Extremophiles (2008) 12:263–270
DOI 10.1007/s00792-007-0125-7

ORIGINAL PAPER

Mutation and reversion frequencies of different *Sulfolobus* species and strains

Silvia Berkner · Georg Lipps

Received: 26 September 2007 / Accepted: 2 November 2007 / Published online: 5 January 2008

Abstract We have determined the apparent and actual spontaneous mutation frequencies and rates for different species and strains of the thermoacidophilic crenarchaeote *Sulfolobus*. The proportion of mutations caused by insertion sequences has also been analyzed. Mutation frequencies for *S. islandicus* (0.08–0.6 mutations per cell division and 10^7 cells) were below those determined for *S. solfataricus* and comparable to or lower than those for *S. acidocaldarius*. The proportion of insertion sequence mutations for the *S. islandicus* strains REN1H1 (9 out of 230) and HVE10/4 (0 out of 24) was found to be considerably lower than in *S. solfataricus* P1 and P2 and also low in comparison to other *S. islandicus* strains. Mutants defective in either the *pyrEF* genes or the *lacS* gene have been isolated. Their growth phenotype on selective and non-selective medium was examined and the inactivating mutations in either of the genes were determined. In addition the reversion frequencies for these mutants were measured and found to be in the range of <0.6–1.5 mutations per cell division and 10^8 cells. However, when being subjected to electroporation as a transformation procedure, increased reversion was observed.

Keywords Mutation frequencies · Reversion frequencies · Uracil selection · Lactose selection · Small-scale fluctuation tests

Communicated by L. Huang.

S. Berkner · G. Lipps (✉)
Department of Biochemistry, University of Bayreuth, Universitätsstr. 30, 95447 Bayreuth, Germany
e-mail: georg.lipps@uni-bayreuth.de

Introduction

Recently it has become obvious that some widely used *Sulfolobus* strains show high genomic plasticity. Especially *S. solfataricus* P2 that is used throughout many biochemical and molecular biology studies, has been shown to be prone to genomic rearrangements (Redder and Garrett 2006). Furthermore, also the strain *S. solfataricus* P1, the parent strain of a recipient mutant for a genetic system for *Sulfolobus* (Jonuscheit et al. 2003) is known to show high spontaneous mutation rates mediated by insertion sequences (Schleper et al. 1994; Martusewitsch et al. 2000). Caution is required when using these *Sulfolobus* strains in molecular biology experiments (Redder and Garret 2006). These strains were mainly used because, *S. solfataricus* P2 was the first *Sulfolobus* strain for which genome sequence information was available (Sensen et al. 1998; Charlebois et al. 2000; She et al. 2001) and *S. solfataricus* P1 was used in pioneering molecular biology studies, e.g. in the demonstration that *Sulfolobus* can be transformed by electroporation (Schleper et al. 1992). With evidence for genomic instabilities in these strains accumulating, it is crucial to identify more genetically stable *Sulfolobus* strains as recipients for genetic systems that are being developed at an increasing pace (Stedman et al. 1999; Jonuscheit et al. 2003; Worthington et al. 2003; Schelert et al. 2004; Kurosawa and Grogan 2005; Aucelli et al. 2006; Berkner et al. 2007a).

The determination of the mutation frequency of the *pyrEF* locus allows us to draw conclusions about the overall genomic stability of a *Sulfolobus* species or strain. For *S. solfataricus* P2, it has been shown that a large number of insertion sequences are present in its genome (She et al. 2001; Brügger et al. 2002; Brügger et al. 2003).

In fact it is the sequenced prokaryotic genome that contains the largest number of insertion sequence elements (Redder and Garrett 2006). The observed genomic instability in *S. solfataricus* P2 has been attributed to the high number of insertion sequences present (Redder and Garrett 2006). On the other hand, *S. acidocaldarius* contains no active insertion sequences (Grogan et al. 2001; Chen et al. 2005) and mutation frequencies determined for the *pyrE* and *pyrF* genes show one to two orders of magnitude lower values than for *S. solfataricus* (Jacobs and Grogan 1997; Martusewitsch et al. 2000). For different *S. islandicus* strains isolated from Iceland (Zillig et al. 1994) that are increasingly being used in biochemical and genetic studies besides *S. solfataricus* and *S. acidocaldarius*, almost no information is available.

