes [14]. These tools, combined with a completed genome sequence...
(http://archaea.ucsc.edu/), form a promising basis for genetic analysis of HR in this organism. In *Sulfolobus solfataricus* strain 98/2, HR has been used to transfer various gene modifications, constructed in vitro, to the host chromosome for the elucidation of gene function [15]. Similar HR events were not detected in *S. solfataricus* isolate P1 [16], however, so it remains unclear whether HR is equally active in all strains of this species.

Another *Sulfolobus* species, *Sulfolobus acidocaldarius*, has been found to transfer DNA directly between cells by a form of conjugation, allowing recombination of the transferred DNA with the recipient chromosome. If the two parental strains contain distinct auxotrophic mutations, this produces selectable genetic recombinants at frequencies of 10^-6-10^-4 per cell [17,18]. The phenomenon, termed ‘marker exchange’, provides a convenient and quantitative assay for HR between chromosomal mutations in *S. acidocaldarius*. Although the mechanistic details of cell pairing and DNA transfer remain undefined, a variety of genetic markers allow certain functional properties of HR to be investigated. In addition to the natural DNA transfer offered by conjugation, electroporation can also introduce DNA into *S. acidocaldarius* cells, which similarly leads to recombination with the chromosome for appropriate sequences [19]. Results of studies using both approaches indicate a mode of HR that operates efficiently on very short DNA sequences.

### Recombination initiated by conjugation

The first indication of this property of *S. acidocaldarius* HR came from matings among independent spontaneous uracil auxotrophs selected by growth medium containing 5-FOA (5-fluoro-orotic acid) plus uracil. Because the selection requires loss of either the orotidylate decarboxylase or the orotate phosphoribosyltransferase of the organism [20], the mutations are confined to the two genes encoding these two enzymes (designated *pyrE* and *pyrF* respectively) or their common promoter. Despite the small size (approx. 1300 bp) of this mutational target, over 90% of mutant pairs yielded recombinants [21]. From this empirical (observed) probability, it was estimated that conjugation was detecting recombination between mutations separated by as few as 15 bp [21]. A later study used sequenced *pyrE* and *pyrF* mutants to measure the efficiency of marker exchange as a function of the distance between the mutations. The resulting frequency against distance behaviour differed from that observed in most microbial models of reciprocal HR [22]. In particular, no ‘minimum efficient processing segment’, representing a type of threshold for HR, was evident from the data, and marker proximity limited recombinant yield only for very close mutations (<50 bp, for example). These and other results, including a lack of genetic linkage between markers spaced only 500–600 bp apart, were interpreted in terms of a non-reciprocal mode of HR in which short segments of DNA from one parent (the donor) become incorporated into the corresponding region of the recipient, without affecting flanking regions [22].

### Recombination involving oligonucleotides

Although conjugation probably has greater relevance for the genetics of natural populations, artificial introduction of defined DNAs has important advantages for quantitative analysis of the recombination process. Electroporation of *S. acidocaldarius pyrE* mutants with corresponding *Pyr^+* sequences has been found to generate prototrophic transformants [19], providing an alternative route to the analysis of HR in this archaeon. Under these conditions, linear DNA is an effective, if not preferred, HR substrate, and even single-stranded synthetic oligonucleotides generate recombinants with reasonable efficiency [19].

The last observation places *S. acidocaldarius* in a small group of micro-organisms shown to be capable of OMT (oligonucleotide-mediated transformation); this group also includes *Saccharomyces cerevisiae* [23] and *Escherichia coli* induced for the bacteriophage λ recombination system [24]. This genetic capability is significant, because it provides an interesting context in which to probe HR and related DNA transactions. In particular, the use of synthetic oligonucleotides grants an unprecedented level of control over the molecular properties of one recombination substrate. Furthermore, because certain molecular features can be confined to the single-stranded substrate, they can block processes, such as ligation or 3'-end extension, essential for the host generally. Thus synthetic ssDNA allows numerous molecular requirements of OMT to be analysed without requiring mutants defective for specific DNA transactions. This is particularly important for *Sulfolobus* spp., as the relevant mutants are generally not available, neither are all the relevant genes even known. In addition, OMT represents a simple mode of HR that has been studied in model micro-organisms, thereby allowing functional properties of the *S. acidocaldarius* system to be compared with corresponding properties of well-studied bacteria and eukaryotes.

