

# High Spontaneous Mutation Rate in the Hyperthermophilic Archaeon *Sulfolobus solfataricus* Is Mediated by Transposable Elements†

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**We have isolated uracil-auxotrophic mutants of the hyperthermophilic archaeon *Sulfolobus solfataricus* in order to explore the genomic stability and mutational frequencies of this organism and to identify completable recipients for a selectable genetic transformation system. Positive selection of spontaneous mutants resistant to 5-fluoroorotate yielded uracil auxotrophs with frequencies of between  $10^{-4}$  and  $10^{-5}$  per sensitive, viable cell. Four different, nonhomologous insertion sequences (ISs) were identified at different positions within the chromosomal *pyrEF* locus of these mutants. They ranged in size from 1,058 to 1,439 bp and possessed properties typical of known transposable elements, i.e., terminal inverted repeats, flanking duplicated target sequences, and putative transposase genes encoding motifs that are indicative of the IS4-IS5 element families. Between 12 and 25 copies of each IS element were found in chromosomal DNAs by Southern analyses. While characteristic fingerprint patterns created by IS element-specific probes were observed with genomic DNA of different *S. solfataricus* strains, no homologous sequences were identified in DNA of other well-characterized strains of the order *Sulfolobales*.**

Small transposable DNA elements are integral parts of most prokaryotic genomes and are also frequently found in eukaryotes (14, 19). The mobile bacterial insertion sequence (IS) elements were initially identified by their capacity to generate mutations or to disseminate antibiotic resistance genes, but more recently, they have frequently been detected in association with pathogenic and virulence functions or degradative and catabolic pathways. Therefore, a general role of transposable elements in assembling and mobilizing sets of accessory functions in bacteria has been proposed (18, 19).

ISs appear also to be widely distributed in the domain *Archaea*. In three of the four completely sequenced archaeal genomes, all of which belong to (hyper)thermophilic organisms of the kingdom *Euryarchaeota*, many families of insertion elements have been identified (4, 15, 16, 31).

The best-studied archaeal IS elements are found in the extremely halophilic euryarchaeote *Halobacterium salinarum* (formerly *Halobacterium halobium*), which exhibits exceptionally high genetic variability due to insertional mutations and DNA rearrangements. Phenotypes of this organism mutate with frequencies of between  $10^{-2}$  and  $10^{-4}$  (21). The IS elements are nonrandomly distributed within the genome, residing mostly on plasmids and in the AT-rich chromosomal regions (21). In *Halobacterium* sp. strain NRC-1, an IS element-mediated process is proposed to account for the movement of chromosomal genes to the 200-kbp megaplasmid pNRC100, which seems to be evolving into a minichromosome (20). A transpositional burst of the IS element ISH27 was observed in *Halobacterium* after plates had been stored for long periods in the cold, suggesting that stress can lead to increased transposition activity (22).

Several IS elements have also been detected in the hyperthermophilic crenarchaeote *Sulfolobus solfataricus*. Spontaneous  $\beta$ -galactosidase mutants have been shown to arise with relatively high frequencies (about  $10^{-4}$  per plated cell [26]). These mutants contained a transposable element in the *lacS* gene ( $\beta$ -galactosidase) with features typical of bacterial and archaeal ISs including terminal inverted repeats, a putative transposase gene, and short direct flanking repeats. Furthermore, in the ongoing genome sequencing project of *S. solfataricus* at least six more classically organized IS elements have so far been detected (28, 29). Finally, putative transposases and at least one insertion element have also been found in the sequence of the promiscuous conjugative plasmid pNOB8 of *Sulfolobus* sp., a close relative of *S. solfataricus* (30), and in strain MT-4 (2). Altogether, these data suggested that several IS elements might be active in *Sulfolobus*.

In this study, we were interested in exploring the genomic stability and mutational frequencies of *S. solfataricus* and in identifying stable auxotrophic mutants that can serve as completable recipients in selectable genetic systems. *S. solfataricus* is becoming a model system to study cellular processes and gene functions in hyperthermophilic crenarchaeota, based on the isolation of several plasmids and viruses (34) and on the demonstration of genetic transformation of this organism (3, 5, 7, 25). Furthermore, the complete genomic sequence of *S. solfataricus* is currently being determined (29).

We have selected for pyrimidine-requiring auxotrophs with 5-fluoroorotic acid (5-FOA). Mutants that are resistant to this drug usually lack either orotidine-5'-monophosphate pyrophosphorylase (PyrE) or orotidine-5'-monophosphate decarboxylase (PyrF) and are thus unable to convert orotate to UMP, which represents the last step in de novo pyrimidine nucleotide biosynthesis. 5-FOA has previously been used for positive selection of uracil auxotrophs in different microorganisms, including the archaeon *Sulfolobus acidocaldarius* (10, 17). Although spontaneous mutational rates of *S. acidocaldarius* were found to be comparable to mutational rates of biosyn-

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thetic genes in *Escherichia coli* in a selection with 5-FOA (13), in this study we show that stable uracil-auxotrophic mutants of *S. solfataricus* arise spontaneously with frequencies at least 1 order of magnitude higher and are caused by transposition of insertion elements.

#### MATERIALS AND METHODS

**Strains, media, and growth conditions.** *S. solfataricus* P1 (DSM 1616), P2 (DSM1617), and PH1 (26); *Sulfolobus shibatae* (DSM 5389); and *S. acidocaldarius* (DSM 639), PIT3, and KAW2 (gifts of Wolfram Zillig) were grown at 80°C and pH 3 in mineral medium supplemented with 0.2 or 0.1% tryptone (Oxoid) and 0.2% (either D-xylose or L-arabinose (Sigma), Gelrite (0.7%; Kelco, San Diego, Calif.) was added to solidify the media for plating as described previously (9). For selection of pyrimidine-auxotrophic mutants, 50 µg of 5-FOA (Sigma) per ml was added as well as 10 µg of uracil per ml. Mutants were grown in media supplemented with 10 µg of uracil per ml.