The *pyrEF* genes are suitable as model genes for the determination of spontaneous mutation frequencies and examination of the activity of insertion sequences, because their mutation frequency can reliably and sensitively be determined (Jacobs and Grogan 1997; Grogan et al. 2001) and insertion sequences can be trapped (Blount and Grogan 2005; Redder and Garrett 2006). The cotranscribed *pyrEF* genes (Thia-Thoong et al. 2002) code for orotate phosphoribosyltransferase (OPRT) and orotidin-5′-monophosphatedecarboxylase (ODC), respectively. These enzymes form part of the de novo uridine-5′-monophosphate (UMP) synthesis pathway (Grogan and Gunsalus 1993). OPRT catalyzes the addition of phosphoribosyl pyrophosphate to orotate, ODC catalyzes the decarboxylation of orotidin-5′-monophosphate to UMP. Loss of function mutations in the *pyrE* or *pyrF* gene leads to uracil auxotrophic mutants. *pyrEF* deficient mutants can be selected for by the use of the fluorinated substrate analogue 5′-fluoro-orotic acid (FOA) (Kondo et al. 1991; Grogan 1991).

We were interested in the mutation frequencies of the *pyrEF* genes of different *Sulfolobus* species for two reasons, namely the assessment of the genetic stability and the isolation and characterization of *pyrEF* and *lacS*-deficient mutant strains with regard to the development of genetic systems for *Sulfolobus spp.* The emphasis was placed on these *Sulfolobus* strains, for which information was lacking, to gain a coherent view for the most important laboratory strains of *S. solfataricus*, *S. acidocaldaricus* and *S. islandicus*.

Experimental

Strains and growth conditions

S. solfataricus strain P1 (Zillig et al. 1980) and PBL2025 (Schelert et al. 2004), *S. islandicus* strains REN1H1, REN2H1 and HVE10/4 (Zillig et al. 1994) and mutant strains REN1H1 R1, R20, R21, R22 (Berkner and Lipps, 2007b), S1, R1S1, REN2H1 DM, and HVE10/4 H1 (see also table 3), and *Sulfolobus acidocaldarius* (Brock et al. 1972) were grown in Brock's basal salts medium at pH 3.5 (Brock et al. 1972) supplemented with 0.1% tryptone and 0.2% arabinose for liquid culture and 0.2% dextrin for plates. For the growth of *pyrEF* mutant strains 20 μg/ml of uracil was added to the medium. The completely uracil-free "20AS" medium is composed of the 20 amino acids (0.5 mM each except for tryptophan (0.25 mM) and tyrosine (0.1 mM)). The lactose medium contained 0.2% lactose as sole carbon and energy source. Plates were solidified by the addition of 0.6% Gelrite (Sigma, USA) and 10 mM $CaCl_2$. Plates and shake flask cultures were incubated at 75°C.

Determination of apparent mutation frequencies

An aliquot of 100 μl of an exponentially growing culture (supplemented with uracil) was plated undiluted on plates containing FOA at concentrations indicated for each experiment. The number of viable cells was determined by plating appropriate dilutions on plates without FOA. The apparent mutation frequency is the number of FOA-resistant cells divided by the number of viable cells. Experiments were conducted at least in triplicate.

