Revealing the essentiality of multiple archaeal *pcna* genes using a mutant propagation assay based on an improved knockout method

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Organisms belonging to the Crenarchaeota lineage contain three proliferating cell nuclear antigen (PCNA) subunits, while those in the Euryarchaeota have only one, as for Eukarya. To study the mechanism of archaeal sliding clamps, we sought to generate knockouts for each *pcna* gene in *Sulfolobus islandicus*, a hyperthermophilic crenarchaeon, but failed with two conventional knockout methods. Then, a new knockout scheme, known as marker insertion and target gene deletion (MID), was developed, with which transformants were obtained for each pMID-pcna plasmid. We found that mutant cells persisted in transformant cultures during incubation of pMID-araS-pcna1 transformants under counter selection. Studying the propagation of mutant cells by semiquantitative PCR analysis of the deleted target gene allele (D<sub>pcna1</sub> or D<sub>pcna3</sub>) revealed that mutant cells could no longer be propagated, demonstrating that these *pcna* genes are absolutely required for host cell viability. Because the only prerequisite for this assay is the generation of a MID transformant, this approach can be applied generally to any micro-organisms proficient in homologous recombination.

INTRODUCTION

Maintaining genome integrity is crucial for any form of cellular life, and all living organisms devote considerable genetic resources to encoding the proteins that ensure accurate duplication and subsequent safeguarding of their genomes. While there is a considerable understanding of DNA replication and repair processes in Bacteria and Eukarya, relatively little is known about these mechanisms in Archaea, the third domain of life, except that all archaeal proteins known to be involved in these molecular processes exhibit more sequence resemblance to their eukaryal counterparts than to those in bacteria (Barry & Bell, 2006; Grabowski & Kelman, 2003). Among all known replication proteins, the sliding clamp, which functions as a docking station or scaffold that hosts diverse cellular activities, is highly conserved structurally in all three domains of life (Indiani & O’Donnell, 2006). In bacteria, the protein is β clamp, which forms a dimer and constitutes an integral part of the bacterial DNA polymerase III holoenzyme, while its archaeal and eukaryal counterparts are the trimeric proliferating cell nuclear antigens (PCNAs). To yield the same overall structure with six structural domains, each bacterial subunit contains three domains, while an archaeal/eukaryal PCNA subunit has two.

The eukaryal PCNA rings in particular have been studied extensively, and they interact with many partners that are involved in DNA transactions as well as other molecular processes (Moldovan et al., 2007; Naryzhny, 2008). All PCNA partners carry a short conserved peptide motif [PCNA-interacting protein (PIP) box] that mediates protein–protein contacts (Tsurimoto, 1999). A similar protein sequence motif is also present in diverse archaeal PCNA-interacting proteins from different archaeal species. The identified archaeal PCNA-interacting proteins include DNA replication and repair polymerases (Cann et al., 1999; Daimon et al., 2002; Dionne et al., 2003; Grüz et al., 2001), DNA ligases (Dionne et al., 2003; Kiyonari et al., 2006;
Mayanagi et al., 2009), nuclease, helicases and DNA glycosylases (Dionne & Bell, 2005; Kiyonari et al., 2008; Yang et al., 2002). Structural analyses of several PCNA– enzyme complexes have yielded considerable insights into the functions of PCNA as a sliding clamp or docking platform (Doré et al., 2006; Matsumiya et al., 2001; Miyata et al., 2005; Xing et al., 2009). Furthermore, archaeal PCNAs have been found to strongly facilitate the endonuclease activity of XPF, a putative nucleotide excision repair enzyme (Hutton et al., 2008), thereby playing a more direct role than forming a platform.

PCNAs from different lineages of life also exhibit interesting differences. While eukaryotes and euryarchaea employ a homotrimeric PCNA ring, crenarchaea encode three different PCNA subunits that may form trimeric rings of different compositions. It has been shown that the heterotrimeric form is the only form that supports DNA replication in vitro (Dionne et al., 2003), although other trimeric forms have also been observed in vitro for Aeropyrum pernix and Sulfolobus tokodaii (Imamura et al., 2007; Lu et al., 2008). This raises an important question as to whether there is functional redundancy or differentiation of crenarchaeal PCNAs.

Recently, versatile genetic tools have been established for the hyperthermophilic crenarchaeon Sulfolobus islandicus (She et al., 2009). In order to study the essentiality of archaeal DNA replication and repair genes systematically, we have designed an improved gene knockout strategy and have found that the novel knockout scheme enables a mutant propagation assay for the study of true gene function. In vitro replication of crenarchaeal PCNAs.

In order to study whether there is functional redundancy or differentiation of crenarchaeal PCNAs.

Recently, versatile genetic tools have been established for the hyperthermophilic crenarchaeon Sulfolobus islandicus (She et al., 2009). In order to study the essentiality of archaeal DNA replication and repair genes systematically, we have designed an improved gene knockout strategy and have found that the novel knockout scheme enables a mutant propagation assay for the study of true gene essentiality. By using these new approaches, we show that the PCNA genes are absolutely required for the viability of S. islandicus.