**Isolation of auxotrophic mutants.** Strains were grown in 50-ml liquid cultures to an optical density of  $A_{600} = 0.9$  to 1.0 (late exponential phase), and approximately  $10^6$  to  $10^7$  cells were plated on solid medium supplemented with 5-FOA and uracil. Controls were plated on media solely containing 5-FOA but no uracil (no growth), and appropriate dilutions were plated on medium without inhibitor to determine viable cell counts as CFU. The plates were incubated for 5 to 7 days, and spontaneous 5-FOA-resistant mutants were isolated and tested by restreaking colonies on fresh plates and/or by inoculating liquid medium with and without uracil.

**Isolation of chromosomal DNA from *Sulfolobus*.** Three milliliters of a late-exponential culture was cooled and centrifuged in a minicentrifuge for 2 min at maximal speed. The cells were resuspended in 500 µl of Tris-EDTA buffer, and *N*-laurylsarcosine (0.8%) and Triton X-100 (0.06%) were added. After incubation at 20°C for 30 min, the lysed cells were extracted with phenol-chloroform and the DNA was precipitated with ethanol. About 50 ng of such preparations was used as templates in PCR amplifications, and about 3 µg was used for restriction enzyme analyses and subsequent Southern hybridizations.

**PCR amplification, cloning, and sequence analysis.** Two primers derived from the DNA sequence located 5' and 3' adjacent to the *pyrEF* gene cluster in *S. solfataricus* P2 (see Fig. 2) that contained *KpnI* restriction sites at their 5' ends were synthesized: *pyr-R*, GCA GGT ACC TCG TGT AGA TTT TCC CC, and *pyr-L*, GCG GTA CCA CTT GCG AAT AAT GCT GC. These primers specifically amplified a 1.7-kbp fragment from chromosomal DNA of *S. solfataricus* P1 and P2 at annealing temperatures of 52°C. Fragments of 2.9 to 3.1 kbp in size were obtained from auxotrophic mutants. Additional primer pairs complementary to regions in the *pyrEF* cluster were used to specifically amplify IS element sequences for the different mutants after their site of integration had been determined. One primer in each set contained a *KpnI* restriction site, and the other contained a *SacII* restriction site. Both enzymes did not cleave within the IS element sequences. The PCR products were purified, cleaved with *KpnI* (or *KpnI* and *SacII*, respectively), and ligated with pBluescript SK(+) cut with the same enzymes. The inserted DNA fragments of the resulting recombinant clones were identified by restriction mapping.

Two primers, *pyrE2Nhe* (TTT TTG CTA GCT TTT TCC AAT ATT TTT C AC) and *pyrE1CLA* (TTT TTA TCG ATG GAT TTC GTG AAA GCT CTA C), were used to amplify the *pyrE* gene of *S. acidocaldarius* (accession no. Y12822, erroneously assigned in that submission to *S. solfataricus*).

Sequence analysis was performed on a Li-Cor DNA sequencer. The University of Wisconsin Genetics Computer Group package (version 10.0) was used for computer-aided editing of the DNA sequences. Amino acid sequences of the putative transposases were compared in a multiple alignment with PILEUP that was corrected manually. Only a few representatives of the transposases from the IS4-IS5 family were chosen for comparison, preferably those that were initially identified as relatives by BLASTP2 searches against the databases.

**Southern hybridization.** Approximately 2 to 3 µg of chromosomal DNAs was cut with *AflIII* and electrophoresed in a 1% agarose gel using standard procedures (24). The DNA was blotted and hybridized according to standard procedures. The *pyrEF*- and IS element-specific probes were prepared by cutting out and purifying the respective inserts from recombinant pBluescript vectors and randomly labeling them using the digoxigenin-DNA labeling and detection kit (Boehringer Mannheim).

**Nucleotide sequence accession numbers.** The DNA sequences of the three novel IS elements characterized in this study have been deposited in GenBank under accession no. AJ270982, AJ270983, and AJ270984.

#### RESULTS

**Isolation of uracil-auxotrophic mutants.** To select spontaneous mutants resistant to 5-FOA, cultures were grown to mid- or late exponential phase and about  $10^6$  to  $10^7$  cells were plated on medium containing 50 µg of the inhibitor per ml and 10 µg of uracil per ml. The inhibitor concentration was about twice

TABLE 1. Estimation of mutation frequencies in *Sulfolobus* spp. by positive selection of uracil auxotrophs on 5-FOA

<i>Sulfolobus</i> sp. (and strain where indicated)	No. of cells <sup>a</sup>		Apparent frequency
	Sensitive	Resistant	
<i>S. solfataricus</i> P1	$4.7 \times 10^6$	113	$2.4 \times 10^{-5}$
	$3.3 \times 10^5$	103	$3.1 \times 10^{-4b}$
<i>S. solfataricus</i> PH1( <i>lacS</i> )	$6.3 \times 10^5$	214	$3.4 \times 10^{-4b}$
	$1.4 \times 10^6$	26	$1.9 \times 10^{-5}$
<i>S. shibatae</i>	$9.7 \times 10^6$	18	$1.8 \times 10^{-6}$
<i>S. islandicus</i> REN	$8.7 \times 10^6$	64	$7.4 \times 10^{-6}$
<i>S. acidocaldarius</i>	$3.2 \times 10^6$	6	$1.8 \times 10^{-6}$
	$4.5 \times 10^5$	2	$4.4 \times 10^{-6}$
	$6.3 \times 10^6$	24	$3.8 \times 10^{-6}$

<sup>a</sup> Cell numbers were calculated from averages of CFU after serial dilutions on Gelrite plates containing tryptone and arabinose (sensitive) or tryptone-arabinose plus 50 µg of 5-FOA per ml and 10 µg of uracil per ml (resistant).

<sup>b</sup> Experiments from which mutants were recovered for this study.

the MIC as determined for *S. acidocaldarius* (10). The same numbers of cells were also plated on medium without uracil. No growth was observed with the four *Sulfolobus* strains that were tested in these control experiments. Appropriate dilutions of the cultures were plated without inhibitor in order to determine viable cell counts and to calculate mutation frequencies per CFU. The results of some representative and independent experiments are shown in Table 1. Resistant colonies of *S. acidocaldarius*, *S. shibatae*, and *Sulfolobus islandicus* REN were recovered at frequencies of  $10^{-6}$  to  $10^{-7}$  per CFU. These results were comparable to those observed in earlier studies for *S. acidocaldarius* ( $2 \times 10^{-7}$  [13]). In contrast, mutational frequencies of *S. solfataricus* appeared to be variable, but in all cases, they were at least 1 order of magnitude higher than those for the other strains. The highest frequencies were around  $3 \times 10^{-4}$ . Enhanced frequencies were also obtained with *S. solfataricus* strain PH1, a β-galactosidase mutant of P1 that contains the insertion element *ISC1217* stably integrated into the *lacS* gene (26).