Small-scale fluctuation tests

A culture was grown without addition of uracil to prevent mutations in the *pyrEF* genes during this culture period. This culture was diluted to contain only 5,000 cells per ml and was divided into ten parallel cultures and cultivated to mid-exponential phase with the addition of uracil to the medium. Then an aliquot of each culture was spread on plates containing 250 μg/ml FOA. Mutation events were determined from these small-scale fluctuation tests (Luria and Delbrück 1943) by the methods described by Lea and Coulson (1949) with the aid of graphs and formulas given in Koch (1982). From the numbers of colonies obtained in the ten parallel cultures, the first, second and third quartiles of the frequency distribution were calculated using spreadsheet software. For each quartile the corresponding number of mutations was deduced from the graphs given in Koch (1982). In case no colonies were observed, the upper limit of the number of mutations was estimated by the p0 method (Lea and Coulson 1949) as described in Koch (1982). Mutational events are reported normalized to the cell number and the number of cell divisions. Cell numbers were determined by plating appropriate dilutions on non-

selective plates. A prerequisite for the applicability of these methods is the equal growth rate of the mutants and wildtype cells. For this reason the medium was supplemented with uracil during growth for the small-scale fluctuation experiments.

Screening and sequencing of *pyrEF* mutants

The *pyrEF* genes from *S. islandicus* REN1H1, REN2H1 and HVE10/4 were amplified by PCR using primers 5′-TTCACCTTTTGCTATCGAAG and 5′-GTTTATAA AGACCGGCTATT yielding a fragment of 1,584 bp containing the promotor region of *pyrB* and the *pyrE* and *pyrF* genes. The different *pyrEF* PCR products were examined for the presence of insertions or deletions by screening for changes in the length of the PCR products on a 1% agarose gel. A total of 230 mutants were examined for *S. islandicus* REN1H1 and 24 mutants for *S. islandicus* HVE10/4. PCR products differing in length from the wild-type PCR fragment were cloned and sequenced.

UV-mutagenesis and Xgal staining

S. islandicus REN1H1 cells were grown to an OD of 0.8, cooled on ice and 1 ml of cells was transferred to different wells of a 24-well plate. The plate was irradiated with UV light (55 W, 10 cm distance to samples, 254 nm) for the indicated periods. Then appropriate dilutions of the cells were plated ($\sim 10^4$ colonies per plate, 120 plates) and screened for white colonies after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal). Plates were sprayed with Xgal solution (20 mg/ml in dimethylformamide) and incubated for up to 30 min at 75°C.

Sequencing of the *lacS* gene

The *lacS* gene from *S. islandicus* REN1H1 and REN2H1 was amplified using primers 5′-AGATACTCGCTCAAA GCTTN and 5′-GAAGGTATGAAAGTATTGCN yielding a 1,602-bp fragment containing 43 bp of upstream sequence. PCR fragments were cloned and sequenced.

Results and discussion

Mutation frequencies of the *pyrEF* genes

The spontaneous mutation rates of the *pyrEF* genes were determined for different *Sulfolobus* strains as apparent mutation rates and as actual mutational events per cell and cell cycle from small-scale fluctuation tests. The apparent mutation rate counts all FOA selectable *pyrEF*-deficient mutants, whereas by small-scale fluctuation tests the overall number of mutants obtained is corrected for the number of mutants that are the descendants of already mutated cells. Small-scale fluctuation tests therefore allow determining the actual number of mutations more precisely. The apparent mutation rate was nevertheless determined to be able to compare the obtained values to published results obtained with either one of the above-mentioned methods.

The apparent mutation frequencies (Table 1) determined for *S. islandicus* species show comparable values for *S. islandicus* REN1H1 and REN2H1. The value for REN1H1 approximately agrees with previous determinations (Martusewitsch et al. 2000). For *S. islandicus* HVE10/4 lower apparent mutation frequencies were determined. The values for *S. islandicus* were one to two orders of magnitude below the values determined for *S. solfataricus* P1 and P2 (Martusewitsch et al. 2006; Redder et al. 2006). However, the apparent mutation frequency for *S. solfataricus* PBL2025 was found to be in the same range as for most *S. islandicus* strains. *S. acidocaldarius* showed comparable apparent mutation frequencies to the *S. islandicus* strains.

