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Paul H. Blum
University of Nebraska-Lincoln, pblum1@unl.edu

Deepak Rudrappa
University of Nebraska–Lincoln, deepakgr@unl.edu

Raghuveer Singh
University of Nebraska - Lincoln, raghuveer@huskers.unl.edu

Samuel McCarthy
University of Nebraska-Lincoln, smccarthy3@unl.edu

Benjamin J. Pavlik
University of Nebraska- Lincoln, benjamin.pavlik@unl.edu

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Experimental Microbial Evolution of Extremophiles

Paul Blum,[1] Deepak Rudrappa,[1] Raghuveer Singh,[1] Samuel McCarthy,[1] and Benjamin Pavlik[2]

1 School of Biological Science, University of Nebraska–Lincoln, Lincoln, NE, USA
2 Department of Chemical and Biomolecular Engineering, University of Nebraska–Lincoln, Lincoln, NE, USA

Corresponding author — P. Blum, email pblum1@unl.edu

1 Experimental Microbial Evolution Theory and Applications in Model Organisms

Experimental microbial evolutions (EME) involves studying closely a microbial population after it has been through a large number of generations under controlled conditions (Kussell 2013). Adaptive laboratory evolution (ALE) selects for fitness under experimentally imposed conditions (Bennett and Hughes 2009; Dragosits and Mattanovich 2013). However, experimental evolution studies focusing on the contributions of genetic drift and natural mutation rates to evolution are conducted under non-selective conditions to avoid changes imposed by selection (Hindré et al. 2012).

To understand the application of experimental evolutionary methods to extremophiles it is essential to consider the recent growth in this field over the last decade using model non-extremophilic microorganisms. This growth reflects both a greater appreciation of the power of experimental evolution for testing evolutionary hypotheses and, especially recently, the new power of genomic methods for analyzing changes in experimentally evolved lineages. Since many crucial processes are driven by microorganisms in nature, it is essential to understand and appreciate how microbial communities function, particularly with relevance to selection. However, many theories developed to understand microbial ecological patterns focus on the distribution and the structure of diversity within a microbial population comprised of single species (Prosser et al. 2007). Therefore an understanding of the concept of species is needed. A common definition of species using a genetic concept

is a group of interbreeding individuals that is isolated from other such groups by barriers of recombination (Prosser et al. 2007). An alternative ecological species concept defines a species as set of individuals that can be considered identical in all relevant ecological traits (Cohan 2001). This is particularly important because of the abundance and deep phylogenetic complexity of microbial communities. Cohan postulated that "bacteria occupy discrete niches and that periodic selection will purge genetic variation within each niche without preventing divergence between the inhabitants of different niches". The importance of gene exchange mechanisms likely in bacteria and archaea and therefore extremophiles, arises from the fact that their genomes are divided into two distinct parts, the core genome and the accessory genome (Cohan 2001). The core genome consists of genes that are crucial for the functioning of an organism and the accessory genome consists of genes that are capable of adapting to the changing ecosystem through gain and loss of function. Strains that belong to the same species can differ in the composition of accessory genes and therefore their capability to adapt to changing ecosystems (Cohan 2001; Tettelin et al. 2005; Gill et al. 2005). Additional ecological diversity exists in plasmids, transposons and pathogenicity islands as they can be easily shared in a favorable environment but still be absent in the same species found elsewhere (Wertz et al. 2003). This poses a major challenge for studying ALE and community microbial ecology indicating a continued need to develop a fitting theory that connects the fluid nature of microbial communities to their ecology (Wertz et al. 2003; Coleman et al. 2006). Understanding the nature and contribution of different processes that determine the frequencies of genes in any population is the biggest concern in population and evolutionary genetics (Prosser et al. 2007) and it is critical for an understanding of experimental evolution. Tatum and Lederberg (Tatum and Lederberg 1947) discovered laterally transferred genes in *E. coli.* However sequencing of the genomes of two isolates of *Helicobacter pylori* revealed that >6 % of the genes are unique (Alm et al. 1999). When recombination is rare and limited to few genes, almost all other genes will be transmitted through vertical inheritance. Mutations will accumulate slowly over the period and will result in irreversible divergence of lineages. In contrast, when recombinational events are common, genes will release themselves from the rest of the genome and diversity in genes linked to adaptive alleles will be purged through selection (Polz et al. 2006). This type of pan-mictic population structure has been reported in *Neisseria gonorrhoeae* and *Rhizobium meliloti* lineages (Smith et al. 1993).

