Evidence for Genetic Drift in the Diversification of a Geographically Isolated Population of the Hyperthermophilic Archaeon *Pyrococcus*

Patricia Escobar-Páramo,* Sulagna Ghosh,† and Jocelyne DiRuggiero*†

*Department of Cell Biology and Molecular Genetics, University of Maryland; and †Center of Marine Biotechnology, University of Maryland Biotechnology Institute

Genetic drift is a mechanism of population divergence that is important in the evolution of plants and animals but is thought to be rare in free-living microorganisms because of their typically large population sizes and unrestricted means of dispersal. We used both phylogenetic and insertion sequence (IS) element analyses in hyperthermophilic archaea of the genus *Pyrococcus* to test the hypothesis that genetic drift played an important role in the diversification of these microorganisms. Multilocus sequence typing of a collection of 36 isolates of *Pyrococcus*, from different hydrothermal systems in the Pacific Ocean and the Mediterranean Sea, revealed that *Pyrococcus* populations from different geographic locations are genetically differentiated. Analysis of IS elements in these isolates exposed their presence in all individuals of only one geographically isolated lineage, that of Vulcano Island in the Mediterranean Sea. Detailed sequence analysis of six selected IS elements in the Vulcano population showed that these elements cause deleterious genomic alterations, including inactivation of gene function. The high frequency of IS elements in the sampled population together with their observed harmful effects in the genome of *Pyrococcus* provide molecular evidence that the Vulcano Island population of *Pyrococcus* is geographically isolated and that those genetic mobile elements have been brought up to high frequency by genetic drift. Thus, genetic drift resulting from physical isolation should be considered as a factor influencing differentiation in prokaryotes.

Introduction

In sexually reproducing species, geographic isolation can generate a barrier to gene flow that can result in genetic drift and/or local adaptation and differentiation (Futuyma 1998). In contrast, microorganisms are generally believed to have unrestricted dispersal capabilities due to their small size, extremely large populations, and highly plastic genomes, providing for worldwide distribution (Finlay 2002). Recent studies have challenged this view and demonstrated that prokaryotes colonizing thermophilic environments undergo genetic differentiation as a result of geographic barriers to dispersal (Papke et al. 2003; Whitaker, Grogan, and Taylor 2003; Papke and Ward 2004). Because nucleotide substitution is a recognized evolutionary mechanism in all organisms, these studies used high-resolution multilocus sequence typing (MLST) analysis of strains isolated from different geographic locations and, in the case of Whitaker, Grogan, and Taylor (2003), calculated the variance of genetic diversity (Cockerham’s *F*<sub>ST</sub> parameter) between groups of strains to show population differentiation between regions. However, an alternative explanation would be that the observed biogeographic patterns are the result of sampling biases toward dominant clones at a particular time, in different locations (Fenchel 2003). Therefore, the ability to detect geographical isolation of microbial population necessarily relies on the use of high-resolution genetic markers that undoubtedly demonstrate the effects of genetic drift on genetic differentiation.

Insertion sequence (IS) elements constitute such molecular markers. IS elements are short segments of DNA, typically 1–2 kb in length, with the ability to move within and between genomes, without a need for DNA homology (Galas and Chandler 1989). Although some elements can persist in the genome because they bring a selective advantage to their host, i.e., antibiotic resistance (Lupski 1987), most IS elements seem to behave as “selfish genes” or parasites, persisting without major consequences or being deleterious to the host genome. The reduced host fitness is then the result of gene inactivation, adjacent gene deletions, and other chromosomal rearrangements (Doolittle and Sapienza 1980; Orgel and Crick 1980; Brookfield 2005). It is known that the frequency of IS elements per site (any locus where an IS is present in at least one of the genomes of the host population) depends on the rates of transposition, excision, and recombination (Langley, Brookfield, and Kaplan 1983). Natural selection limits the spread of IS by transposition due to the deleterious fitness effects associated with gene disruption and inactivation, and an equilibrium is reached when the effects of transposition and selection are balanced (B. Charlesworth and D. Charlesworth 1983). In large populations the number of sites for transpositions is high enough that finding elements linked to a particular site is an unlikely event. In other words, the probability of finding two individuals with the same IS at a particular site is much higher in a small population than in a large population (Slatkin 1985). Therefore, stochastic processes rather than selection better explain the presence and maintenance at high frequencies of IS elements in natural populations.