**METHODS**

**Archaeal and bacterial strains, media and growth conditions.** S. islandicus strains REY15A and E233S (ApyrEF ΔlacS) have been described previously (Deng et al., 2009) and were used as the genetic hosts. These strains, S. islandicus transformants and the Δxpf deletion mutant are listed in Table 1.

All Sulfolobus media contained the same composition of mineral salts as described previously (Deng et al., 2009). Additionally, the rich TYS medium contained 0.1 % tryptone, 0.05 % yeast extract and 0.2 % sucrose. The selective media ACVY and SCVY were prepared by mixing 0.2 % D-arabinose (ACVY) or sucrose (SCVY), 0.2 % (w/v) vitamin-free Casamino acids (Difco Vitamin Assay, BD), 0.005 % yeast extract and a mixed vitamin solution (Deng et al., 2009) with mineral salts. The final pH value of each medium was adjusted to about 3.5, using concentrated H2SO4.

Sulfolobus strains were grown in liquid or solid media, as described previously (Deng et al., 2009). Strains or transformants to be selected by uracil dropout selection were grown in SCVY, whereas pyrEF-deficient strains were cultured in either TYS or SCVY supplemented with uracil (20 μg ml−1). When specified, 5’-fluoroorotic acid (5-FOA) was added to a concentration of 50 μg ml−1 to yield counter selection.

**General DNA manipulations.** Restriction and modification enzymes were purchased from Fermentas, New England Biolabs or Takara Bio. Plasmid DNAs were isolated from Escherichia coli or Sulfolobus cells using the QIAprep Spin Miniprep kit. Total genomic DNA was prepared either with the Qiagen DNeasy kit or by extraction with phenol/chloroform after lysing Sulfolobus cells with SDS and protease K (Contursi et al., 2006). General DNA manipulation was carried out as described previously (Sambrook & Russell, 2001; She et al., 2001). DNA sequencing was performed using the dye-terminator chemistry and a MegaBACE 1000 sequencer. DNA oligonucleotides used for PCR were synthesized at TAG Copenhagen.

**Plasmid constructs**

The genome sequence of the genetic host S. islandicus REY15A has been determined (our unpublished data). Genes encoding each PCNA were identified from the genome sequence by BLAST searches against those encoded in Sulfolobus solfataricus (She et al., 2001). The genomic regions flanking each pcna gene (+ 2 kb) were retrieved and used to design homologous sequence arms for knockout plasmid construction. Three classes of knockout plasmids were constructed: (1) plasmids for allelic replacement (AR) manipulation, pMRL plasmids for marker replacement and looping out (MRL), and pMID plasmids (including pMID-araS) for marker insertion and target gene deletion. The recombination module for each knockout method is illustrated in Fig. 1.

**Table 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and features</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. islandicus</td>
<td>Wild-type</td>
<td>Deng et al. (2009)</td>
</tr>
<tr>
<td>S. islandicus</td>
<td>ApyrEF</td>
<td>Deng et al. (2009)</td>
</tr>
<tr>
<td>S. islandicus</td>
<td>ApyrEF ΔlacS</td>
<td>Deng et al. (2009)</td>
</tr>
<tr>
<td>S. islandicus</td>
<td>E233S double-crossover transformant generated with pMID-pcna1 via upstream insertion,</td>
<td>This work</td>
</tr>
<tr>
<td>pcna1-T</td>
<td>harbouring In-arm:: Out-arm pryEF lacS Tg-arm</td>
<td></td>
</tr>
<tr>
<td>S. islandicus</td>
<td>E233S double-crossover transformant generated with pMID-pcna2 as for the pcna1-T strain</td>
<td>This work</td>
</tr>
<tr>
<td>pcna2-T</td>
<td>E233S double-crossover transformant generated with pMID-pcna3 as for the pcna1-T strain</td>
<td>This work</td>
</tr>
<tr>
<td>S. islandicus</td>
<td>E233S double-crossover transformant generated with pMID-araS-pcna1 as for the pcna1-T strain</td>
<td>This work</td>
</tr>
<tr>
<td>pcna1-T</td>
<td>E233S double-crossover transformant generated with pMID-araS-pcna1 as for the pcna1-T strain</td>
<td>This work</td>
</tr>
<tr>
<td>S. islandicus</td>
<td>E233S double-crossover transformant generated with pMID-xpf via downstream insertion,</td>
<td>This work</td>
</tr>
<tr>
<td>pMID-xpf-T</td>
<td>harbouring Tg-arm pryEF lacS Out-arm:: In-arm</td>
<td></td>
</tr>
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in one reaction or stepwise by cloning of each PCR product as constructed either by ligation of multiple fragments with pUC19 facilitate the cloning process, and knockout plasmids were contains a unique restriction site (Supplementary Table S1) to