Comparisons with founder or starting strains are made to quantify genotypic and phenotypic changes for example, DNA sequencing of specific genes or entire genomes is used to determine the mutational differences among evolved strains (Kussell 2013). Adaptive laboratory evolution (ALE) is a common method in EME studies to gain insights into the basic mechanisms of molecular evolution and adaptive landscapes that accumulate in microbial populations during long term selection under predefined growth conditions (Bennett et al. 1990). Over the past two to three decades, there have been an increasing number of experiments to understand the adaptive landscapes during ALE especially with *E. coli* and *Saccharomyces cerevisiae*

(Bennett et al. 1990; Paquin and Adams 1983). Microbial adaptation to new environmental conditions mainly occurs via two different mechanisms: alteration of gene regulation without any heritable genetic change, or, selection of novel adaptive phenotypes conferred by stable mutations. Experimental evolution allows phenotypic changes to be associated with growth conditions that eventually select for traits (Hardison 2003). EME studies have led to significant insights and experimental proof for evolutionary biology (Bennett et al. 1990; Paquin and Adams 1983). The long term EME study of *E. coli* by Lenski's group remains at the forefront of these studies where a single parallel *E. coli* adaptation experiment has exceeded 50,000 generations (Sniegowski et al. 1997; Lenski et al. 1998; Cooper and Lenski 2000). These studies along with similar experiments conducted by others have provided insights into the genetic basis of increased microbial fitness (Barrick et al. 2009), their implications during evolution (Woods et al. 2011), understanding of population size, evolvability (Bloom et al. 2007; Elena et al. 2007; Draghi et al. 2010) and clonal interference (Kao and Sherlock 2008).

Experimental evolution allows the microorganisms to evolve through propagation thus relying on the inherent capacity of the organism to introduce mutations (Sauer 2001). Fast generation time, ease of maintaining large population sizes and storing them make microorganisms well suited to the study of ALE in a laboratory setting (Elena and Lenski 2003). However extensive cultivation periods are required for selecting a desired phenotype with a limited natural mutation rate. Adding to this, identifying mutations necessary for conferring a particular phenotype is difficult since neutral mutations end-accumulate thus making an "omic" based approach necessary to understand the observed phenotype (Bro and Nielsen 2004). Furthermore, experimental evolution provides an advantage over reverse genetics-based approaches that employ targeted activation or inactivation of genes in that ALE can result in occurrence of mutations of unexpected composition that provide gain of function for the organism (Conrad et al. 2011). Growing knowledge about the biochemical and physiological natures of some of the model organisms along with the use of genetic and mapping techniques developed in the 1980s and 1990s, has opened up new avenues (Helling et al. 1987; Rosenzweig et al. 1994; Treves et al. 1998; Kinnersley et al. 2014; Ferenci 2007; Adams et al. 1992). These same approaches have been used to test both experimentally-evolved and naturally occurring microorganisms for genome amplification, deletion, insertion and rearrangement of genes or sequences (Cooper et al. 2003; Philippe et al. 2007; Kadam et al. 2008; Bachmann et al. 2012; Gresham et al. 2008; Wenger et al. 2011).

2 Technologies Relevant to Performing Experimental Microbial Evolution

For both extremophilic and non-extremophilic microbes, methods of cultivation are fundamental, as they dictate physiologic status and therefore mutation formation. For this reason, they are critical to the field of experimental evolution. Extended laboratory timescales of microbial growth in selective environments are also crucial.

Under these conditions, microbes are advantageous as evolutionary models due to their large population size, short generation time, relatively small genome and generally high supply of mutations (Gresham and Dunham 2014). Extremophiles may exhibit elongated generation times and but maintain normal mutation rates (Grogan et al. 2001), necessitating longer culture durations to achieve equivalent degrees of mutation formation. The understanding and application of continuous cultivation and serial batch culture passage has expanded the use of microbial systems to address evolutionary questions. Although paramount to experimental evolution, these culturing technologies have remained relatively unused in the ALE of extremophiles and future research is warranted.