Sixteen isolates of *S. solfataricus* P1 or PH1 and two of *S. acidocaldarius* were subsequently checked for their auxotrophic phenotype in liquid medium. While all isolates grew in the presence of uracil with rates comparable to those of wild-type (wt) cells, 14 out of the 16 drug-resistant strains of *S. solfataricus* did not grow in uracil-free medium (Fig. 1), nor did the two isolated mutants of *S. acidocaldarius*. By analogy to earlier studies (10, 17), we therefore concluded that the FOA-resistant phenotype was most probably based on auxotrophs lacking biosynthetic conversion between orotate and UMP.

On solid medium, however, to which no uracil was added, we observed weak growth of the mutants. Very small colonies developed after 4 days but ceased growth upon further incubation, while wt cells grew to large colonies (of about 2 to 3 mm in diameter) within 6 to 7 days. We therefore suspect that the gellan gum used for solidification of the medium might be slightly contaminated with nucleic acids that allowed some initial growth of the mutants until it was depleted. No revertants were observed from the auxotrophic isolates, either in liquid media or on plates (in the latter case, large colonies should have developed).

**Genetic characterization of uracil-auxotrophic mutants.** We have used PCR amplification and chromosomal Southern anal-

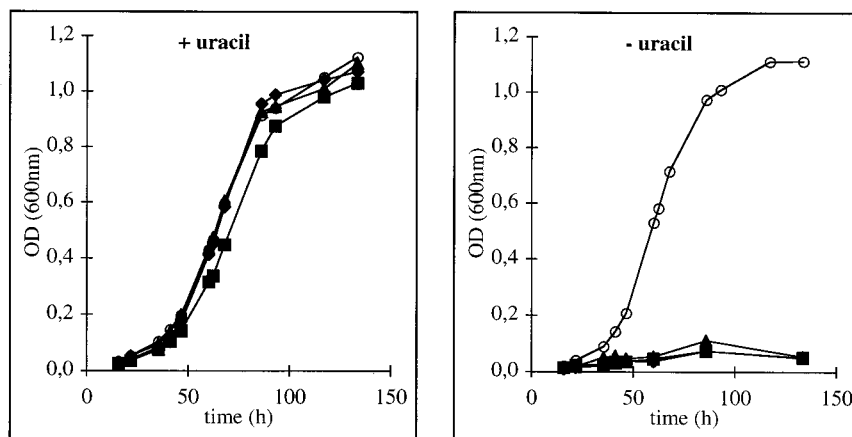


FIG. 1. Growth of *S. solfataricus* P1 wt (open circles) and of 5-FOA-resistant mutants (filled symbols; triangles, P1-18; squares, P1-17; diamonds, PH1-15) on medium containing 0.2% D-arabinose and 0.1% tryptone as carbon sources with (10 µg/ml) or without uracil. OD, optical density.

yses in order to study the *pyrE-pyrF* locus in the auxotrophic isolates. In the genomic sequence of *S. solfataricus* P2, the two genes lie adjacent to each other with the open reading frame (ORF) of *pyrE* overlapping *pyrF* by 14 bp (Fig. 2). Further homologs of pyrimidine synthesis genes (*pyrBCD*) were found directly upstream of *pyrEF* in the opposite orientation. The intergenic region of the two divergently oriented gene clusters was 63 bp long and contained a sequence in its center that follows the consensus of box A elements of archaeal promoters on both strands (TTTAAA). Based on the sequence information, two oligonucleotides were designed to amplify the *pyrEF* region from chromosomal DNA including 404 bp upstream of the putative *pyrE* gene and 78 bp downstream of *pyrF* (Fig. 2). The PCR amplification from *S. solfataricus* P1 DNA yielded a product of 1.7 kbp as predicted from the genomic sequence of

the closely related strain P2. In contrast, all PCR products obtained from the auxotrophic isolates of strains P1 and PH1 were 1 to 1.4 kbp larger, suggesting that a genetic change caused by insertional mutations in the *pyrEF* region was responsible for the mutant phenotypes (data not shown). For comparison, two mutants of *S. acidocaldarius* were also analyzed by using primers derived from the *pyrE* gene sequence of this strain. In both cases, fragments of 550 bp were obtained, corresponding to the size of the predicted wt fragments (data not shown).

In order to verify these results, chromosomal DNA from seven mutants of *S. solfataricus* P1 and PH1 was isolated, and a probe spanning almost the complete *pyrEF* wt region (positions 394 to 1493 in Fig. 2) was used in Southern analysis (Fig. 3). While a single 2.7-kbp fragment was detected in wt DNA