Small-scale fluctuation tests were performed to be able to measure more accurately the number of mutations that is occurring in the different strains (Table 2). The highest mutation rate was again determined for *S. solfataricus*. It becomes obvious that the difference between *S. solfataricus* and *S. islandicus* strains is overestimated by determining only the apparent mutation frequency. However with both methods the same ranking order of the mutation frequencies was obtained. *S. islandicus* REN2H1 shows a slightly lower frequency than REN1H1 followed by HVE10/4, which again showed the lowest frequency observed. In comparison to previously conducted small-scale fluctuation for isolates typed as conspecific to *S. islandicus* from Yellowstone, Lassen and Kamtchatka (Blount and Grogan 2005) the icelandic *S. islandicus* strains showed lower mutation frequencies and also lower fractions of insertion sequence mutations.

We cannot exclude that different FOA concentrations may have an effect on the results of the different small-scale fluctuation studies. For that reason only studies are compared that used approximately the same FOA concentrations (Table 2). However, from the determinations of apparent and actual mutation frequencies it can be deduced that *S. solfataricus* shows the highest mutation rates equaled by some of the *S. islandicus* isolates from Blount and Grogan (2005).

Table 1 Apparent mutation frequencies of the *pyrEF* genes in different *Sulfolobus* species and strains

Sulfolobus species and strain	Apparent mutation frequency of *pyrEF* genes	FOA/uracil concentration (μg/ml)	Fraction of insertion elements (%)	Reference
S. solfataricus P2	1.3×10^{-4}	50/20	~67	Redder and Garrett (2006)
S. solfataricus P1	$2.4–31 \times 10^{-5}$	50/10	100	Martusewitsch et al. (2000)
S. solfataricus PH1	$1.9–34 \times 10^{-5}$	50/10	100	Martusewitsch et al. (2000)
S. solfataricus PBL2025	3×10^{-6}	50/20	nd	This study
S. acidocaldarius	$1.8–4.4 \times 10^{-6}$	50/10	nd	Martusewitsch et al. (2000)
	8×10^{-7}	50/20	nd	This study
S. islandicus REN1H1	8.7×10^{-6}	50/10	nd	Martusewitsch et al. (2000)
	2×10^{-6}	50/20	4	This study
S. islandicus REN2H1	1×10^{-6}	50/20	nd	This study
S. islandicus HVE10/4	5×10^{-8}	50/20	<4	This study

nd not determined

Table 2 Actual mutation frequencies determined by small-scale fluctuation tests for different *Sulfolobus* species and strains

Sulfolobus species and strain	Mutation frequency of *pyrEF* genes (mutations per cell division)	FOA/uracil concentration (μg/ml)	Fraction of insertion elements (%)	Reference
S. solfataricus P1	$1.4–1.7 \times 10^{-7}$	250/20	nd	This study
S. islandicus REN1H1	$0.5–0.6 \times 10^{-7}$		4	
S. islandicus REN2H1	0.2×10^{-7}		nd	
S. islandicus HVE10/4	0.08×10^{-7}		< 4	
S. islandicus isolates	$1.3–37 \times 10^{-7}$	150/20	5.6-88.9	Blount and Grogan (2005)
S. acidocaldarius	3.37×10^{-7}	50/20	0	Grogan et al. (2001)

nd not determined

Isolation and characterization of *pyrEF* and *lacS* mutants

To obtain suitable recipient strains for uracil selection or lactose selection or for the use in reporter gene experiments, mutants defective in the *pyrEF* genes and/or defective in the *lacS* gene were isolated.