Continuous long term culturing techniques for the purpose of mutation accumulation and strain evolution was popularized in the mid-twentieth century. The chemostat is a continuous culturing device first described by (Monod 1942). The modern chemostat was introduced by Novick and Szilard and applied to the study of genetic changes of microorganisms (Novick and Szilard 1950). The basic concept of this culturing device is the continuous removal of medium with concurrent replacement of fresh medium at a defined rate. Microbial populations will grow in proportion to this rate. Contemporary versions of this device have been scaled downwards to achieve larger numbers of experimental replicates or to the point of single cell analysis (Dénervaud et al. 2013). Discoveries made using chemostats are rooted in physiological responses to environmental stresses. The nature of some of these studies include: variation between and within species, mutation rates, mutational takeovers, population changes, quorum sensing, genomic rearrangements, emerging diversity, metabolism and energetics, and membrane transport (Ferenci 2008). Chemostats present a research opportunity for extremophilic experimental evolution in that the current use is mainly in bioprocess applications (Lorantfy et al. 2014).

Similar to the chemostat is the turbidostat, popularized for experimental microbial evolution by (Bryson and Szybalski 1952). The main difference is that nutrients are not limited, and that fresh medium introduction is proportional to maximum culture turbidity, resulting in maximum growth rates of microorganisms in mid- exponential phase (Gresham and Dunham 2014). Both the chemostat and turbidostat maintain a steady-state environment, but represent minimal or optimal resource conditions, respectively. These conditions result in different types of mutations based on nutrient stress or growth enhancement and overall strain fitness improvement. Turbidostats have been employed in the study of evolutionary responses to anti-microbial compounds (Avrahami-Moyal et al. 2012; Toprak et al. 2012). As with chemostats, turbidostats have seen limited use in the experimental evolution of extremophiles.

Long term batch culture intended for the evolution of microbes requires serial dilution or passaging of cells at a known dilution and duration of culture (Atwood et al. 1951). This technique provides dynamic environment for microbial growth that is less conditionally consistent than continuous culture. Under this regimen, cells may be selected during all phases of cell growth. Moreover, this introduces a complex environment for non-continuous selection as in the case of continuous culturing

devices. This approach has recently been introduced in the experimental evolution of the extremophile *Sulfolobus solfataricus* and is a prudent approach to the ALE of extremophiles without knowing the physiological responses required to design feeding regimes in continuous cultures (McCarthy 2015).

2.1 High-Throughput Methods Evaluate Genotypic and Phenotypic Evolution in Extremophiles

The method of analysis of evolutionary changes in microbial genomes has increased in throughput and resolution through the recent development of "omic" technologies, and must be considered regardless of the species of microorganism. High-throughput technologies such as whole genome sequencing, transcriptomics, microarrays and chromatin immunoprecipitation (ChIP-Seq) have enhanced resolution of the relationships between observable strain characteristics (traits) and the underlying molecular mechanisms of evolution. Understandably, high-throughput technologies have become indispensable to the study of EME. Advances in DNA sequencing throughput has expanded the research potential of experimental microbial evolution in extremophiles (Araya et al. 2010; Barrick et al. 2009; Hong and Gresham 2014; Kvitek and Sherlock 2013; Lang et al. 2013; Herring et al. 2006). Since the first archaeal genome was sequenced for *Methanocaldococcus jannaschii* (Bult et al. 1996), many more genomes have become available for extremophiles (Allers and Mevarech 2005). Gene expression data can also be obtained in high throughput formats using RNA sequencing (RNA-seq) or microarrays (DeRisi et al. 1997; Lashkari et al. 1997; Dunham et al. 2002; Gresham et al. 2006; Wurtzel et al. 2010), or proteomics (Rigaut et al. 1999). In addition protein-protein interactions (Schwikowski et al. 2000) and protein-DNA interactions (ChIP-seq) have been developed for EME model organisms and extended to extremophiles (Gresham et al. 2006; Dunham et al. 2002; Wilbanks et al. 2012)

3 Extending Experimental Evolution to Extremophiles

3.1 Specific Challenges Associated with Extremophiles

The application of experimental evolution to extremophiles must overcome specific difficulties associated with their cultivation and biophysical issues pertaining to their macromolecules arising from their extremophilic environments. Working with extremophiles in the laboratory requires special techniques and equipment to maintain their extreme environments and complex nutritional requirements. Many extremophiles have relatively slow growth rates compared to other microbial model systems. This means that ALE studies require longer time periods to achieve equivalent number of generations. For example, this is especially true for extremophiles cultivated under lithoautotrophic conditions where energy limitation constrains replication rate (McCarthy et al. 2014; Maezato et al. 2012).