Fig. 1. Recombination modules of each knockout plasmid. Three series of knockout plasmids were constructed. The pAR and pMRL plasmids contained the recombination modules I and II, respectively. The pMID plasmids contained the recombination modules I and II, respec-

The last group of knockout plasmids, denoted the pMID-araS-pcna series, were constructed by replacing each Tg-arm in the pMID-pcna plasmids with the corresponding araS-pcna arm. A promoter fragment of about 400 bp derived from the araS gene, encoding an arabinose-binding protein as well as each target gene (pcna1, pcna2, or pcna3), was amplified by PCR with the primers listed in Supplementary Table S1, and the resulting PCR products were digested with SalI plus MluI for Tg-arms, and XhoI and MluI for Out-arms (Supplementary Table S1). Furthermore, the xpf gene, encoding an endonuclease implicated in nucleotide excision repair, was also identified, and a knockout plasmid, pMID-xpf, was constructed as described above.

pAR knockout plasmids. Upstream and downstream sequences (0.8–1 kb) flanking each pcna gene were amplified by PCR using pAR-pcna primers (Supplementary Table S1), which yielded the left and right homologous sequences (L-arm and R-arm). A restriction site was included in each primer to facilitate cloning. Sall and XhoI/MluI sites were used for each L-arm, whereas NcoI and SphI sites were employed

for each R-arm. The marker cassette contained pyrEF and lacS genes, encoding orotate phosphoribosyltransferase, orotidine-5’-monophosphate decarboxylase and β-glycosidase, respectively, and was amplified by PCR from pHZ2lacS (Deng et al., 2009) using pyrEF-lacS Fwd and pyrEF-lacS Rev primers (Supplementary Table S1). PCR was carried out as described below and the resulting products were purified with the Qiagen PCR purification kit, cleaved with MluI and SphI, and cloned into the E. coli vector pUC19 in two steps. First, each L-arm and the pyrEF-lacS marker were ligated with pUC19 at the same sites by a triple ligation. Then, each R-arm was inserted at the NcoI and SphI sites of the respective pUC19-L-arm-pyrEF-lacS plasmid, yielding three pAR-pcna knockout plasmids.

pMRL knockout plasmids. In order to construct knockout plasmids for MRL, a second R-arm containing XhoI and MluI sites at the 5’ and 3’ ends, respectively, was prepared by PCR using the corresponding R-arm primers (Supplementary Table S1). The resulting PCR products were inserted into each pAR-pcna plasmid at the same sites and this yielded three pMRL-pcna knockout plasmids.

pMID knockout plasmids. A prerequisite for the pMID recombination scheme is that an active target gene should be maintained in the transformants. Thus, each S. islandicus pcna gene was analysed for its putative promoter. Promoter-like sequences were identified for the pcna1 and -2 genes, and these sequences were included in each target gene arm (Tg-arm). As a result, a 50 and a 34 bp sequence repeat was produced for the Tg-arm and the In-arm in the pMID-pcna1 and pMID-pcna2 plasmids, respectively, which has an important implication for the mutant propagation assay (see Results). The pcna3 gene appears to lack its own promoter and forms an operon with the upstream and downstream adjoining genes, since the intergenic sequence is only 4 nt. A putative promoter sequence (39 bp) was identified for the operon and fused onto the pcna3 gene to yield the Tg-arm. To facilitate cloning, different restriction sites were included in the pMID primers. Sall and XhoI/MluI were used for In-arms, NcoI and SphI for Tg-arms, and XhoI and MluI for Out-arms (Supplementary Table S1). Furthermore, the xpf gene, encoding an endonuclease implicated in nucleotide excision repair, was also identified, and a knockout plasmid, pMID-xpf, was constructed as described above.

Transformation of Sulfolobus and isolation of unmarked mutants. S. islandicus E233S was transformed with linearized plasmid DNA (0.5–1 µg) by electroporation, as described previously (Deng et al., 2009). Cell suspension (0.1–1 ml) was then plated onto the SCVy medium solidified with 0.8 % Gelrite and incubated at 75 °C. Colonies appeared on plates after 7–10 days. They were then stained with X-Gal. Since all colonies appeared blue, caused by the indicator marker lacS, they were all true transformants. A few colonies were then picked up, transferred into SCVy liquid medium (6 ml) in a test tube, and incubated at 78 °C for 3-7 days. When they had grown, Sulfolobus cells were collected and genomic DNA was prepared.
Different target gene alleles present in transformant cultures were identified by PCR with flanking primers designed for each pcna gene (Supplementary Table S1). Since relatively large PCR products (>8 kb) were expected, the LA Taq DNA polymerase (Takara Bio) was employed and PCR was performed under the following conditions: 94 °C for 3 min; 30 cycles of 94 °C for 15 s, 53 °C for 30 s and 72 °C for 10 min (with a 5 s increase for each cycle for the last 20 cycles), with a final extension at 72 °C for 10 min. Transformants were considered as pure if no wild-type target gene allele was detected by PCR. Purified transformants were then grown in liquid medium. Growing the transformant cells with counter selection led to colony formation by mutants on the plates containing uracil and 5-FOA if the target gene was non-essential.