Isolation of *pyrEF* mutants was done by picking single colonies from FOA-containing plates (250 μg/ml) and subsequently characterizing the mutants phenotypically and genotypically to determine their suitability to serve as potential recipient strains in the establishment of genetic systems. Two criteria that suitable mutants should meet were, (1) comparable growth to wild type strains under non-selective conditions and (2) most preferably negligible growth under selective conditions. Strains that showed the best behavior in growth tests (Fig. 1) were then chosen and the *pyrEF* genes were sequenced to determine the mutations responsible for the inactivation of the *pyrEF* genes. We noticed that the tested mutants, as well as other *pyrEF* mutants (e.g. pH 1–16, Martusewitsch et al. 2000) showed residual growth in tryptone medium, probably because traces of uracil are present in tryptone (Fig. 1). For that reason a medium composed of 20 amino acids (20AS, see Methods) was used. Cells grew slower in this medium and only when a vitamin solution (Wolin et al. 1963) was added. However, this completely uracil-free medium considerably reduced the background growth observed with tryptone containing medium (Fig. 1). As reliable uracil selection was a prerequisite for determining the reversion frequencies of the *pyrEF* deficient mutants, we examined the efficiency of this selection method with different media. A low number of uracil prototrophic cells was plated mixed with an approximately six orders of magnitude higher number of uracil auxotrophic cells on tryptone/dextrin plates and on 20AS/dextrine plates (Fig. 2). If the selection was reliable and efficient the number of recovered colonies should correspond to the number of prototrophic cells plated. This was observed for all tested *Sulfolobus* species for the dextrin/20AS plates (recovery >90%), but not for the tryptone/dextrin plates where almost only background growth was observed and only very few, very small colonies.

As no selection procedure exists to isolate *lacS*-deficient mutants comparable to the FOA selection for *pyrEF* mutants, cells were mutagenized to increase the probability of obtaining a *lacS*-deficient mutant. Mutagenesis was done by irradiation with UV-light. First, the survival rate of cells was determined for different periods of irradiation (Fig. 3 a). The final irradiation time was chosen to yield a fraction

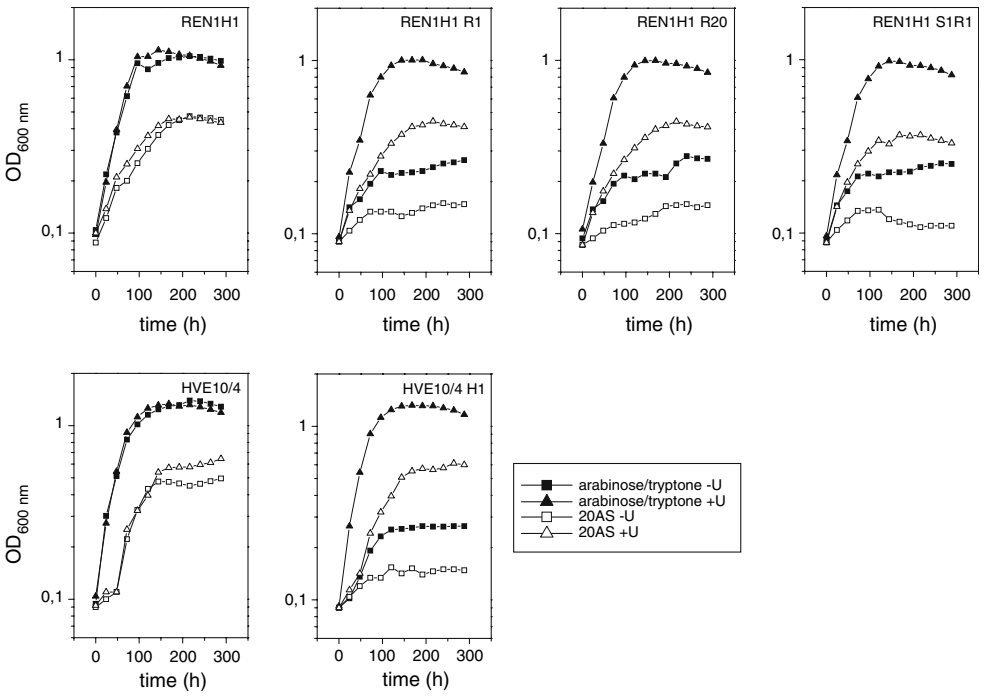

Fig. 1 Growth curves for *S. islandicus* wild type and *pyrEF* deficient mutant strains in tryptone containing medium +/- uracil (U) and in more selective amino acid medium (20AS) +/- uracil