In the context of macromolecules, where EME impacts their identities, proteins constitute a specific barrier while lipids and various classes of small molecules can also present challenges. Proteins derived from hyperthermophilic extremophiles require high temperature for analysis and therefore the use of specialized instrumentation. Proteins derived from halophilic extremophiles are particularly difficult to purify and characterize because they are unstable in low salt concentrations and their fractionation is impeded by the incompatibility of methods such as electrophoresis and ion-exchange with high salt concentrations (Anfinsen et al. 1995). Recombinant expression of extremophile proteins in non-extremophile hosts such as *E. coli* often face a similar problem: outside of the extreme conditions of their native environments halophilic proteins have a tendency to misfold and aggregate (Allers 2010). For these reasons, examining the performance of evolved extremophilic macromolecules within the native extremophile host becomes critical and depends on the availability of genetic systems (Maezato et al. 2012).

3.2 Cultivation and Preservation of Extremophiles

One example of an extremophilic life style is a hyperthermophilic anaerobic bacterium, *Thermotoga maritima* that was isolated from geothermal heated marine sediment at Vulcano, Italy (Huber and Stetter 1998). While most of the members of Thermotogales were isolated from hot springs and deep-sea hydrothermal vents, few of the members are able to cope with partially oxidative condition due to the partially-oxygenated hot sediments and fluids in hydrothermal vent ecosystems (Rusch et al. 2005). *T. maritima,* despite it description as a strict anaerobe, has been reported to grow in the presence of 0.5 % v/v oxygen (Le Fourn et al. 2008). Lack of abundant carbon in its marine thermal vent environment likely led to the ability of this organism to metabolize a broad diversity of sugars without apparent selectivity. For example this organism possess a significantly higher number of ABC-type substrate transporters than most other organisms (Nelson et al. 1999; Ren et al. 2007) *T. maritima* and its closely related relatives, *T. neapolitana, Thermotoga sp. strain RQ2, T. naphthophila* and *T. petrophila* thrive between 55 °C and 90 °C and pH range 5.5–9.5 using simple and complex carbohydrates (Chhabra et al. 2003; Conners et al. 2005). *T. maritima* can be cultivated in batch culture in biological replicates using Hungate tubes or serum bottles supplemented with diverse carbon sources. Tubes can be sealed with butyl rubber stoppers (Bellco Biotechnology), crimped with metal collars and the headspace can be exchanged with nitrogen gas. Medium inoculation can use sterile 1 cc syringes attached to 20½ G needles and cultures incubated anaerobically at 80 °C typically overnight.

T. maritima is also of interest due to an apparent extensive degree of lateral gene transfer from an archaeal donor and a tendency to undergo continued genome size reduction (Nelson et al. 1999; Singh et al. 2015). Moreover, it is of applied interest due to its ability to produce molecular hydrogen at rates surpassing those of other microorganisms (Schröder et al. 1994). Ongoing experimental evolution

studies concerned with expanding this capacity through the use of transient gene inactivation by targeted chromosomal recombination combined with purifying selection have established novel cell lines with unique properties (Singh et al. 2015). The term transient gene inactivation refers to the use of temporary gene disruption of a chromosomal locus resulting from consecutive single crossover events. The term purifying selection is a classic genetic method that involves maintaining selective growth conditions while isolating clonal populations.

Another example of an extremophile is the thermoacidophile *Sulfolobus solfataricus* which was isolated from volcanic hot springs in both Italy and the United States (Brock et al. 1972). This organism grows from a temperature range of 65–90 °C and a pH range of 2.5–5.0 with optimum growth conditions of 80 °C and pH 3.0 (Brock et al. 1972; Grogan 1989). In the laboratory this organism is cultivated in a modified basal salts medium (Brock et al. 1972): complex media is supplemented with 0.2 % tryptone and minimal media is supplemented with 0.2 % sugars such as glucose. Cultures are incubated at 80 °C in glass screw-capped flasks with aeration in orbital baths (Rolfsmeier and Blum 1995; Bini et al. 2002; Worthington et al. 2003) and growth is monitored by light absorption at a wavelength of 540 nm. *S. solfataricus* is a model organism in the archaea and it has an established genetic system (Maezato et al. 2011). This makes it ideal for ALE studies as genetic changes seen in experimental evolution experiments with this organism can be reconstructed in wild-type cell lines to verify their effects. *S. solfataricus* is of applied interest as a source of heat and acid stable enzymes for various industrial processes such as trehalose production and cellulose degradation (Antranikian et al. 2005). Ongoing experimental evolution studies in this organism have generated cell lines with increased acid stability (McCarthy 2015).