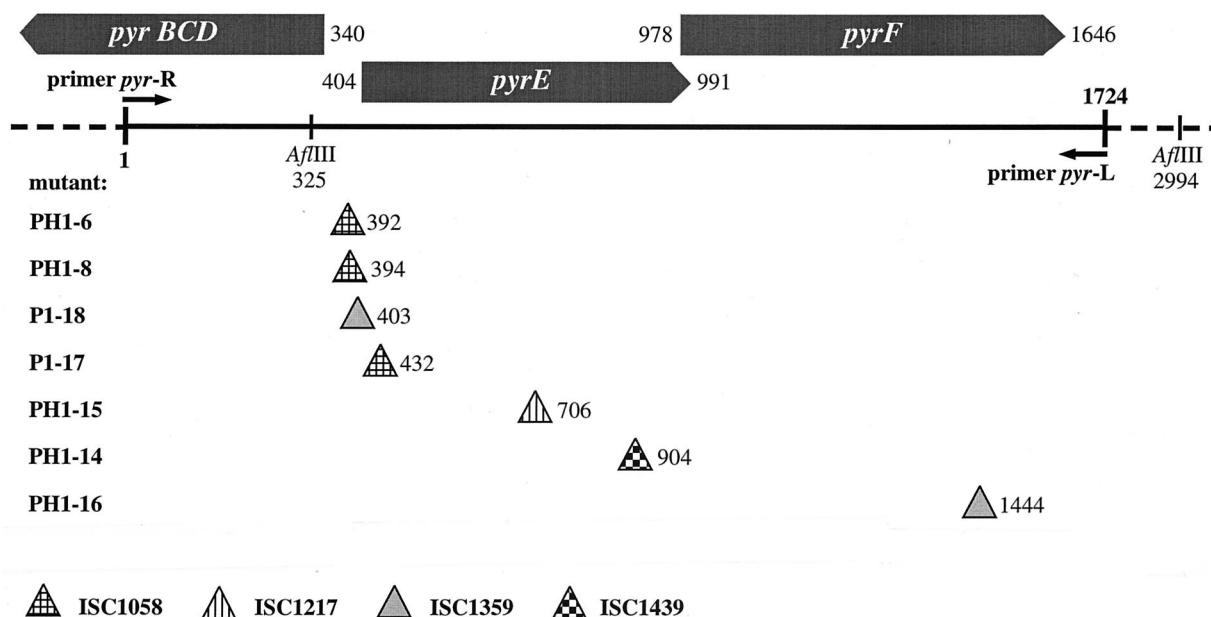


FIG. 2. Genomic region of pyrimidine biosynthesis genes in *S. solfataricus* P2. The positions of the primers *pyr-R* and *pyr-L*, used to amplify the *pyrEF* genes from wt and mutant DNA, are indicated, as well as the sites of insertion of the four different IS elements found in seven different mutants. The two *AflIII* sites within this region are also shown. *pyrE* and *pyrF* are genes coding for putative orotidine-5'-monophosphate pyrophosphorylase (PyrE) or orotidine-5'-monophosphate decarboxylase (PyrF) as identified by homology to known protein sequences from the database.

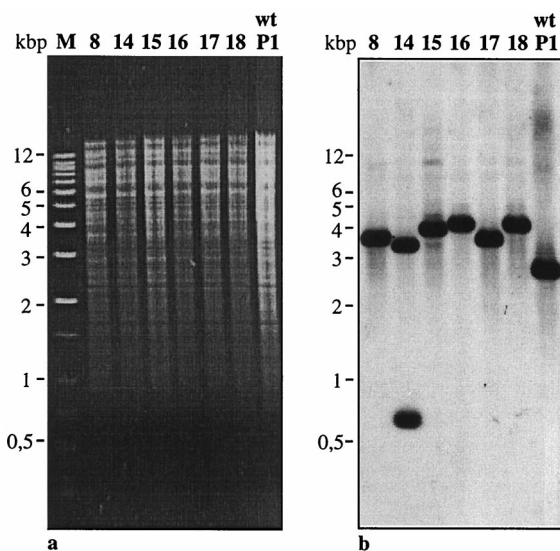


FIG. 3. Agarose gel electrophoresis (a) and corresponding Southern hybridization (b) of *AflIII*-digested genomic DNAs identifying the *pyrEF* loci in *S. solfataricus* P1 (wt) and in uracil-auxotrophic mutants. The *pyrEF* probe was obtained by PCR amplification of a 1,099-bp fragment (position 394 through 1493 in Fig. 2). M, markers.

cut with *AflIII*, six of seven DNAs isolated from the mutants showed a hybridizing fragment that was enlarged by about 1 to 1.4 kbp, resulting in fragments of approximately 3.7 to 4.1 kbp in length. Mutant PH1-14 showed a hybridizing fragment of approximately 3.7 kbp and a second fragment of 750 bp (Fig. 3b) apparently caused by the presence of an additional *AflIII* site within the enlarged *pyrEF* locus.

**Detection of IS elements in the *pyrEF* locus of uracil-auxotrophic mutants.** The *pyrEF* loci of seven mutants were directly amplified from chromosomal DNA and cloned into pBluescript. The recombinant fragments were cut with various restriction enzymes, and the patterns were compared to those of the wt fragment in order to localize insertions within the mutated *pyrEF* regions (data not shown). The exact positions of the insertions were then determined by sequence analysis and shown to be located in different sites in each of the seven mutants analyzed (Fig. 2). In three cases, the insertion was found within 12 bp immediately upstream of ORF *pyrE* (mutants PH1-6, PH1-8, and P1-18), three further insertions were found within ORF *pyrE* (mutants PH1-14, PH1-15, and P1-17), and one insertion was in ORF *pyrF* (mutant PH1-16). Oligonucleotides complementary to *pyrEF* sequences directly flanking each insertional site were used for PCR amplification of the ISs from the chromosomal DNA of the mutants. The seven fragments were cloned into pBluescript and sequenced from both sites. Four different ISs, of 1,058, 1,147, 1,359, and 1,439 bp in length, were identified (Fig. 4). The IS elements had terminal inverted repeats of 13, 19, 20, and 21 bp, respectively, and contained ORFs of 273 to 354 amino acids that potentially encoded transposases. Each IS was flanked by a directly repeated sequence of diagnostic length which was duplicated upon transposition of the respective element (Fig. 4 and Table 2). *ISC1058* was found in three mutants at different target sites (PH1-6, PH1-8, and P1-17 [Fig. 2]). The 9-bp duplicated target sequences were different but shared identical bases in their last two positions (AG). *ISC1359* was found in two mutants with different but similar flanks of only 4 bp (PH1-16, P1-18). *ISC1439* was found in mutant PH1-14 with a 9-bp direct repeat. The insertion element of PH1-15 was identical to *ISC1217*, which had been detected earlier in a  $\beta$ -galactosidase

