Establishing a Markerless Genetic Exchange System for Methanosarcina mazei Strain Göl for Constructing Chromosomal Mutants of Small RNA Genes

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1. Introduction
Methanosarcina mazei strain Göl belongs to the methylothrophic methanogenic Archaea and, due to its role in methane production, is of high ecological relevance [1]. It serves as an archael model for investigating nitrogen stress responses, salt adaptation, methane production from different substrates, energy metabolism, as well as analyzing the role of small RNAs as regulatory elements in stress responses [2–7]. Although it only grows under strictly anaerobic conditions, the organism is genetically tractable and single colonies can be obtained on agar plates, a general requirement for genetic studies [8, 9]. However, genetic manipulation is restricted due to the fact that puromycin is the only selectable marker commercially available for methanoarchaeal, which complicates generation of multiple mutations or even complementation experiments. Using Methanosarcina acetivorans, Metcalf and coworkers developed a so-called markerless exchange method using the hpt gene encoding hypoxanthine phosphoribosyltransferase as a counterselectable marker [10]. A Δhpt strain which shows resistance towards the toxic purine analog 8-aza-2,6-diaminopurine (8-ADP) can be used for counterselection following integration of an nonreplicable plasmid containing the wild-type hpt gene and the desired mutation with flanking regions for recombination. The complete plasmid is integrated into the site of the desired mutation (pop-in) in the chromosome by a single homologous recombination event, making the strain sensitive to 8-ADP and allowing selection for puromycin resistance. The presence of 8-ADP permits selection for removal of the plasmid-based hpt gene (in concert with the vector backbone) by another single homologous recombination (pop-out) event. During this latter event, the gene of interest can be exchanged by the mutant construct [10]. Theoretically, allelic exchange takes place with a chance of 50% resulting in the desired mutant strain.

The goal of this study was to establish this method for M. mazei in order to allow markerless chromosomal deletion or point mutations of small regulatory RNA genes. To set up the system, a Δhpt strain as well as the allelic exchange
vector containing the wild-type hpt gene for counterselection was generated. To validate the method, we deleted the small noncoding RNA sRNA154. This sRNA has been identified in a genome wide RNA-seq screen and shown to be differentially transcribed dependent on nitrogen availability [7]. We suggest that sRNA154 plays a central role in nitrogen regulation in M. mazei, potentially adding another level of regulation to the known regulatory mechanism via the general nitrogen transcriptional repressor NrpR [11, 12]. sRNA154 is located in the intergenic region of MM3337 and MM3338 encoding a conserved and a hypothetical protein, respectively [7, 13]. A potential NrpRI operator (GGTA-N6-TACC) has been identified in the promoter region of sRNA154 gene implying that this small RNA is under direct control of the global nitrogen regulator NrpRI [7].

2. Materials and Methods

2.1. Bacterial Strains and Plasmids. Strains and plasmids used in this study are listed in Table 1. Plasmid DNA was transformed into E. coli according to the method of Inoue et al. [14] and into M. mazei using liposome-mediated transformation as described recently [8, 15].

2.2. Growth. M. mazei wild-type and mutant strains were grown in minimal medium under a nitrogen gas atmosphere in 5 or 50 mL closed growth tubes, which were incubated at 37 °C without shaking [18, 19]. To screen on 8-ADP, however, the concentration of yeast extract in the minimal medium was reduced from 2 g/L to 0.5 g/L. In general, the medium was supplemented with 150 mM methanol or 25 mM trimethylamine (TMA) and 40 mM acetate as carbon sources and reduced with 2 mM cystein and 1 mM sodium sulfide. For nitrogen limited growth, ammonium was omitted from the media; molecular nitrogen in the gas phase served as sole nitrogen source [19]. In general, the Methanosarcina cultures were supplemented with 100 μg/mL ampicillin to prevent bacterial contamination. For mutant selection, puromycin (5 μg/mL) was added to the medium, for counterselection during markerless exchange the medium was supplemented with 8-ADP (20 μg/mL). Growth was monitored by determining the optical density of the cultures at 600 nm (O.D.600). M. mazei wild-type and mutant strains were grown on solid medium by carefully spreading the cells on 1.5% bottom agar containing 25 mM TMA as carbon source and incubated in an intrachamber incubator under a gas atmosphere consisting of 79.9% N2, 20% CO2, and 0.1% H2S. Mutants were selected by adding 5 μg/mL puromycin or 20 μg/mL 8-ADP (final concentration) to the agar. To identify positive pop-out mutants, single colonies derived in the presence of 8-ADP were streaked in parallel on plates complemented with puromycin and 8-ADP, respectively, to screen for puromycin sensitivity and 8-ADP resistance.