Hyperthermophilic archaea of the genus *Pyrococcus* are found in deep sea hydrothermal vents and shallow marine hot springs and can only grow above 70°C and under anaerobic conditions (Fiala and Stetter 1986; Lepage et al. 2004). Their environment displays “island-like” characteristics with high-temperature niches (hot springs) separated by large areas of inhospitable conditions (cold oceans). The genus comprises three species *Pyrococcus furiosis*, *Pyrococcus abyssi*, and *Pyrococcus horikoshii* for which complete genome sequences were obtained (Kawarabayasi et al. 1998; Robb et al. 2001; Cohen et al. 2003). Full-length IS elements are found in the genome *P. furiosis* but are absent from that of *P. abyssi* and *P. horikoshii* (DiRuggiero et al. 2000; Lecompte et al. 2001). *P. furiosis* IS elements belong...
to a novel family of transposable elements and can be classified into three major groups based on nucleotide identity (83%–84%): IS-pf1u (11 copies), IS-pf2u (8 copies), and IS-pf3 (4 copies) (Kanoksilapatham et al. 2004). Each IS element contains a transposase gene (≈702 bp), a short spacer (54–57 bp), and two flanking 16-bp inverted repeats (fig. 3).

We used both, MLST and IS element analyses, in hyperthermophilic archaea of the genus *Pyrococcus* to test the hypothesis that genetic drift played an important role in the diversification of these microorganisms.

**Materials and Methods**

**Strain Isolation and Growth Conditions**

Water and sediment samples were obtained from mud pools and underwater hot springs in Vulcano Island, Italy, in fall 2002 and 2003. Samples were stored in reduced media and anaerobic conditions until further processing. Enrichment cultures were grown in 50–60 ml liquid *P. furiosus* medium inoculated with 1–2 ml, as described previously (Lepage et al. 2004). Following incubation at 90°C, cell densities were determined using acridine orange direct counts (Darzynkiewicz 1990). Cell-containing enrichment cultures were plated on solid gel media as described in Lepage et al. (2004) and incubated at 90°C in anaerobic jars for 3–4 days. Eight individual colonies from the 2002 sampling (VB81, VB82, VB83, VB85, VB96, VB93, VB112, VB113) and 11 from the 2003 sampling (V61, V62, V63, V72, V73, V211, V212, V221, V222, V231, V323) were selected and subjected to another round of liquid and solid media growth before final culture in liquid medium and storage at 4°C. Isolates from the Juan de Fuca Ridge (MZ4 from 1995; MV4 and MV7 from 1996; AV5 from 1999; EX2 from 2002; and JT1 from 1982) and the East Pacific Ridge (12/1, 30/3, 30/4, 31/2, 32/1, 32/2, 32/3, 32/4 from sampling in 1999) in the Pacific Ocean were kindly supplied by J. Baross (University of Washington, personal communication), T. Tuttle (Tuttle and DiRuggiero, personal communication), and P. Forterre (Lepage et al. 2004), respectively.