RESULTS

Crenarchaeal PCNAs and their knockout analyses in S. islandicus

In the recently completed genome sequence of the crenarchaeon S. islandicus REY15A (L. Guo and others, unpublished results), three pcna genes have been identified, as in other known crenarchaea. The encoded proteins (PCNA1, -2 and -3) are almost identical in amino acid sequence to those from other S. islandicus stains (Reno et al., 2009), highly similar to those from S. solfataricus (89–93 % identity for protein sequence) (She et al., 2001), and more distantly related to those from Sulfolobus acidocaldarius, Sulfolobus tokodaii and Metallophaga sedula (41–63 % sequence identity) (Auernik et al., 2008; Chen et al., 2005; Kawarabayasi et al., 2001), three other members of the order Sulfolobales. Moreover, these S. islandicus PCNAs show no significant sequence similarity to those of A. pernix (~21 %), a distantly related crenarchaeon (Daimon et al., 2002). Pairwise sequence comparisons between PCNA subunits from the same crenarchaeon yielded sequence identities of 20–21 %, indicating a rapid evolution and diversification of the crenarchaeal PCNA subunits. Intriguingly, while a single subunit forms exclusively a homotrimeric PCNA ring in eukaryotes and euryarchaea, three different PCNAs are potentially capable of generating different forms of sliding clamps in crenarchaea, thereby posing an important question as to whether these crenarchaeal pcna genes are essential.

To answer this question, the following two conventional gene knockout methods were employed for the analysis of each S. islandicus pcna gene: (i) AR, which replaces the target gene with a marker in the mutants, and (ii) MRL, which yields unmarked deletion mutants. Two classes of knockout plasmids, namely pAR-pcna1, -2 and -3, and pMRL-pcna1, -2 and -3, were constructed and used to transform S. islandicus E233S (see Methods). Transformation failed to yield any transformant colonies in several experiments for both types of knockout plasmids, although pMRL-pcna plasmids occasionally yielded a few colonies. However, colonies were never observed in transformation with a PAR-pcna plasmid.

Previously, we showed that transforming S. islandicus with an MRL knockout plasmid always yields two different types of transformant. One carries a partial diploid that contains the marker cassette lacking the target gene (MRL transformants), while the other is formed via marker circularization and integration (MCI) and harbours a marker cassette in either the free or the integrated form (Deng et al., 2009). The latter was characterized by maintaining an active target gene and was unable to form a deletion mutant. If the target gene is essential or imposes a slow-growth phenotype upon inactivation, only MCI transformants would be expected.

Analyses of each pMRL-pcna transformant revealed that they carried the respective pcna::pyrEF-lacS alleles and the circular marker (data not shown), which was indicative of MCI transformants. The Δpcna::pyrEF-lacS or Δpcna alleles which result from an MRL recombination were absent. Taken together, both AR and MRL knockout analyses indicated that each S. islandicus pcna gene is either essential to, or very important for, host viability. However, neither experiment excluded the possibility that the resultant deletion mutants were viable but incapable of colony formation on selective plates under the experimental conditions.

Development of an improved unmarked gene deletion method

Notably, both the MCI and MRL recombination schemes exhibit special features. Transformants are always produced from MCI because an active target gene is maintained after transformation and, further, the designed deletion mutants represent the only genotype to be specifically selected in the second step of MRL (Deng et al., 2009). Intriguingly, replacing one of the repeated arms in the MRL module with a target gene sequence was able to yield a new knockout plasmid (Fig. 1), which led, in turn, to a new recombination scheme. This new scheme, denoted ‘marker insertion and unmarked target gene deletion’ (MID), exhibits two important features. First, as for MCI, an active target gene is generated after transformation and, as for MRL, only the mutant cells are specifically selected for, and transformants are selectively killed by counter-selecting pyrEF with 5-FOA (Fig. 2).

MID knockout was performed for three pcna genes and xpf, the last of which encodes an endonuclease implicated in nucleotide excision repair in archaea (Roberts et al., 2003). Transforming S. islandicus E233S with pMID-pcna1, -2, -3 or pMID-xpf by electroporation yielded colonies of transformants on SCVγ plates, as would be expected, and the transformation efficiency was 14–27 colonies per microgram DNA. Two colonies were picked for each construct and purified by repeated streaking of single colonies. To isolate unmarked deletion mutants, each purified transformant was grown to early exponential phase in SCVγ and plated onto solid SCVγ medium containing 5-FOA and uracil. While the number of 5-FOA-resistant colonies formed up to 1 % of the viable counts in the pMID-xpf transformant culture, the number...
of 5-FOA-resistant colonies was >1000-fold lower for each pMID-pcna transformant culture (Table 2). When incubated with X-Gal, colonies with different phenotypes were observed: (i) all colonies formed by the pMID-pcna3 transformant were blue; (ii) both blue and white colonies appeared on the selective medium plated with either the pMID-pcna1 or the pcna2 transformant; and (iii) most colonies produced from the pMID-xpf transformant were white.