Extremophiles can be stored for long periods through the preparation of a frozen permanent. This becomes essential during ALE to preserve intermediate stages of evolutionary changes resident within distinct microbial populations. To achieve this end, mid-exponential phase cultures are collected by centrifugation, washed with fresh medium, amended with 7 % (v/v) dimethyl sulfoxide (DMSO). The sample is then mixed and flash frozen using an ethanol-dry ice bath. Storage at −80 °C provides a long term method to preserve culturability (Maezato et al. 2012).

4 Examples of Experimental Microbial Evolution Using Extremophiles

A broad range of genome changes underlie evolutionary changes in non extremophilic microbes. Gene duplication, deletion (Kunin and Ouzounis 2003), translocation, inversion (Suyama and Bork 2001), lateral gene transfer (Garcia-Vallvé et al. 2000; Nelson et al. 1999) and transposition (Kidwell and Lisch 2000) are some of the phenomenon that result in genome evolution (Fraser-Liggett 2005). Genome evolution in extremophiles can occur in a similar fashion because various genetic elements such as direct repeats (DR), inverted repeats (IR) and transposable elements exist in extremophiles. Since DRs and IRs have the potential to manipulate

the dynamics of a genome (Ussery et al. 2004), extremophiles should be capable of undergoing processes involving these sequences to result in genome evolution. The origins of DRs in non-thermophiles have been proposed to arise from lateral gene transfer, slip strand pairing and genome hopping (Achaz et al. 2002; Romero and Palacios 1997). As lateral gene transfer in Thermotogales has been described, these extremophiles can be used to study genome evolution. Close repeats or tandem repeats with smaller spacer regions are removed by RecA independent recombination (Bi and Liu 1994; Lovett et al. 1993) whereas direct repeats with a longer spacer depending on selection pressure may be stably maintained in the genome and are less likely to be deleted by illegitimate recombination (Lovett et al. 1994; Chédin et al. 1994). The biological relevance of repetitive sequence is to provide phenotypic variation in two ways. Repeats located in the regulatory region of a gene can modulate the expression of the gene (van Ham et al. 1993) and location inside a coding region could result in protein truncation when gene rearrangement results in loss of the repeat. Repetitive sequence retention also depends on selective pressure and this aspect of genomic instability can be experimentally tested.

Transposable elements are hypothesized to be both harmful and occasionally beneficial to their hosts (Schneider and Lenski 2004) by providing a source of genetic diversity through mutations, duplications, and genome rearrangements (Kidwell and Lisch 2000). Since transposable elements are broadly distributed in extremophiles their activity is again relevant to evolutionary change. In Lenski's long-term *E. coli* evolution experiments several pivotal changes in the different lineages of evolved cell lines were linked to transpositions, however the transposition rates did not correlate with the rates of adaptation: fitness showed a rapid increase in early generations and then sharply decelerated over time, while the rate of transposition remained relatively constant (Barrick et al. 2009; Schneider and Lenski 2004). In another EME study on thermal adaptation in *E. coli* the phenotypes of half of the derived heat-adapted cell lines could be explained by duplication events in a similar genomic region and a corresponding up-regulation of the duplicated genes. However, the other half of the adapted cell lines showed no duplications or transposition events and no change in expression of those same genes, indicating that these adapted through a different but unknown pathway (Riehle et al. 2001). These studies may be predictive of analogous efforts using extremophiles.

4.1 The Hyperthermophilic Bacterial Anaerobe Thermotoga maritima *and Deletion Formation*

T. maritima is one of the most well characterized members of the bacterial phylum Thermotogae. This phylum contains a broad range of bacterial extremophiles noted for their thermophilicity and anaerobiosis (Huber and Stetter 1998). *T. maritima* has been reported to undergone lateral gene transfer from an archaeon (Nelson et al. 1999), making it a promising model system to investigate experimental genome evolution, and an 8 kb deletion in the type strain supports the occurrence of