**Strain Characterization and Phylogenetic Analysis**

Genomic DNA was extracted from 60 ml of liquid cultures using the BIO 101 Genome Kit (Q-BioGene, Irvine, Calif.) as described by the manufacturer. Specific 16S rDNA primers, Arch0333aF15 (5′-TCCAGGCCCTACGGG-3′) and Arch0958R19 (5′-YCCGGCGTTGAMTCCTAATT-3′) were used to obtain polymerase chain reaction (PCR) products from all the Vulcano isolates. All loci were amplified by PCR in 12.5 μl reactions containing 10 pmoles of primers, 3.0 mM MgCl2, 50 mM KCl, 10 mM Tris, 0.2 mM deoxyribonucleoside triphosphates, and 1 U of AmpliTag DNA polymerase (Fermentas, Hanover, Md.). PCR conditions were as follows: 94°C for 2 min and 30 cycles of 94°C for 30 s, 55°C or 60°C for 30 s, and 72°C for 30 s. Sequences were obtained by direct sequencing of PCR products purified using exoSAP (USB, Cleveland, Ohio) and sequenced using Big Dye terminator reactions (Perkin Elmer, Boston, Mass.) and an ABI3100 automated sequencer (Applied BioSystems, Foster City, Calif.). Sequences for 16S rDNA, approximately 700 bp in length, were aligned with those of closely related species of Thermococcales (as listed in the GenBank database [http://www.ncbi.nlm.nih.gov]) with the ClustalV program (Higgins, Bleasby, and Fuchs 1992) from the Sequence Navigator package and used to generate the tree shown in Figure S1 (Supplementary Material online) with the Neighbor-joining algorithm from PAUP* 4.0b10 (Swofford 1998). Sequences from the other loci were edited and aligned using the same software. Phylogenetic reconstruction of the evolutionary history of 36 *Pyrococcus* strains and one *Thermococcus* strain was performed by simultaneous analysis of the DNA sequences of four loci homologous to *P. furiosus* 16S rDNA synthetase (PF0290), putative DNA helicase (PF0572), hypothetical protein PF1459, and α-glucan phosphorylase (PF1536) + hypothetical protein PF1537 (I–IV; table 1). Three additional loci prephenate dehydratase (PF0291) + deoxypentosylase synthase (PF0292), 3-hydroxyisobutyrat dehydration enzyme (PF0716), and a putative protease PF1905 (I–VI; table 1) were used to expand on the phylogenetic relationship among the 19 Vulcano isolates and *P. furiosus* (I–VII; table 1). These loci were selected to represent regions of high-nucleotide variation among the three reference strains and include protein-encoding genes and intergenic regions. Primers used to amplify these loci are reported in Table S1 (Supplementary Material online). Phylogenetic analyses were performed using the maximum likelihood analysis from PAUP* 4.0b10 (Swofford 1998). In the likelihood analysis, the general time reversible model with invariant site and gamma correction was used (GTR + I + G) with four rate categories. Likelihood and parsimony bootstrap proportions were calculated from 1,000 iterations.

**IS Analysis**

Six primer pairs were designed to amplify specific IS elements homologous to *P. furiosus* PF0069, PF0242, PF0946, PF0536, PF1736, and PF0898, in all Vulcano strains (Table S1, Supplementary Material online). The PCR and sequencing conditions used were similar to those described above. Sequences were aligned using the ClustalV program in the Sequence Navigator package (Applied BioSystems).

**Southern Blot Analysis**

Genomic DNA was isolated as described above. Restriction digest with *HindIII* and Southern hybridizations were performed according to the procedure of Sambrook, Fritsch, and Maniatis (1989). The probe, 210 bp in length containing *P. furiosus* transposase sequence, was generated by PCR and labeled using the DIG probe synthesis kit (Roche Diagnostics Inc., Indianapolis, Ind.) as described by the manufacturer.

**Results and Discussion**

**Phylogenetic Analysis**

We evaluated the existence of geographic genetic differentiation by MLST of 36 *Pyrococcus* strains from three geographical locations (fig. 1) comprising 3 reference
strains fully sequenced, *P. furiosus* DSM3638 (Fiala and Stetter 1986), *P. horikoshii* OT3 (Gonzalez et al. 1998), and *P. abyssi* GE5 (Erauso et al. 1993), 19 strains isolated from shallow hot spring samples collected in Vulcano Island (Italy) by our group in 2002 and 2003 and identified as *Pyrococcus* sp. based on 16S rDNA sequence analysis (fig. S1, Supplementary Material online), and 14 *Pyrococcus* strains previously isolated in the Pacific Ocean from the Juan de Fuca Ridge and the East Pacific Ridge (Lepage et al. 2000; Lecompte et al. 2001), suggesting that IS elements may have been present in the *Pyrococcus* strains. Six major lineages of *Pyrococcus*, corresponding to the main geographical locations considered in the study, were resolved in the maximum likelihood phylogenetic tree derived from the simultaneous analysis of nucleotide sequence data for four loci (I–IV; table 1) from 36 strains (fig. 1). Isolates from the East Pacific Ridge formed the most ancestral group followed by the group of isolates from the Juan de Fuca Ridge (fig. 1). The reference strains *P. abyssi* and *P. horikoshii* (isolated from the North Fiji Basin and the Okinawa Trough in the Pacific Ocean, respectively) stand alone as separate lineages, whereas *P. furiosus* and the isolates collected from the shallow hot springs of Vulcano Island (Italy) in the Mediterranean Sea constitute a more recent group. How-