These colonies were studied further. White colonies resulting from the pMID-xpf transformant only contained the Δxpf allele (data not shown), as would be expected for the deletion of a non-essential gene. By contrast, the white colonies derived from pMID-pcna1 and -pcna2 transformants were not deletion mutants but rather revertants containing each wild-type target gene (see below). Blue colonies derived from the pMID-pcna3 transformant appeared to result from spontaneous mutation in the

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**Fig. 2.** Flow chart of unmarked mutant construction via MID. Locations of the three homologous sequences, In-, Out- and Tg-arm, are illustrated on the wild-type target gene locus in the host chromosome. After transformation, double-crossover recombination between the linearized pMID plasmid and the host chromosome at the Tg-arm and the In-arm leads to the marker cassette insertion at the target gene locus. Recombination between the two Out-arms loops out the target gene and marker cassette, yielding an unmarked knockout mutant that is counter-selectable on a plate containing 5-FOA.
**Table 2. 5-FOA-resistance frequency of S. islandicus strains determined by colony formation**

*S. islandicus* strains were grown in SCVy alone or supplemented with uracil (+ U) and then plated onto two sets of solid media, namely SCVy + uracil and SCVy + 5-FOA + uracil. The 5-FOA-resistance frequency for each strain was expressed as the ratio between the numbers of colonies formed on the two different types of plate. The different genotypes were revealed by incubation of colonies with X-Gal and by PCR analyses of the target gene alleles. NA, Not available.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spontaneous mutation</th>
<th>Reversion</th>
<th>Mutant formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>REY15A (wild-type)</td>
<td>3.86 × 10⁻⁶</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pMID-pcna1*</td>
<td>2.04 × 10⁻⁵ (+ U)</td>
<td>7.20 × 10⁻⁷</td>
<td>NA</td>
</tr>
<tr>
<td>pMID-pcna2*</td>
<td>1.33 × 10⁻⁶ (+ U)</td>
<td>1.45 × 10⁻⁶</td>
<td>(- U)</td>
</tr>
<tr>
<td>pMID-pcna3T</td>
<td>5.41 × 10⁻⁷ (- U)</td>
<td>8.36 × 10⁻⁸</td>
<td>(- U)</td>
</tr>
<tr>
<td>pMID-araS-pcna1-T</td>
<td>4.23 × 10⁻⁶ (+ U)</td>
<td>9.98 × 10⁻⁷</td>
<td>(- U)</td>
</tr>
<tr>
<td>pMID-araS-pcna2-T*</td>
<td>2.17 × 10⁻⁶ (- U)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pMID-araS-pcna3, respectively (see Methods)</td>
<td>4.75 × 10⁻⁶ ( + U)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pMID-araS-pcna3, respectively (see Methods)</td>
<td>15.6 × 10⁻⁶ ( + U)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pMID-xpf-T†</td>
<td>1.82 × 10⁻⁶ ( + U)</td>
<td>1.58 × 10⁻³</td>
<td>( + U)</td>
</tr>
<tr>
<td>pMID-xpf-T†</td>
<td>7.10 × 10⁻⁶ ( + U)</td>
<td>8.34 × 10⁻³</td>
<td>( + U)</td>
</tr>
</tbody>
</table>

*Generating cells with the wild-type target gene allele that form colonies.
†Generating target gene deletant mutant cells that form colonies.

**pyrEF marker**, as suggested by their efficiency of formation.

We then looked for the cause of the revertant formation from the pMID-pcna1 and -pcna2 transformants. Since a pMID transformant was produced by double crossover at a Tg-arm and an In-arm between a pMID plasmid and the host chromosome, any recombination between the Tg-arm and the In-arm in the transformant would revert the transformation recombination, yielding the wild-type revertant (Fig. 2). As indicated in Methods, short sequence repeats were introduced into these arms on pMID-pcna1 (50 bp) and pMID-pcna2 (34 bp). The revertant should appear as white colonies in the presence of X-Gal because lacS would be removed together with the *pyrEF* marker. Thus, blue colonies carried a spontaneous mutation in the *pyrEF* marker, while white colonies were the wild-type revertant.

Colonies were counted on these plates individually and the results were used to estimate the relative recombination efficiency at the short sequence repeats. The recombination efficiency at 50 nt (pMID-pcna1) was at the level of the spontaneous mutation rate, which was eightfold more active than that for a 34 nt repeat (pMID-pcna2) (compare reversion in the absence of uracil in Table 2). Thus, homologous recombination occurred efficiently for short DNA sequence repeats in *S. islandicus*. The frequencies of occurrence of different genotypes for the studied pMID transformants are summarized in Table 2.