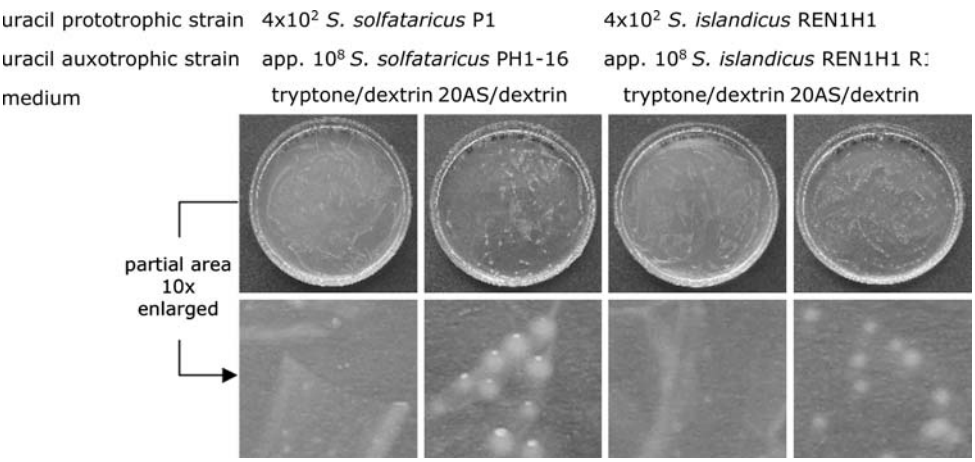

Fig. 2 Competition experiments to determine the efficiency of uracil selection on different media. The indicated numbers of uracil prototrophic and auxotrophic cells were mixed and plated on tryptone/dextrin plates and 20AS/dextrin plates

of 10^{-4} surviving cells. 1.2×10^6 cells (OD 0.8) were plated and plates were sprayed with Xgal solution and screened for white colonies. Only one white colony could be isolated. For this colony (*S. islandicus* REN1H1 S1) the growth on selective lactose and non-selective tryptone/arabinose medium was examined. Only very faint growth was observed in lactose medium (Fig. 3b) and no color developed when tested for prolonged incubation times by Xgal staining.

So far no genome sequence for *S. islandicus* is available. However it is known, that its sequence is close to that of *S. solfataricus* (Zillig et al. 1994). We determined the sequence of the *pyrEF* genes for *S. islandicus* REN1H1, REN2H1 and HVE10/4. For *S. islandicus* REN2H1 seven exchanged nucleotides were detected, the sequence from HVE10/4 differs only by four nucleotides from the REN1H1 sequence. We also determined the *lacS* sequence for *S. islandicus* REN1H1 and found it to correspond to that of REN2H1. The *lac*S gene comprises 1,467 nucleotides. The sequence similarity to the *S. solfataricus* P2 *lacS* sequence is only 85%. The *pyrEF* and *lacS* sequences have been deposited in Gene Bank (accession numbers EU252578-EU252581).

Mutational spectrum

The mutations found in the *pyrEF* genes or the *lacS* gene are summarized in Table 3. Point mutations occurred frequently. Insertion sequence mutants were also detected.

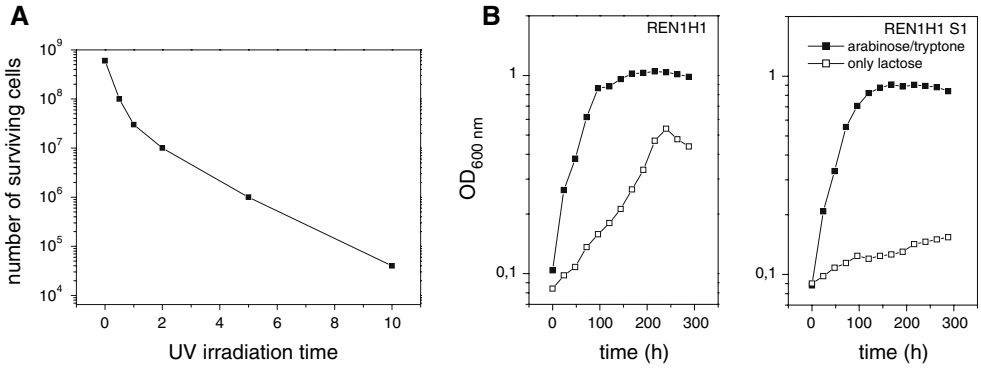

Fig. 3 A: Number of surviving cells after exposure to UV irradiation for *S. islandicus* REN1H1 at OD 0.8. B: Growth curves for the *lacS* deficient mutant strain REN1H1 S1 in tryptone/arabinose containing medium and in lactose only containing medium