Table 1  
Loci Considered in the Phylogenetic (I–VII) and IS Elements (VIII–XIII) Analyses  

<table>
<thead>
<tr>
<th>Loci</th>
<th>ORF Numbers</th>
<th>Genome Locations</th>
<th>Description</th>
<th>Segment Analyzed (bp)</th>
<th>Variable Sites (nucleotide substitution rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>PF0290</td>
<td>304315–306990</td>
<td>Valyl-tRNA synthetase</td>
<td>654</td>
<td>252 (38.5%) 77 (12%)</td>
</tr>
<tr>
<td>II</td>
<td>PF0572</td>
<td>592552–594519</td>
<td>Putative DNA helicase</td>
<td>543</td>
<td>310 (57%) 119 (21%)</td>
</tr>
<tr>
<td>III</td>
<td>PF1459</td>
<td>1364290–1365701</td>
<td>Hypothetical protein</td>
<td>773</td>
<td>394 (50%) 40 (5%)</td>
</tr>
<tr>
<td>IV</td>
<td>PF1535 +</td>
<td>1432910–1435938</td>
<td>α-Glucan phosphorilase + hypothetical protein</td>
<td>718</td>
<td>383 (53%) 40 (5%)</td>
</tr>
<tr>
<td></td>
<td>PF1536</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>PF0291 +</td>
<td>307149–308978</td>
<td>Prephenate dehydratase + deoxyhypusine synthase</td>
<td>778</td>
<td>N/A 116 (14%)</td>
</tr>
<tr>
<td></td>
<td>PF0292</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>PF0716</td>
<td>715679–716515</td>
<td>3-Hydroxyisobutyrate dehydrogenase</td>
<td>699</td>
<td>N/A 13 (1.8%)</td>
</tr>
<tr>
<td>VII</td>
<td>PF1905</td>
<td>1755914–1757236</td>
<td>Putative protease</td>
<td>1,182</td>
<td>N/A 141 (12%)</td>
</tr>
<tr>
<td>VIII</td>
<td>PF0069</td>
<td>72993–73694</td>
<td>Transposase</td>
<td>651</td>
<td>N/A 82 (13%)</td>
</tr>
<tr>
<td>IX</td>
<td>PF0242</td>
<td>253396–253584</td>
<td>Transposase</td>
<td>583</td>
<td>N/A 68 (12%)</td>
</tr>
<tr>
<td>X</td>
<td>PF0946</td>
<td>903695–910639</td>
<td>Transposase</td>
<td>730</td>
<td>N/A 30 (4%)</td>
</tr>
<tr>
<td>XI</td>
<td>PF0536</td>
<td>552553–553254</td>
<td>Transposase</td>
<td>702</td>
<td>N/A 2 (0.3%)</td>
</tr>
<tr>
<td>XII</td>
<td>PF1736</td>
<td>1612383–1613132</td>
<td>Transposase</td>
<td>750</td>
<td>N/A 24 (3%)</td>
</tr>
<tr>
<td>XIII</td>
<td>PF0898</td>
<td>870647–871348</td>
<td>Transposase</td>
<td>702</td>
<td>N/A N/A</td>
</tr>
</tbody>
</table>

Note:—N/A: not applicable.
a Genome locations in *P. furiosus*.  
b Number of variable nucleotides per analyzed segment.  
c Thirty-six strains considered.  
d Twenty strains considered unless indicated.  
e Eight strains considered.  
f Six strains considered.