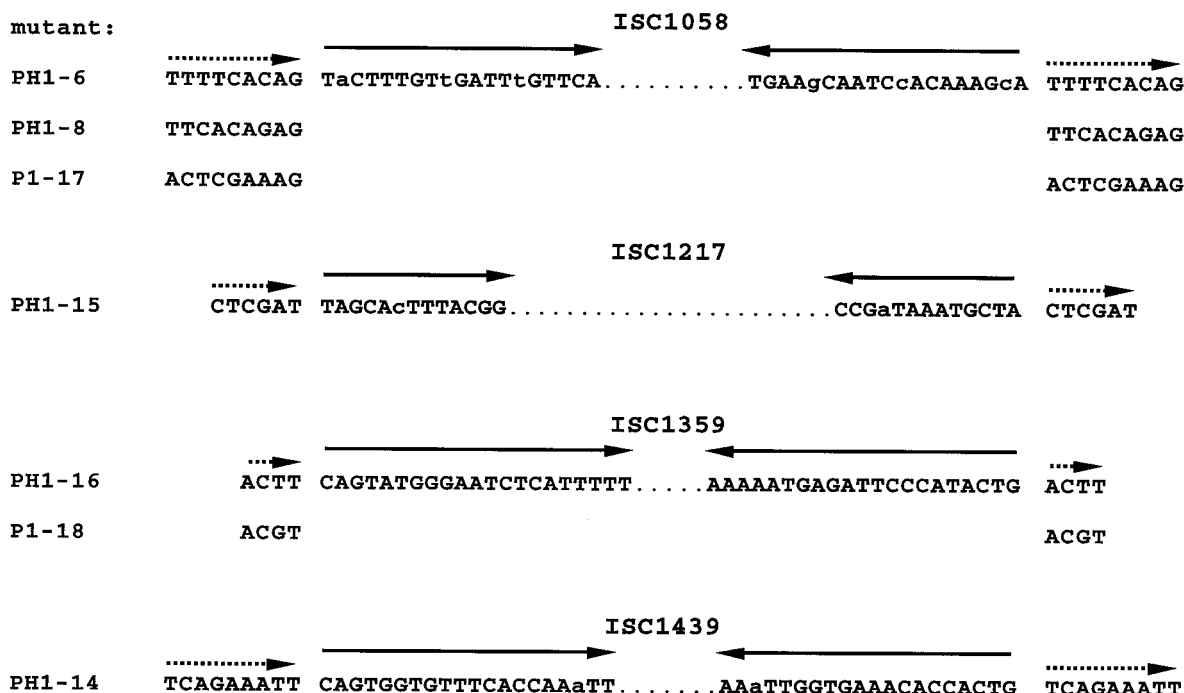


FIG. 4. Sequences of structural features of the four IS elements. Dotted arrows, duplicated target sequences (direct repeats) in the *pyrEF* genes flanking the IS element in each mutant; solid arrows, inverted repeats at termini of IS elements (lowercase letters represent mismatches within the inverted repeat).



TABLE 2. Features of IS elements isolated from *pyrEF* mutants of *S. solfataricus*

Feature	ISC1058	ISC1217 <sup>a</sup>	ISC1359	ISC1439
Size (bp)	1,058	1,147	1,359	1,439
Size of inverted repeat (bp)	19	13	21	20
Size of direct repeat (bp)	9	6	4	9
Size of largest ORF (amino acids)	299	354	273	321
Mutated gene(s)	<i>pyrE</i> prom- <i>pyrEF</i> <sup>b</sup>	<i>pyrE lacS</i>	prom- <i>pyrEF pyrF</i>	<i>pyrE</i>
Copy no. in:				
<i>S. solfataricus</i> P1	>17	>12	>13	>20
<i>S. solfataricus</i> P2	>14	>10	>13	>24
PIT3	>13	>15	>15	>18
KAW2	4	0	0	0

<sup>a</sup> Data are from reference 26.

<sup>b</sup> prom-*pyrEF*, IS element in promoter region.

mutant (26). The insertion elements were also found in the partial genome sequence of strain P2 (see below).

**Sequence similarity to known IS elements.** No apparent DNA sequence similarity of the four identified IS elements was found to other transposable elements available in the databases. Some similarities to transposases, however, were found when the deduced amino acid sequences of the largest ORF were used in the searches. While *ISC1359* and *ISC1439* showed limited similarities to elements of the *IS4* family, *ISC1058* was more similar to *IS903* of the *IS5* family. A closer inspection of the amino acid sequences revealed in all four cases the conserved motif Y-N<sub>2</sub>-R-N<sub>3</sub>-E-N<sub>6</sub>-K of the C1 region typical of transposases from the *IS4-IS5* families (19, 23) (Fig. 5). Furthermore, the second conserved motif of the N3 region, which is also indicative of *IS4* or *IS5* elements (D-N-G/A-Y/F), was identified. Based on these two motifs, many of the known bacterial and archaeal IS elements, i.e., those of members of the genera *Halobacterium* and *Haloferax*, have been combined in these families. Members of these groups also share the conserved D-D-E motif of different families of prokaryotic and

eukaryotic elements (i.e., transposases and retroviral integrases [19]).

**Abundance of insertion elements in the *S. solfataricus* genome and in auxotrophic mutants.** Southern analyses of chromosomal DNA were used to examine the copy number and distribution of the four IS elements in the *S. solfataricus* genome and mutant strains. Randomly labeled probes spanning the entire length of each IS element were prepared from the recombinant pBluescript plasmids and were used for hybridization against total DNA cut with *AflIII* (Fig. 6). The restriction enzyme did not cut within the sequences of *ISC1058*, *-1217*, and *-1359*. In *ISC1439*, two recognition sequences for *AflIII* were found at positions 1170 and 1330 of the element, but no hybridization was observed from the internal 160-bp fragment and from the remaining 109 bp of the labeled fragment (compare Fig. 3 and 6). We therefore assumed that the number of chromosomal fragments hybridizing to each IS element probe represented a minimal estimate of the copy number within the genomic DNAs.