To resolve the problem of homologous recombination at short repeats (<50 bp) in the MID procedure, and to permit the study of *pcna* expression under the control of an inducible promoter, we replaced the original promoters of the *pcna1*, -2 and -3 genes with the promoter sequence of *S. solfataricus araS*, which encodes an arabinose-binding protein, and this yielded pMID-araS-pcna1, -pcna2 and -pcna3, respectively (see Methods). Following electroporation, the only viable transformant was that transformed with pMID-araS-pcna1. When the transformant was grown under the conditions for counter selection with 5-FOA, only spontaneous *pyrEF* mutants were enriched, as observed for the pMID-pcna3 transformant. The absence of the wild-type revertant in the pMID-araS-pcna1 transformant culture reinforces the conclusion that the short repeat sequence shared between the Tg-arm and the In-arm is responsible for the generation of the wild-type revertants in the pMID-pcna1 transformants.

**Demonstrating *pcna* gene essentiality using a mutant propagation assay**

Taking advantage of the fact that 5-FOA selectively kills *pMID* transformant cells and allows deletion mutant cells to grow in a *pMID* transformant culture, we studied whether the mutant cells generated from each *pMID*-pcna transformant could propagate. All *pMID* transformants were used in this experiment with the wild-type strain *S. islandicus* REY15A as a control. Purified transformants were inoculated into a uracil-containing medium and grown to OD₆₀₀ 0.35, and enrichment cultivation was started by adding 50 μg 5-FOA ml⁻¹ to each culture. These cultures were incubated to allow the optical density to peak. At this point, the growth of any cell carrying *pyrEF*
genes was inhibited completely, resulting in enrichment culture 1 or En1. En1 was diluted with fresh medium of the same composition to OD600 0.08. Incubation was for 7 days to give En2, during which period only cells that either were devoid of pyrEF genes or carried a pyrEF mutant gene could propagate. Then, the entire En2 was collected for each culture by centrifugation, and transferred into fresh medium of the same composition. In the subsequent enrichment, the pMID-xpf transformant grew first, followed 2 days later by the pMID-pcna1 and pMID-pcna2 transformants as well as REY15A, and 4 days later by the pMID-pcna3 and pMID-araS-pcna1 transformants (Fig. 3). All of the En3 cultures were then diluted 10-fold with fresh medium, and growth of these diluted cultures yielded En4. Samples were taken from each En1, En3 and En4 culture, and genomic DNA was prepared and used as the template for subsequent PCR analyses to study the composition of each enrichment culture and to assess mutant propagation.

The increase of the OD600 value of the REY15A culture in the presence of 5-FOA marked the point at which spontaneous pyrEF mutants outgrew the wild-type strain under 5-FOA selection (Fig. 3). Thus, it was interesting to note that pMID-pcna transformants grew differently: pMID-pcna1 and pMID-pcna2 transformants grew just before REY15A, while the growth of pMID-pcna3 and pMID-araS-pcna1 transformants was significantly delayed (Fig. 3).

To investigate what occurred in the pMID-pcna transformant cultures during the entire enrichment procedure, their target gene alleles were analysed by PCR with the respective flanking primers, and this revealed that the wild-type cells outgrew the transformant and its spontaneous mutants (PyrEF- LacS+) both in the pMID-pcna1 and in the pMID-pcna2 cultures. In fact, PCR with the flanking primers only detected the wild-type alleles of the pcna1 and pcna2 genes in En3 and En4 (Fig. 4a, b). By contrast, the same analysis revealed that the mutant alleles persisted in, and the wild-type alleles were always absent from, the cultures of the pMID-pcna3 and pMID-araS-pcna1 transformants (Fig. 4c, d). These results indicated that only pMID-pcna3 and pMID-araS-pcna1 transformants were useful in studying the ability of the mutant to propagate, because the wild-type target gene allele, which could interfere with PCR quantification of the corresponding deletant allele, did not exist in the culture.