IS Analysis  
The relationship between geographical isolation and genetic divergence in the *Pyrococcus* populations was further supported by our observation that *P. furiosus*–like IS elements were present in all the isolates from Vulcano Island (Mediterranean Sea) and completely absent from that of the Pacific Ocean (Fig. S2, Supplementary Material online). We used standard Southern blot hybridization and a 210-bp probe of highly conserved sequences for *P. furiosus* ISs (DiRuggiero et al. 2000). The number of bands visualized by this method in the Vulcano isolates ranged from 15 to 23, and the band patterns differed among isolates and with that of *P. furiosus* illustrating diversity in the number and chromosomal positions of ISs among isolates. Sequence analysis of the complete genome sequence of *P. abyssi* and *P. horikoshii* revealed the presence of a 211-bp DNA fragment at position 761435 and a 244-bp fragment at position 368535 in each genome, respectively. Both fragments have high level of nucleotide identity (89% and 91%) to *P. furiosus* transposase sequences (DiRuggiero et al. 2000; Lecompte et al. 2001), suggesting that IS elements might have been present in the *Pyrococcus* populations of the Pacific Ocean at some point in time and were subsequently lost. IS element diversity in the Vulcano isolates was further analyzed by direct sequencing of six selected ISs and their flanking regions (table 1). We found three categories of IS element’s evolutionary dynamics based on our sequence analysis (fig. 2). In the first category, ISs and
their flanking open reading frames (ORFs) were found in the same order in all the Vulcano isolates and in P. furiosus (PF0069, PF0242, PF0946), and ISs accumulated neutral point mutations at the same level as protein-encoding genes (table 1) indicative of coevolution with their host. In the second category, ISs were present in some isolates but absent in others and/or showed modifications of the flanking ORFs (PF0536, PF1736), suggesting potential selection against the retention of those mobile elements (fig. 2). For example, in the genome of P. furiosus, IS-pfu2 PF1736 flanks one side of a composite transposon, with IS-pfu2 PF1752 flanking the other side. This composite transposon contains a 16-kb fragment acquired by horizontal gene transfer and carrying an actively transcribed ABC transport system for maltose and trehalose (DiRuggiero et al. 2000). This 16-kb DNA fragment together with PF1752 were absent from all the Vulcano isolates (from 2002 to 2003) and PF1736 was absent from 13 isolates (fig. 2), suggesting a short-term selective advantage for these newly acquired genes. In the third category, ISs of one type were replaced by ISs, or fragments of ISs, of another type with significant alterations of the flanking regions (PF0898; fig. 3, D, E, and F). We also found extensive rearrangement within the sequence of IS-pfu3 PF0898 by replacement of part of or of the whole transposase gene, including in some cases the spacer and left inverted repeat (fig. 3, A, C, E, and F). The observed rearrangements associated with IS elements were the result of both transposition and intragenic recombination events, and the high number of insertions/deletions among closely related isolates suggests that these regions are hot spots for genome shuffling. It was indeed suggested that IS elements were the cause of major genomic rearrangements in the genome of P. furiosus when compared to that of P. abyssi and P. horikoshii (Lecompte et al. 2001; Zivanovic et al. 2002). The deleterious effects of IS elements observed in the genomes of Pyrococcus are related to their movements and include gene deletions (fig. 3), chromosomal rearrangements (DiRuggiero et al. 2000; Lecompte et al. 2001), and gene inactivation such as the napA gene in Pyrococcus woesei (Kanoksilapatham et al. 2004). In this Pyrococcus strain, also isolated from

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**Fig. 1.—** Maximum likelihood tree inferred from the analysis of a concatenated nucleotide alignment of four loci: valyl-tRNA synthetase (PF0290), putative DNA helicase (PF0572), hypothetical protein PF1459, and α-glucan phosphorylase (PF1536) + hypothetical protein PF1537 (I–IV; table 1) in 36 Pyrococcus strains and 1 Thermococcus strain used as the out-group. (a) World map indicating the locations where the samples were collected. (b) The tree was constructed using PAUP* 4.0b10 (Swofford 1998) with a GTR + I + Γ4 model of sequence evolution. Numbers above branches indicate maximum likelihood (>60%)/maximum parsimony bootstrap proportions (>60%) when the same branches were recovered. Location names to the right of the strains indicate their origin.
Fig. 2.—Maximum likelihood tree inferred from the analysis of a concatenated nucleotide alignment of seven loci (prephenate dehydratase [PF0291] + deoxyhypusine synthase [PF0292], 3-hydroxyisobutyrate dehydrogenase [PF0716], and a putative protease PF1905 in addition to the four genes from fig. 1) (I–VI; table 1) in the 19 Vulcano isolates and Pyrococcus furiosus. (a) The tree was constructed as described in figure 1. Numbers to the right indicate collection years. (b) Summary of the analysis of six P. furiosus-like IS elements in all isolates. Numerals I, II, III indicate the type of IS element: IS-pfa1, IS-pfa2, and IS-pfa3, respectively; D indicates truncated IS elements of approximately 300–700 bp, found in the genome of P. furiosus (four copies) and classified as IS-D (Kanoksilapatham et al. 2004); “-” indicates absence of IS; and ND = nondetermined. Letters in PF0898 are according to figure 3.