Table 3 Characterization of the genotype of *pyrEF* and *lacS*-deficient mutants

Sulfolobus islandicus strain	Gene	Type of mutation	Nucleotide change	Amino acid change	Reason for inactivation
REN1H1R1	*pyr E*	Point mutation	223 G→A	A75→T	A75 is conserved
		Silent mutation	336 T→C	–	
REN1H1 R20, R21, R22	promoter *pyrEF*	Insertion sequence	SMN1 in TATA-Box	–	negative influence on transcription
REN1H1 S1	*lacS*	Frameshift mutation	648 T is lacking	From V216 10 changed aa, then stop codon	lacking C-terminal 274 aa
REN1H1 S1R1	*lacS*	Same as mutant S1			
	pyrE	Point mutation	350 G→A	G117→E	G117 is situated in the vicinity of the active site
	pyrF	Point mutation	245 T→C	F82→S	
REN2H1 DM	*lacS*	Point mutation	1280 T→A	L427→stop	Lacking C-terminal 63 aa
	pyrF	Frameshift mutation	A inserted behind nt 346	Stop codon 1 AS later	Lacking C-terminal 109 aa
HVE10/4 H1	*pyrE*	Point mutation	223 G→T	A75→S	A75 is conserved
		Point mutation	586 C→T	Q196→stop	Lacking C-terminal 7 aa
	pyrF	Silent mutation	12 C→T		
		Point mutation	26 C→T	A9→V	
		Silent mutation	339 G→A		
		Point mutation	430 A→G	T144→A	
		Point mutation	549 T→A	Y183→stop	Lacking C-terminal 40 aa

Nucleotide positions for *pyrE*, *pyrF*, and *lacS* refer to the A of the ATG start codon of the respective gene as position 1; *aa* amino acids

S. islandicus REN1H1 R20–R22 contain the element SMN1 that inserted into the promoter region of the *pyrEF* operon. A total of nine mutants contained insertion sequences. Apart from SMN1, elements related to ISC796 and ISC735 were detected for *S. islandicus* REN1H1 (Berkner and Lipps 2007b). A one bp deletion and a 1-bp insertion were found in the *lacS* gene of *S. islandicus* REN1H1 S1 and the *pyrF* gene of *S. islandicus* REN2H1 DM. Two of the point mutation mutants showed more than one mutation per gene, and the same two mutants also exhibited silent mutations. Mutations with high probability to cause a loss of function are specified in Table 3.

With the additional information gained on *S. islandicus* strains, it becomes obvious that there are three types of different mutational spectra. *S. solfataricus* P1 and P2 show high spontaneous mutation frequencies associated with a high to very high fraction of insertion sequence mutants and a lower fraction of point mutants (Martusewitsch et al. 2000; Redder and Garrett 2006). In contrast, *S. acidocaldarius* shows lower spontaneous mutation frequencies and no active insertion elements (Jacobs and Grogan 1997; Grogan et al. 2001). The icelandic *S. islandicus* isolates show mutation frequencies in the same range or below the values for *S. acidocaldarius* but also contain active insertion sequences. Therefore, the presence of active insertion sequences does not necessarily lead to high spontaneous mutation frequencies. However in *S. islandicus* no mutants with larger partial (>50 bp) or complete deletions of the

Table 4 Reversion frequencies for *pyrEF* and *lacS*-deficient mutants determined by small-scale fluctuation tests