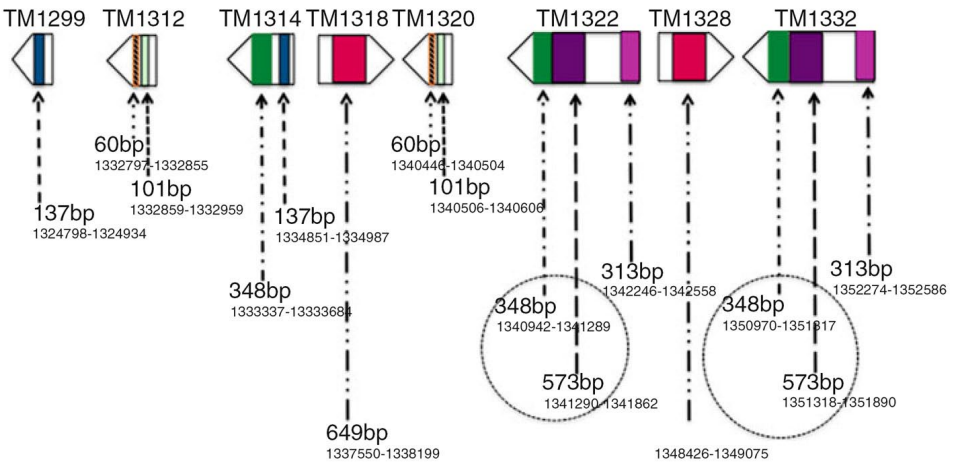

Fig. 1. A potential hot spot of genome evolution in *T. maritima*. A specific colored block represents direct repeats of conserved length. Genomic coordinates and gene locus tags are shown according to the genome of *T. maritima* reported (Nelson et al. 1999)

genome evolution in this organism (Zhaxybayeva et al. 2009). Bioinformatic analysis to find direct repeats (DRs) confirms the presence of various small and larger DRs in this organism that could contribute to genome evolution (Fig. 1). To identify the location of DRs that might contribute to genome evolution, the *T. maritima* genome was scanned to find those DRs ranging from 50 to 1500 bp using REPuter (Kurtz et al. 2001). The *T. maritima* genome reported by Nelson (Nelson et al. 1999) was used to describe the coordinates and gene locus tags. In *T. maritima,* repetitive sequences surround a variable spacer region and clustering of various DRs has generated a larger DR. This was evident when a region between TM1299 and TM1332 where a bigger DR generated via clustering, was identified as a potential hot spot of genome evolution. Various arrangements of *T. maritima* DRs are presented (Fig. 2). The biggest DRs of 921 bp exist in TM1322 (coordinates; 1340942–1341862) and TM1332 (1350970–1351890) surrounding a 10 kb spacer region. Considering the occurrence of large DRs (921 bp) in the genome, their location could constitute a hot spot for deletion of the intervening region. Such events would continue genome evolution in this bacterium. While the organism has been shown to evolve by deleting an 8 kb gene naturally, an experimental evolutionary approach used to manipulate metabolite formation resulted in deletion of genes (Singh et al. 2015). A transient gene inactivation by targeted chromosomal recombination combined with purifying selection established novel cell lines with unique properties (Singh). Genome resequencing identified a 10 kb deletion between TM1322 and TM1332 that resulted from crossover between flanking 921 bp DRs that deleted the intervening region (1341863–1351890). This is the first report of experimental microbial genome evolution in *T. maritima*. The 10 kb deletion strain of *T. maritima* was named Tma200 and due to its altered phenotype of hydrogen production may provide a strategy for further evolution of its extremophilic traits (Singh et al. 2015).