Vulcano and considered to be a subspecies of P. furiosus (Zillig et al. 1987), the putative Na\(^+\)/H\(^+\) antiporter (napA) gene was disrupted by a IS-pfu3, whereas the napA gene from P. furiosus remained intact. Inactivation of gene function by transposition events in the domain Archaea is not unique (Schleper et al. 1994) and has been shown to occur in Sulfolobus solfataricus (Schleper et al. 1994) and Halobacteria (DasSarma, RajBhandary, and Khorana 1983).

The frequency of IS elements per site in a population is the result of their rate of proliferation through transposition and their removal by natural selection, given the deleterious fitness effects caused by their presence (B. Charlesworth and D. Charlesworth 1983). In large host populations the number of sites available for transposition is high, producing low-allele frequencies per site (Slatkin 1985). Therefore, high-allele frequencies are only possible in a small host population due to stochastic drift (B. Charlesworth and D. Charlesworth 1983; Langley, Brookfield, and Kaplan 1983; Slatkin 1985; Brookfield 1986), which is what we observed in Vulcano Island (fig. 2). This brings the question as to whether the presence of IS elements only in the Vulcano Island population is the result of local adaptation through selective advantage from harboring IS elements or the result of random genetic drift. Given the high frequency per site of the IS elements analyzed here and their potentially deleterious fitness effects in the genome of Pyrococcus, we suggest that in the case of the population of Vulcano Island these genetic mobile elements were present in the colonizing population and brought up to high frequency by genetic drift, whereas they disappeared from the Pacific Ocean populations. Advantages associated with the potential acquisition of novel genes through horizontal gene transfer, possibly to cope with the highly dynamic environment of the shallow waters of Vulcano Island (J. P. Amend, A. C. Amend, and Valenza 1998; Aiuppa et al. 2000) and the constant input of xenobiotic compounds in the ecosystem, are only transient and are not sufficient to explain the invasion of these genetic elements in the population (Top and Springael 2003). In contrast, the arms race against the invasion of the Pyrococcus genome by these “hostile” IS elements may explain the rapid genetic evolution of specific regions of the genome (Lecompte et al. 2001) as a mechanism to disrupt their optimal transposition site sequences. This strategy has been shown in bacteria where fast-evolving DNA regions were shown to be associated with genes involved in defense against bacteriophage invasion (Zheng, Roberts, and Kasif 2004). The observed temporal clustering between Pyrococcus isolates from 2002 (8 isolates) and 2003 (11 isolates) (fig. 2), P. furiosus isolated in 1986, might be the result of a similar process. Indeed, selective sweep cannot explain the observed temporal variation as the diversity of the strains is high from one year to the next.

**Conclusion**

The presence of IS elements in only one population of Pyrococcus, together with MLST, revealed genetic divergence between populations occupying different geographic locations. Physical barriers to their dispersal might be the
result of severe constraints on their growth conditions together with the discontinuous nature of their environment. The observed high frequency of IS elements and evidence of their deleterious effects strongly suggest that genetic drift occurred in the Vulcano Island population and that it is an important mechanism of genetic divergence in *Pyrococcus*. Thus, these observations and the island-like nature of their environment make hyperthermophiles good candidates to be the microbial counterpart of the giant tortoise of the Galapagos (Fenchel 2003).

**Supplementary Material**

Table S1 and Figures S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

**Acknowledgments**

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