A semiquantitative PCR procedure was employed to investigate the propagation of Δpcna3 and Δpcna1 mutant cells. Fig. 5 shows an example of the semiquantitative PCR analysis of the Δpcna3 allele during enrichment. The pcna3 flanking primers, which amplify both the wild-type allele (2.7 kb) and the mutant allele (2.0 kb), were used to estimate the relative proportion of cells carrying each genotype in the transformant cultures and the wild-type strain REY15A, respectively. This was done by dilution of the template DNAs to 16 different concentrations ranging from 100 to 0.001 ng, each of which was used as a template to carry out PCR amplification with the pcna3 flanking primers. While it required a minimum of 0.002 ng DNA of the wild-type strain to yield a PCR product of the pcna3 allele, about a 100-fold higher amount of DNA (0.2 ng) was required to yield a visible PCR product of the Δpcna3 allele in the pMID-pcna3 transformant culture (Fig. 5a). These results suggested that the mutant cells formed about 1 % of the cells in the transformant culture. After 10 days’...
Fig. 4. Analysis of target gene alleles of pMID-pcna transformant cultures. Genomic DNAs were isolated from the indicated cell samples (see also Fig. 3) and used for PCR with the flanking and internal primers whose PCR products are indicated on the right-hand side of the agarose gel panels. T, transformant; En1, enrichment 1; En3, enrichment 3. En4 was obtained by diluting En3 10-fold followed by growth to high optical density (OD$_{600}$ > 1.0). Total DNAs were prepared and PCR was conducted with two primer sets, the FlankP and IntP primers. Each pcna allele, namely wild-type (i, iii), mutant (ii) or recombinant (iv), is indicated. The target genes are pcna1 (a, d), pcna2 (b) and pcna3 (c). The size ranges for the PCR products are: wild-type gene, (i) 0.74–0.78 kb and (iii) 2.7–3.4 kb; mutant allele (ii), 2.0–2.6 kb; recombinant allele (iv), 7.1–8.3 kb.
incubation in the presence of 5-FOA, which allowed only mutant cells to duplicate, the proportion of ∆pcna3 cells did not change in the culture (comparing pcna3-En1 and Pcna3-En3 in Fig. 5a), indicating that ∆pcna3 cells did not propagate during the incubation period. For comparison, when the same enrichment procedure was conducted for the pMID-xpf transformant from which the knockout was obtainable (data not shown), the ∆xpf allele was amplified as a PCR product of 2.4 kb from 0.002 ng DNA template derived from pMID-xpf En3, but this was only yielded with a 100-fold higher amount of DNA from the pMID-pcna transformant or En1 (0.2 ng). These results indicated that ∆xpf cells increased by about 100-fold (Fig. 5b) during the enrichment. When the mutant propagation assay was conducted for the pMID-araS-pcna1 transformant, it was revealed that ∆pcna1 cells also did not propagate (data not shown). In conclusion, conducting the mutant propagation assay based on the MID knockout scheme allows the genes that are absolutely required for microbial growth to be identified.

**DISCUSSION**

A new gene deletion scheme designated marker integration and unmarked target gene deletion (MID) was developed and used for genetic manipulation in *S. islandicus*, and unmarked mutants were obtained for several genes involved in DNA transactions, including *xpf*, which encodes an endonuclease implicated in the archaeal nucleotide excision repair pathway (unpublished results). Further, we have demonstrated that pMID transformant cultures can be used to study the ability of mutant cells to propagate. This is because pMID transformant cultures contain cells of only two genotypes. As a consequence, the number of mutant cells can be determined by quantifying the mutant target gene allele, and doing so for an extended period under counter selection leads to an assessment of the change in the number of mutant cells that reflects the ability of the mutant to propagate. Using this method we have demonstrated that *pcna1* and *pcna3* mutant cells have lost the capability for duplication and that therefore these
genes belong to the category of essential genes in *S. islandicus*.

All known crenarchaea contain three *pcna* genes, except for *Pyrobaculum aerophilum*, in which there are two (Barry & Bell, 2006). Determination of the content of each PCNA protein by Western blot analysis has indicated that the three PCNA proteins are expressed at different levels in the crenarchaeon *A. pereix* (Imamura *et al*., 2007). Doing the same for *S. islandicus* has yielded similar results (unpublished data). This implies that PCNA proteins must form different PCNA heterotrimers in *S. islandicus*, because only heterotrimers have been found for *S. solfataricus* (Dionne *et al*., 2003) and for *S. tokodaii* (Lu *et al*., 2008). Together with the *pcna1* and *pcna3* gene essentiality revealed in this work, this supports the earlier hypothesis that a heterotrimer of the PCNA clamp with three different subunits is responsible for DNA replication in *Sulfolobus* species (Dionne *et al*., 2003).

The occurrence of different PCNA clamps suggests that their gene expression must be controlled very strictly, because if these PCNA trimers have different functions, an altered cellular content of any subunit would yield a change in the dynamic population of PCNA clamps in *vivo*, disturbing DNA transaction processes. We have obtained some indirect evidence that supports strictly controlled transcriptional regulation for each *S. islandicus pcna* gene. First, rescuing *pcna* mutant cells in pMID transformant cultures by transforming MID cultures with *Sulfolobus*-*E. coli* shuttle vectors carrying each *pcna* gene but devoid of the *pyrEF* marker did not yield any true transformants because the colonies formed were spontaneous mutants carrying the Δ*pcna::pyrEF-lacS* allele, although *pcna* deletion mutants should yield up to 1000-fold more colonies after transformation (data not shown). Second, in knockout experiments with pMID-araS plasmids in which each *pcna* gene was placed under the control of the arabinose-inducible promoter *araS* (Jonuscheit *et al*., 2003), only transformants for pMID-araS-pcna1 were obtained in *S. islandicus*, suggesting that the *araS* promoter employed can only mimic expression from *pcna1*. For comparison, constructing gene knockouts with pMID-araS plasmids has yielded transformants for several other DNA replication and repair genes, including those encoding reverse gyrase, RadA and RadA-like proteins (unpublished data). The expression of *pcna* genes could also be regulated at the post-transcriptional and/or translational levels. In future work, we will use genetic systems to address the regulation of PCNA expression, including the *Sulfolobus* reporter gene system recently (Peng *et al*., 2009) and the pMID-araS-pcna1 transformant obtained in this work.