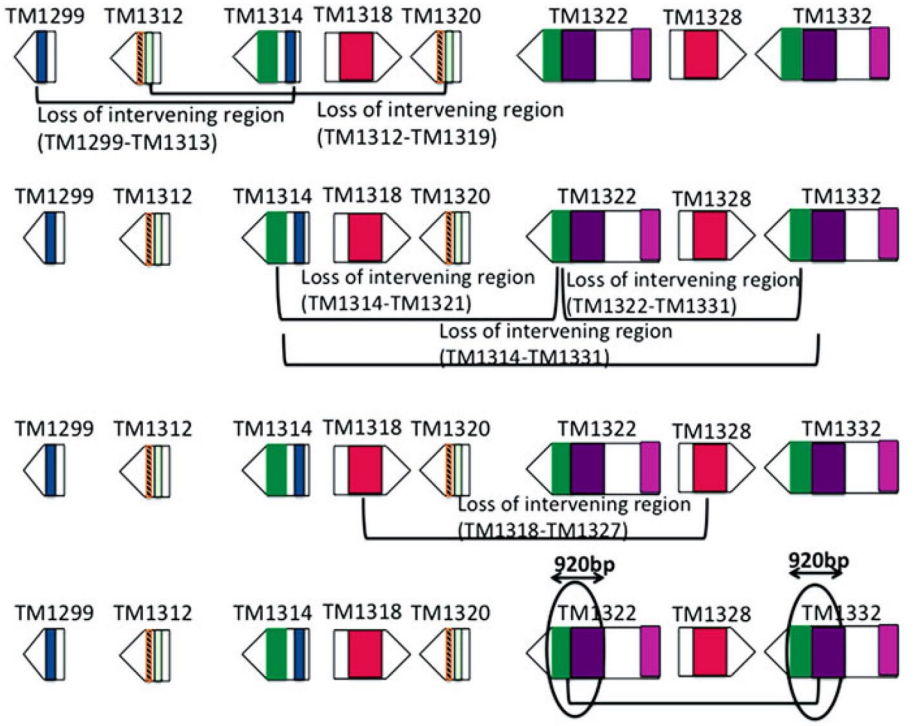

Fig. 2. A proposed schematic of genome deletions mediated via cross-over between various direct repeats. Specific colored block represents direct repeats of conserved length. Identical direct repeats that could undergo homologous recombination are presented as a bracketed regions.

4.2 The Archaeon Sulfolobus solfataricus *and the Role if Insertion Sequence Elements*

Sulfolobus solfataricus is an extremely thermoacidophilic member of the Crenarchaeotal phylum of the Archaea. Ongoing studies concerned with evolving new cell lines with increased thermoacidophily have generated a series of novel lineages (McCarthy). Aspects of these lineages pertaining to ALE are presented here. Unlike other species in this genus, *S. solfataricus* has a genome that is rich in insertion sequence (IS) elements with over 200 IS elements (10 % of genome) and its genome is predicted to be continually changing due rearrangements caused by these transposable elements (Redder and Garrett 2006; Brügger et al. 2004). It undergoes a high frequency of transposition and its gene order (synteny) is very different from other members of the *Sulfolobus* genus, indicating that it has undergone multiple rearrangements (Brügger et al. 2004).

Several evolved *S. solfataricus* cell lines were isolated by extensive serial passage to select for increased acid resistance in an experimental evolution experiment (Fig. 3) (McCarthy). Intermediate isolates were purified to clonality using a solid complex medium and the clonal isolates were re-screened for the acid resistance phenotype. These cell lines maintained acid resistance after passaging at pH 3.0,

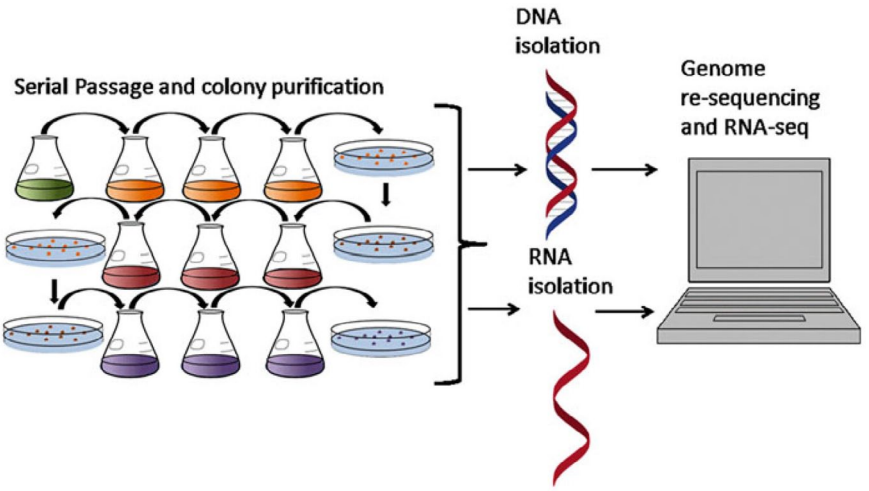

Fig. 3. Experimental microbial evolution by serial passage. Cells are sub-cultured into a slightly more extreme condition (indicated by changing coloration of the medium), passaged several times until they adapt to this condition, and then the condition is adjusted and the process is repeated. Colonies are isolated from adapted cultures and screened for the unique phenotype. The genomes and transcriptomes of experimentally evolved colonies are then examined to determine its cause.

indicating that their phenotype was stable and heritable rather than a transient response or stress-induced trait (McCarthy). The acid adapted *S. solfataricus* cell lines were resistant to a 150-fold greater acid concentration than the wild-type and was predicted to have a significant number of adaptive mutations based on transcriptomic studies. RNA-seq analysis showed high (+5-fold or more) up-regulation of 27 transposons and transposases throughout the entire genome in the adapted strains at low pH compared to the transcriptomes of wild-type cell lines growing at optimal pH. Eighteen of these elements were up-regulated tenfold or more and eight were up-regulated over 30-fold. The most up-regulated IS elements were all members of the same families including transposases ISC1217, 1234/ST1916, and IS1 (Fig. 4). Curiously however, genome re-sequencing of these strains showed very few transposition events. Two of the three adapted cell lines had a single event each and the third showed no transposition. The frequency of transposition in the adapted cell lines appeared to decrease, even though the expression of many transposase genes was increased at low pH. The lack of transpositions in the evolved cell lines indicates that these were not a source of adaptation and this was surprising given the high frequency of IS elements in the genome.