A recent study took this approach to compare the molecular requirements of the *Sulfolobus* OMT system with those of bacteriophage λ-induced *E. coli* [25]. To make the comparison as precise as possible, corresponding selections were set up in these two highly divergent organisms (Figure 1). In each case, oligonucleotides representing the wild-type sequence were electroporated into cells of auxotrophic mutants, where they would restore the ability to grow on selective (unsupplemented) media, if incorporated at the corresponding sites of the recipient chromosome. The efficiency of this recombination, measured as the number of transformants per pmol of DNA, increased similarly in both systems with increasing length of ssDNA, but could be detected for ssDNA substrates as short as 20 nt centred over the mutation being replaced. Longer ssDNA tolerated considerable skew in their positions, such that 2–5 nt at the limiting end promoted low, but measurable, levels of recombination [25]. The similar responses of the two systems to length and position of the ssDNA substrates suggests a corresponding similarity in the stability of ssDNA annealing to the *S. acidocaldarius* and *E. coli* genomes in vivo, despite
the dramatic molecular and physiological differences between these two organisms and their genomes.

Effects of certain chemical modifications of the ends of the ssDNA substrates were also evaluated, and reveal apparent differences of oligonucleotide processing in the E. coli and Sulfolobus acidocaldarius systems. PT (phosphorothioate) linkages incorporated into either or both ends of the ssDNA generally enhanced Sulfolobus OMT, consistent with protection of the oligonucleotides against exonucleases in vivo [25]. In E. coli, however, 5’ PT essentially abolished OMT by 5’-limited transforming DNA; this was investigated and attributed to a requirement for a 5’ phosphate on the ssDNA in E. coli [25]. The fact that the corresponding requirement was not evident in S. acidocaldarius suggests the existence of a kinase activity capable of phosphorylating the 5’ end of ssDNA.

Implications for genetic stability and diversification

Observations that S. acidocaldarius recombines short ssDNA containing a single base difference into its genome without any special genetic manipulation (in contrast with OMT in E. coli) raises various questions regarding whether this or other archaeal species may undergo similar DNA transactions normally. For example, many sequences of 40 nt or less are repeated in the S. acidocaldarius genome, and thus represent potential substrates for deleterious ectopic recombination events. How frequent are such events, and what mechanisms limit them? In bacteria and eukaryotes, OMT is greatly attenuated by DNA MMR (mismatch repair) [26,27], presumably because the intermediate of OMT (a short heteroduplex formed at a single-strand gap in the recipient chromosome) mimics the natural substrate of MMR. Sulfolobus species and other hyperthermophilic archaea lack any identifiable MMR genes, however [28]. This raises the possibility, which remains challenging to test experimentally, that the ease of transforming S. acidocaldarius by ssDNA derives, in part, from the absence of post-replication MMR.

In any event, the minimal length requirements exhibited by OMT seem consistent with functional properties of the other manifestations of HR that have been analysed in S. acidocaldarius, including conjugational marker exchange and transformation by linear dsDNA (double-stranded DNA). Do all of these recombination phenomena reflect a common set of DNA transactions and proteins? Conceivably, for example, conjugation would produce a short-patch pattern of marker exchange if chromosomal DNA were fragmented into short pieces during cell-to-cell transfer. The OMT data seem consistent with this, since even rather short oligonucleotides can recombine into the S. acidocaldarius genome [25].

Potential sources of the short-patch nature of HR in marker exchange are being investigated by electroporation of a double-stranded, full-length, functional pyrE gene into S. acidocaldarius pyrE mutants. In this system, the exogenous donor DNA contains sequence markers, in the
form of synonymous substitutions distributed throughout the pyrE gene, that create or destroy recognition sites for restriction endonucleases (Figure 2). As a result, each Pyr+ clone generated by HR of the donor DNA with the host chromosome can be analysed to identify the intervals (replacement tracts) transferred from the introduced DNA to the recipient chromosome. Preliminary results reveal various examples of single replacement tracts spanning the selected region (Figure 2), but these represent the minority situation. Most recombines, in contrast, contain multiple short blocks of donor markers interrupted by intervals of receptor sequence (J. Rockwood and D.W. Grogan, unpublished work). The effects of various parameters, including the physical state of donor DNA and the position of the selected region within the pyrE gene, remain to be tested. It seems likely, however, that functional properties observed previously for HR initiated by conjugation (i.e. the minimal possibility that a short-patch pattern of recombination results from the HR system operating on longer DNA remains an exquisite question for future research on this and other hyperthermophilic archaea.

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