Hybridization with the *ISC1217* probe (of mutant PH1-15)

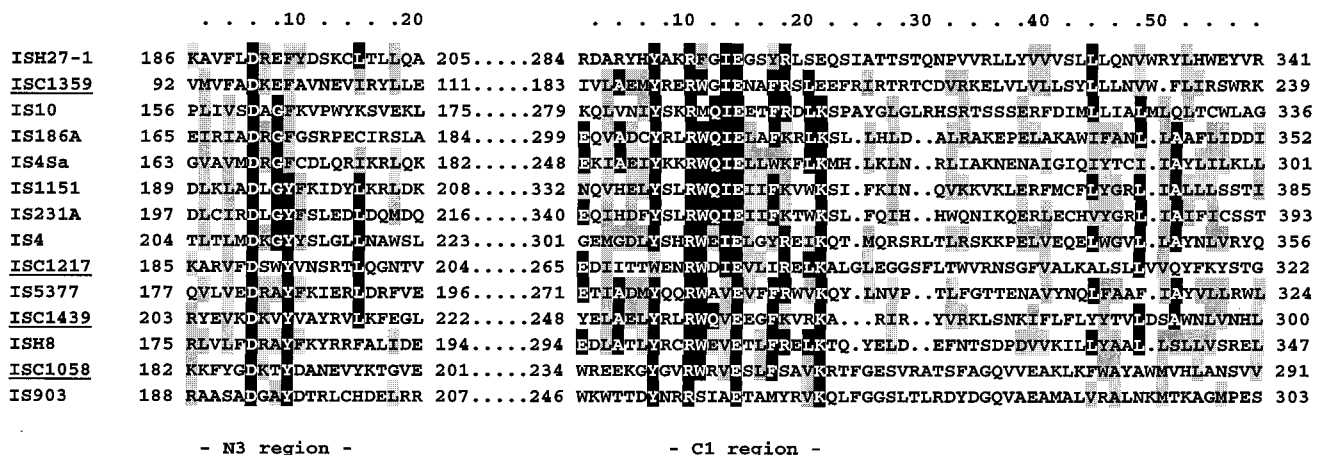


FIG. 5. Sequence similarity of the deduced amino acid sequences of the largest ORFs to representative transposases of the *IS4-IS5* family of insertion sequences. The two conserved signatures, D-N-G/A-Y/F and Y-N<sub>2</sub>-R-N<sub>3</sub>-E-N<sub>6</sub>-K, of the N3 and the C1 region, respectively, as described by Rezsözhazy et al. (23), were found in the putative transposases of the *S. solfataricus* elements. Identical amino acids are shaded in black; similar amino acids are shaded in grey (with a 40% cutoff). Sources of known IS elements (accession numbers are given in brackets) from archaea: ISH27-1, *H. salinarum* PHH1 (X54432); ISH8, *H. salinarum* SD108 (M58557). Those for bacteria: IS10, *Salmonella enterica* serovar Typhimurium Tn10 (J01829); IS186A, *E. coli* RR1 (M11300); IS4Sa, *Synechocystis* sp. strain PCC6803 (U38915); IS1151, *Clostridium perfringens* NCTC2062 (Z18246); IS231A, *Bacillus thuringiensis* (X03397); IS4, *E. coli* K-12 (J01733); IS5377, *Bacillus stearothermophilus* CU21 (X67862); IS903, *E. coli* (J01839). IS elements from this study are underlined.

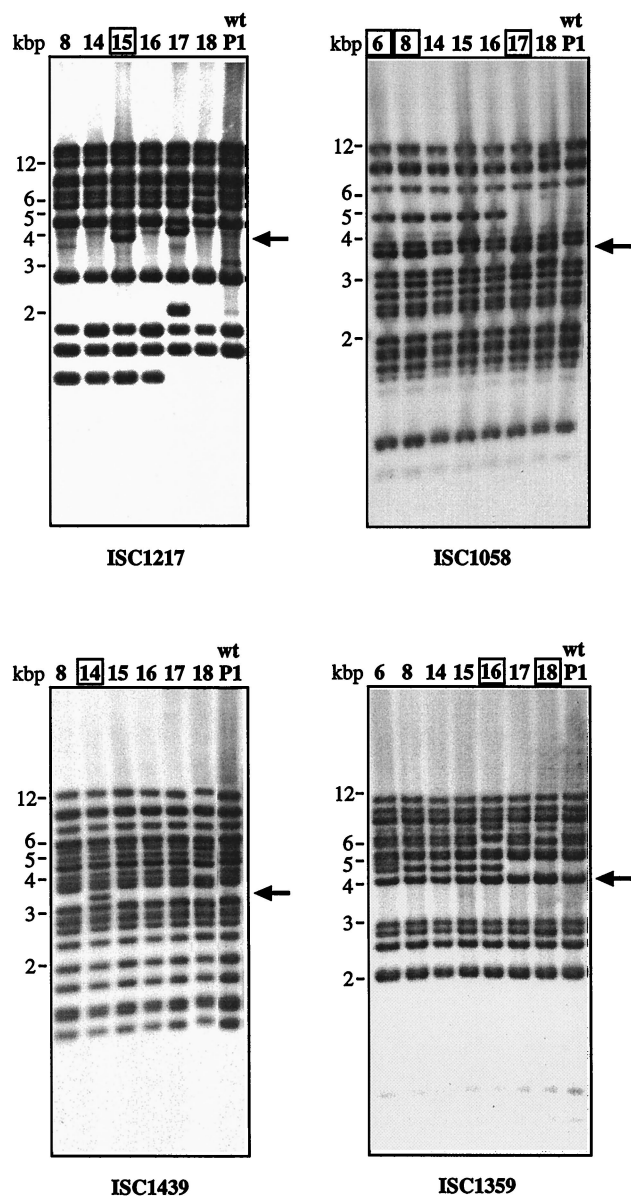


FIG. 6. Southern hybridization using IS element-specific probes and *Afl*III-digested chromosomal DNA of *S. solfataricus* P1 (wt) and uracil-auxotrophic mutants derived from P1 (17 and 18) or PH1 (6, 8, 14, 15, and 16;  $\beta$ -galactosidase mutant with a copy of *ISC1217* in *lacS* [26]). Each hybridizing fragment corresponds to at least one IS element copy, because *Afl*III does not cut within *ISC1058*, *ISC1217*, and *ISC1359* and only close to the terminus of *ISC1439* (Fig. 3b). Large arrows indicate the positions of fragments containing the IS element in the *pyrEF* region (as in Fig. 3, detected with the *pyrEF* probe). The respective mutants containing the IS element in this region are boxed. Note additional fragments in several mutants caused by multiple transposition events.