The overall transcriptomes of the acid-adapted cell lines gave additional clues to their mechanism of acid resistance. Many changes were observed in expression of putative transporters and genes predicted to be involved in signal transduction as well as overall metabolism (Fig. 5). Examining transcriptomics using RNAseq is relatively new in prokaryotes and combining this approach with genomics of ALE represents a powerful approach for looking at the relative contributions of mutations and changes in gene expression to adaptation and the interaction between them.

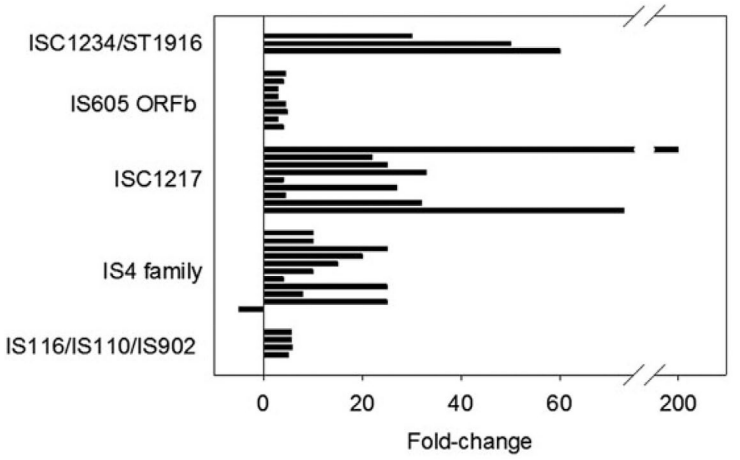

Fig. 4. Transcriptomic profile of insertion elements in *S. solfataricus* evolved to strong acid resistance by EME. Bars represent the expression changes of individual transposase genes relative to the expression in pH 3.00-grown wild-type *S. solfataricus*. Most of these elements were upregulated in the evolved cell line, with the greatest changes seen in ISC1217 and ISC1234/ST1916 family transposable elements.

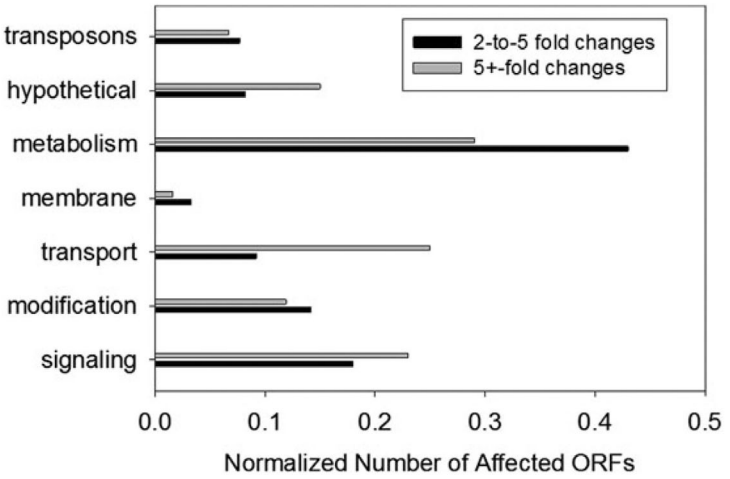

Fig. 5. Classes of ORFs with altered expression in experimentally evolved *S. solfataricus*. The number of ORFs in several functional categories that had small (two-to-fivefold) and large (5+-fold) expression changes in a pH 1.00-grown evolved cell line normalized to the total number of ORFs in each category in the genome are shown. The fold-changes are relative to pH 3.00- grown wild-type *S. solfataricus*. Biological replicates of all RNA-seq samples had Pearson correlation coefficients greater than 0.96.

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Conflict of Interest — Paul Blum, Deepak Rudrappa, Raghuveer Singh, Samuel McCarthy and Benjamin Pavlik declare that they have no conflict of interest.

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