Several gene knockout methods have recently been developed for archael genetics, first for mesophilic archaea (Allers & Mevarech, 2005), then for hyperthermophilic ones (Sato *et al*., 2005; She *et al*., 2009; Worthington *et al*., 2003). When they are used to assess gene essentiality, the conclusion is exclusively based on the colony formation capability of the resulting mutants. By this criterion, some non-essential genes will inevitably be misclassified into the essential group because due to retarded growth, their mutants will not form colonies under the experimental conditions employed. Only directly investigating mutant propagation can distinguish gene essentiality from retarded growth.

To date, only two genetic procedures yield transformants that can be used for mutant propagation assays; these are MID and plasmid integration and segregation (PIS) (also called popping in and popping out) method. Deletion mutant cells yielded by the latter have not been investigated for their ability to propagate, although there has been a report that addresses rescuing the mutant cells via complementation (Berquist *et al*., 2007). In a PIS culture, three different genotypes are formed: the transformant harbouring the partial diploid target gene allele, the wild-type revertant and the unmarked deletion mutant, two of which, namely the revertant and deletant, will survive counter selection. Because revertant cells interfere with the assessment of mutant propagation, PIS transformants are not suitable for studying gene essentiality, and this has been demonstrated in our recent work in which we constructed deletion mutants for *tfb2* and *tfb3* that encoded two of the three orthologues of the general transcription factor B. Since mutants were not obtainable for these genes, the pPIS-tfb2 and pPIS-tfb3 transformants were used to investigate mutant propagation together with the pPIS-lacS transformant that has yielded deletion mutants (Deng *et al*., 2009). We found that these PIS cultures grow readily after the addition of 5-FOA, the counter-selection agent, regardless of whether mutants are obtainable or not (data not shown). By contrast, MID transformant cultures contain cells of two genotypes, namely transformants and unmarked deletion mutants, because the formation of any revertant is prevented by designing homologous arms without any repeated sequence. Consequently, only mutant cells are capable of growing in a MID transformant culture, and this makes prolonged incubation of MID transformants possible, enabling the assessment of mutant propagation over a relatively long period.

It is important to point out that only a carefully designed MID scheme enables successful transformation with pMID and the counter selection of the deletion mutants obtained in the case of a gene essentiality study. The precautions that should be taken include the following: (i) insertion of the marker cassette should allow proper expression of the target gene; (ii) expression of flanking genes should be ensured; and (iii) sequence repeats between the In-arm and the Tg-arm should be avoided. However, addressing the first two points often compromises the third, because including the promoter of a target gene in a Tg-arm almost invariably introduces a short sequence repeat between the Tg-arm and the In-arm. The mutant propagation experiment failed for the pMID-pcna1 and -2 transformants because their pMID plasmids contained short repetitive
DNA sequences between the Tg-arm and the In-arm (50 and 34 bp, respectively). Looping out the marker cassette at the short DNA sequence repeats yields the original host genotype that predominates in the culture upon enrichment incubation (Fig. 4a, b). So far, we have not systematically analysed the minimal length of sequence repeat that is allowed in the MID scheme. However, using a repeat of less than 20 bp in a few other experiments has not given any problems in the mutant propagation assay (unpublished data).

Studying gene essentiality has a manifold purpose. Knowledge of gene essentiality provides important insights into the in vivo roles of a gene. Once the essentiality of a gene is established, alternative in vivo investigations can be initiated, such as utilizing an inducible promoter for controlled expression of the gene and knockdown of its expression, all of which yield further insight into the function of the essential gene. Essential genes also provide important targets for the development of antibacterial and antifungal drugs against human pathogens, many of which grow only slowly in the laboratory. In this regard, using a colony formation experiment to infer gene essentiality can be misleading and dangerous. The MID method developed in this work, combined with the mutant propagation assay demonstrated, can in principle be applied to any microorganisms that exhibit efficient homologous recombination. This technique either yields unmarked mutants or demonstrates gene essentiality for a target gene. Moreover, discriminating the inhibitors of essential gene products that can mimic the phenotype of a gene knockout from those that only knock down the activity of the gene product will eventually lead to the discovery of the drugs that stop the growth of pathogenic microorganisms completely.

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