detected at least 11 fragments in chromosomal DNA of the wt strain (lane P1). An additional fragment of approximately 1.2 kbp was observed in the mutants derived from strain PH1 that contains an extra copy of this IS element within the *lacS* gene (Fig. 6) (PH1-8, PH1-14, PH1-15, and PH1-16). As expected, an extra fragment of 3.9 kbp hybridized with DNA of mutant PH1-15 showing the IS element copy within the *pyrEF* region (Fig. 6, arrow). However, an additional fragment of 4.5 kbp hybridized in PH1-15 and two additional fragments were also observed in mutant P1-17. Similarly, mutant P1-18 showed an

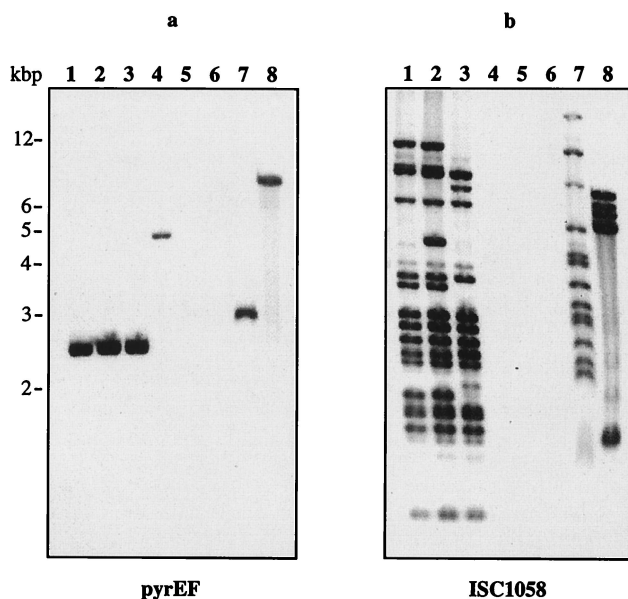


FIG. 7. Southern hybridization with *pyrEF* (a)- and IS element (b)-specific probe of *ISC1058* to *Afl*III-digested chromosomal DNAs of *S. solfataricus* P1 (lanes 1), the  $\beta$ -galactosidase mutant PH1 (lanes 2), strain P2 (lanes 3), *S. shibatae* (lanes 4), *S. acidocaldarius* (lanes 5), *A. ambivalens* (lanes 6), isolate PIT3 (lanes 7), and isolate KAW2 (lanes 8).

additional hybridizing fragment (of approximately 5 kbp) at the expense of a larger one (approximately 6 kbp). We concluded that additional transposition events of *ISC1217* apparently occurred in the chromosomal DNA of these mutants, as the latter two were identified through transposition of a different IS element into the *pyrEF* region (*ISC1058* and *ISC1359*, respectively). Multiple transposition events were also observed with the other IS element-specific probes (see below).

About 20 fragments hybridized to probe *ISC1439* in *S. solfataricus* P1, and the additional 3.4-kbp fragment of mutant PH1-14 showed the insertion of the element in *pyrEF*, while loss of one signal was observed in mutant P1-18.

Seventeen fragments hybridized to probe *ISC1058* in *S. solfataricus* P1 (wt) DNA, and 13 fragments were detected with probe *ISC1359*. The additional copies of these two IS elements in the *pyrEF* region were seen as bands of stronger intensities at 3.7 kbp for *ISC1058* (in mutants PH1-6, PH1-8, and P1-17) and at 4.0 kbp for *ISC1359* (in mutants PH1-16 and P1-18). They could be visualized as separate bands when the chromosomal DNAs were cut with a different restriction enzyme (*Hinc*II [data not shown]). At least one additional copy of *ISC1058* and *ISC1359* was detected in the mutants derived from strain PH1 (4.9- and 4.5-kbp fragments, respectively, in Fig. 6), suggesting that both elements were active in the *lacS* mutant at an earlier stage. Two or three additional fragments hybridized to *ISC1058* in mutant P1-18. One additional fragment was observed with *ISC1359* in mutant PH1-6, while a larger fragment appeared in PH1-16 and P1-18 at the expense of a smaller one.

In summary, four out of the seven mutants showed transpositional events or chromosomal variations in addition to the insertional recombination of the *pyrEF* region. Only the patterns of mutants PH1-8 and PH1-14 (Fig. 6) appeared to be identical to that of the original strain PH1 (as shown for *ISC1058* in Fig. 7b, lane 2).

### Distribution of the four IS elements in *Sulfolobus* strains.

Known strains of the order *Sulfolobales* (i.e., *S. shibatae*, *S. acidocaldarius*, and *Acidianus ambivalens*) and novel isolates that most probably represent close relatives of *S. solfataricus* were investigated in hybridization experiments to study their phylogenetic relationships and the occurrence of homologous IS elements. Strain PIT3 was isolated in 1998 from the same solfataric field in Pisciarelli, Italy, from which *S. solfataricus* P1 and P2 had been obtained in 1980 (33; personal communication). Strain KAW2 was isolated in Kawaii, northern Japan. Under stringent hybridization conditions, fragments homologous to *pyrEF* and of equal size were detected in the chromosomal DNA of *S. solfataricus* strains P1, PH1, and P2 (Fig. 7a, lanes 1 to 3). While no hybridization was observed with DNA from *S. acidocaldarius* and *A. ambivalens*, fragments of different sizes derived from *S. shibatae* and isolates PIT3 and KAW2 hybridized to this probe (Fig. 7a, lanes 4, 7, and 8). This result reflects the closer phylogenetic relationship of the three latter organisms to *S. solfataricus* as determined by 16S rRNA analysis or chromosomal restriction fragment patterns (8; W. Zillig, personal communication).

With each of the four IS element-specific probes, similar but slightly distinct hybridization patterns were observed against chromosomal DNA of *S. solfataricus* strains P1, PH1, and P2 (as shown for *ISC1058* in Fig. 7b, lanes 1 to 3). The differences in their patterns could be accounted for by restriction fragment length polymorphism in the two strains as well as by different activities of the IS elements.