Sulfolobus islandicus strain	Selection/detection used	Reversions per generation and 10^8 cells
REN1H1 R1	Growth on uracil free medium	<0.6
REN1H1 R20	Growth on uracil free medium	<0.6
REN1H1 R21	Growth on uracil free medium	<0.6
REN1H1 R22	Growth on uracil free medium	<0.6
REN1H1 S1	Screening with Xgal	$<6 \times 10^2$
REN1H1 S1R1	Growth on uracil free medium	<0.6
HVE10/4 H1	Growth on uracil free medium	1.0–1.5

The range given comprises the lowest and highest value obtained by using the first, second or third quartile of the revertant frequency distributions, when no revertants were observed the upper limit was estimated using the p0 method (see experimental)

genes of interest were detected, despite screening a high number of mutants. Considering the apparent and actual mutation frequencies for *S. islandicus* REN1H1, REN2H1 and HVE10/4, these strains are closer to *S. acidocaldarius*, but they show also similarities to *S. solfataricus* P1 and P2 because insertion sequence mutants are observed, although at much lower frequencies (Table 1). The *S. islandicus* strains seem to be genetically more stable than *S. solfataricus* P1 and P2 but less stable than *S. acidocaldarius*.

Reversion frequencies

Next the reversion frequencies for the mutants were determined, to be able to judge their suitability as stable recipient mutants for the development of genetic systems. *S. islandicus* REN2H1 mutants were not further characterized in detail because we found that this strain contains a restriction/modification system (Söllner et al. 2006). For the mutants of *S. islandicus* REN1H1 and HVE10/4 the reversion frequencies were determined by small-scale fluctuation tests (Table 4).

The reversion frequencies were very low. With the exception of HVE10/4 H1 no revertants could be detected. The reversion frequencies are in the same range as the ones determined by Blount and Grogan (Blount and Grogan 2005) for *S. islandicus* insertion sequence mutants (0.19–<0.02 reversions per generation and 10^8 cells). The exception is the relatively high reversion frequency for HVE10/4 H1. HVE10/4 showed the lowest mutation frequency (Tables 1, 2) but the highest reversion frequency. The reason for this behavior is unknown. It is nevertheless possible that HVE10/4 H1 is a mutator mutant that would explain the multiple mutations found in this mutant and its high reversion frequency. Therefore these results should not be considered to be generally true for other HVE10/4 derived mutants.

Because of the low reversion frequencies all the *S. islandicus* REN1H1 mutants were considered to be suitable recipient strains, e.g. for transformation of shuttle vector constructs using the *pyrEF* genes as selectable marker.

Increased reversion frequencies after electroporation

When using the different *S. islandicus* mutants in electroporation experiments with constructs containing the intact *pyrEF* genes from *S. solfataricus* P2 as selectable marker, however, considerably higher reversion frequencies were detected. When plating a 10^{-4} dilution of cells directly after electroporation (3 h of regeneration in tryptone medium, shorter than the generation time of *S. islandicus* of ~6 h under these growth conditions, total duration of cultivation comparable to small-scale fluctuation experiments), tens to several hundreds of colonies were observed on selective plates without uracil either on tryptone or on 20AS medium. From the small-scale fluctuation experiments we would expect no colonies to arise at that dilution. We verified by PCR and sequencing that reversion was the cause for the restored ability to grow on medium without uracil. As this behavior was observed in transformation experiments with constructs containing the *pyrEF* genes as well as in control electroporations without the presence of a vector, the reversion seems to be caused by the stress and/or the stress response that electroporation imposes on the cells. This unexpected high background of revertants after electroporation precludes point mutants and insertion sequence mutants from being used as recipient strains for shuttle vector constructs that are not self-spreading. These findings indicate that only mutants with larger deletions are suitable for the use with genetic systems that require high selective pressure for stable maintenance in the recipient strain.

Acknowledgments We are grateful to Christa Schleper for providing us with the *pyrEF/lacS* mutant *S. islandicus* REN2H1 DM and to Paul Blum for the *lacS*-deletion mutant *S. solfataricus* PBL2025. This work was supported by the DFG (Grant Li 913/3 to G.L.).

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