No hybridization to IS element-specific probes was observed with DNA from other known members of the *Sulfolobales*, i.e., *S. shibatae*, *S. acidocaldarius*, and *A. ambivalens*. However, hybridization patterns of complexity similar to that found in *S. solfataricus* were observed with DNA from isolate PIT3 (as shown for *ISC1058* in Fig. 7b, lane 7), suggesting that homologous IS elements of all four types occur with similar frequency in this strain. Interestingly, hybridization of *ISC1058* to DNA of KAW2 (Fig. 7b, lane 8) and other isolates (data not shown) suggests that members of this IS element family or an element with high similarity occurs in relatives from Japanese hot springs. The other three ISC element-specific probes did not hybridize to DNA of these isolates (data not shown). No hybridization was observed with DNA of 20 other strains recently isolated from solfataras in New Zealand and Iceland (data not shown).

## DISCUSSION

The results in this study suggest that transposon mutagenesis is a dominant mechanism of mutation in the hyperthermophile *S. solfataricus* and that IS elements may play an important role in genome dynamics of this organism. We have found four different active IS elements that inserted stably into the *pyrEF* locus in seven independent transposition events. In six out of seven cases, the insertion was found in the *pyrE* gene or immediately upstream of it, and only in one case was it found in *pyrF*. Similarly, Jacobs and Grogan (13) described an up-to-fivefold-higher mutational rate for *pyrE* than for *pyrF* in *S. acidocaldarius*. Mutants of *S. acidocaldarius* have not yet been analyzed on the genetic level, and IS elements have not been described for this organism. The apparent mutational frequency of *pyrEF* in that organism was  $5 \times 10^{-6}$  (10; this study). In contrast, spontaneous mutations in *S. solfataricus* that were created by IS elements arose with variable frequencies of between  $10^{-4}$  and  $10^{-5}$  per plated cell, i.e., they were at least 50 to 60 times higher than those in *S. acidocaldarius*. It is not clear yet which factors have influenced the rates of transposition

events in our experiments. Potentially, small differences in growth conditions or conditions that cause stress reactions might induce transpositions. Such observations have been made earlier in the study of  $\beta$ -galactosidase mutants, where spontaneous mutants arose with frequencies of around  $10^{-4}$  (26).

The hybridization patterns of the uracil-auxotrophic mutants revealed that insertions in *pyrEF* were not the only recent transposition events. In five out of seven *pyrEF* mutants analyzed, we detected between one and five additional or different fragments hybridizing to IS-specific probes besides the additional IS element copy in *pyrEF* (Fig. 6). Similarly, an additional fragment hybridized to *ISC1058* or *ISC1359* in the genome of all PH1 mutants, indicating earlier transposition events during the selection of the *lacS* mutant, which was created by insertion of *ISC1217* into the *lacS* gene (Fig. 7b). This represents a minimal number of transposition events, since multiple insertions into the same large DNA fragment or into large DNA fragments of similar size would not have been detectable in our study. Our findings suggest that the activity of each of these IS elements may be regulated by similar factors, making simultaneous transposition events more probable.

All four IS elements were found in high copy numbers not only in the genomes of *S. solfataricus* P1 and P2 but also in those of related strains that have only recently been isolated from the same solfataric field (Fig. 7 for strain PIT3; others are not shown), suggesting that the IS elements occur in high abundance also in natural strains and not only in isolates that have been propagated over several years under artificial laboratory conditions. The partial genome sequence of *S. solfataricus* P2 (29) so far has revealed at least three more IS elements that do not show any homology to each other and to the four elements described here. Within about 2.7 Mbp of the genome sequence, there were 12 copies of *ISC1058*, 5 copies of *ISC1359*, 13 copies of *ISC1439*, and 8 copies of *ISC1217*. Except for one region, we did not see any areas in the genome that contained a significant accumulation of IS elements. It seemed rather that transposition targets were randomly distributed (see also the website <http://niji.imb.nrc.ca/sulfolobus>). As reported earlier for other elements (29), individual copies of the different IS elements or IS families vary in their sequences either by carrying a few point mutations or by showing larger deletions. This suggests that the elements decay over long periods in the genome. It may explain how the organism copes with the large number of IS elements in the genome and is reminiscent of the occurrence of the IS element families in halobacteria (6, 21).

The IS elements seem to be specific for *S. solfataricus* or closely related strains and could be useful as markers in restriction fragment length polymorphism studies for strain typing and for the study of geographical distributions, because they detect relatively recent evolutionary divergence. Interestingly, one element, *ISC1058* or a close relative thereof, was also detected in isolates from Japanese hot springs (Fig. 7b). Horizontal spread of IS elements among different strains is quite possible, because a number of viruses and plasmids have been described for *Sulfolobus* (34) and conjugation has been demonstrated for *S. acidocaldarius* and *S. solfataricus* (12, 27). Furthermore, IS elements and transposases have been identified on several large plasmids that were shown to propagate by conjugation (30; W. Zillig, personal communication). Also, the relatively high G+C content of the IS elements (up to 15% higher than the average G+C content of the genome) suggests that IS elements in *S. solfataricus* may have been acquired by horizontal gene transfer from another organism.

The isolation of auxotrophic mutants from *S. solfataricus*



opens new possibilities for the development of genetic tools. So far, two selectable markers, an alcohol dehydrogenase (3) and a hygromycin pyrophosphatase (5), that confer resistance to specific inhibitors have been used in *Sulfolobus*. Similarly, the wt *pyrEF* genes could now be used as a selectable marker together with uracil-auxotrophic mutants. This marker should function in low-copy-number vectors as well as in single copy upon integration into the chromosome. Although insertional transposition was the cause of the mutant phenotypes, all auxotrophs seemed to be quite stable and should therefore be suitable recipients. Initial transformation experiments based on a recombinant plasmid derived from the *S. shibatae* virus SSV1 (32) allowed complementation to prototrophy with this self-spreading vector (unpublished data). With this system, it should be possible to demonstrate and optimize conditions for transformation by homologous recombination in *Sulfolobus* and to use the *pyrEF* marker in low-copy-number vectors. In addition, conjugation mechanisms and transfer of chromosomal markers can now be studied in *S. solfataricus* by mating different uracil auxotrophs with strains containing conjugative plasmids. It might also be possible to develop a transposon-based mutagenesis procedure by using transformation vectors that contain IS elements in combination with a selectable marker.

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