2.3. Construction of Plasmids. All primers used in this study are listed in Supplementary Table 1. The plasmid for generating an M. mazei hpt null mutant was constructed as follows: the sequences 800 bp down- and upstream of the hpt gene were amplified using chromosomal M. mazei DNA and the primer sets Mm 201 800 up.f/or/Mm 201 800 up.rev and Mm 201 800 down.f/or/Mm 201 800 down.rev, respectively. The PCR products obtained contained additional synthetic primer-mediated restriction sites which, for the 800 up stream product included a BamHI at the 5’ end and EcoRI site at the 3’ end and for the 800 downstream fragment an EcoRI site at the 5’ end and KpnI site at the 3’ end. Both fragments were restricted using BamHI/EcoRI and EcoRI/KpnI, respectively, and cloned into pBSK+ (Stratagene, La Jolla, Calif, USA) yielding plasmid pRS283. The allelic exchange vector for the markerless exchange was generated by amplifying the hpt gene from chromosomal M. mazei DNA using the primers Mm hpt for and Mm hpt rev with additional BamHI and XhoI sites, respectively. The PCR fragment was digested

| Table 1: Strains and plasmids used in this study. |
|-----------------------------------------------|
| **Strains**                                      |
| Methanosarcina mazei strain G61   Wild type            DSM No. 3647          |
| M. mazei Δhpt                M. mazei, with hpt deletion          This study       |
| M. mazei ΔsRNA154             M. mazei, with sRNA154 deletion        This study       |
| E. coli DH5a                 general cloning strain      Stratagene, La Jolla, US |
| E. coli DH5a/Apir            general cloning strain      Stratagene, La Jolla, US |
|                               |                               |                               |
| **Plasmids**                                      |
| pMCI210                      general cloning vector          [17]           |
| pBSK+                        general cloning vector          Stratagene, La Jolla, US |
| pDRIVE                       general cloning vector          Qiagen, Hilden, Germany |
| pRS207                       pac-resistance cassette in pSL1180       [8]           |
| pRS269                       pmcr of M. voltae in pDRIVE              This study       |
| pRS283                       M. mazei hpt deletion construct in pBSK+         This study       |
| pRS311                       pBSK+ plus M. mazei hpt gene            This study       |
| pRS320                       pRS311 with pmcr upstream of hpt                This study       |
| pRS345                       pRS311 with pac-resistance cassette       This study       |
| pRS606                       pMCL210 with 930 bp of sRNA154 upstream region       This study       |
| pRS631                       pMCI210 plus sRNA154 deletion construct       This study       |
| pRS632                       (pRS631) inserted into the Apal site of pRS345         This study       |
using BamHI and XhoI and ligated to BamHI and XhoI linearized pBSK+ to generate plasmid pRS311. In order to provide the hpt gene of pRS311 with a strong archaeal promoter, the known pmcr promoter of Methanococcus voltae [9] was cloned upstream of the gene. This was achieved by amplifying pmcr with the primers pmcr BamHI and pmcr XhoI using pRS207 [8] as template. The PCR product was cloned into TOPO-TA-cloning vector pDRIVE (Qiagen, Hilden, Germany) yielding plasmid pRS269. Digestion of pRS269 into TOPO-TA-cloning vector pDRIVE (Qiagen, Hilden, Germany) resulted in excision of the pDRIVE vector. This was achieved by ligating the PCR product (937 bp) to TOPO-TA-cloning vector pDRIVE (Qiagen, Hilden, Germany) yielding plasmid pRS269. Digestion of pRS269 with BamHI resulted in excision of the pmcr promoter that was cloned into the BamHI site located directly upstream of the hpt gene of plasmid pRS311, resulting in pRS320. Finally, the 1.7 kbp EcoRI fragment from pRS204 containing the pac-cassette under the control of the constitutive promoter (pmcr) and terminator (pmcr) from the mcr-gene of M. voltae was cloned into the unique NotI site of pRS320, generating plasmid pRS345. This plasmid was used for markerless allelic exchanges by cloning the desired mutation into its unique Apal site. To construct the M. mazei sRNA154 deletion mutant, approximately 1000 bp of the upstream-flanking region of the small RNA was amplified using the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to generate the primer pair pac1 and pac2 was used to 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2.4. PCR Analysis for Mutant Strain Confirmation. Verification of sRNA154 deletion was performed using the primer pair Mm s154_1 and Mm s154_seq rev A ~250 bp product of the bla gene was amplified using the primers bla rev. and bla for. The primer pair pac1 and pac2 was used to generate a ~300 bp product of the pac-cassette. Generally, 2 ng of chromosomal DNA was used as template.

2.5. RNA Preparation and Northern Blot Analysis. Total RNA isolations and Northern blot analyses were performed essentially as described before [7], except that Isol-RNA Lysis Reagent (5 PRIME GmbH, Hamburg, Germany) was used for total RNA preparation.

2.6. Cell Extracts. M. mazei cell extracts were prepared as described previously [20]. 65 μg of M. mazei wild type, Δhpt and ΔsRNA154 crude extracts were separated by 12.5% SDS-PAGE.

3. Results and Discussion

As mentioned above, markerless exchange of alleles originally developed for M. acetivorans was applied for M. mazei using the hpt gene as counterselection marker [10] and successfully generated a null mutant of the gene encoding sRNA154.

3.1. Setting up a Markerless Exchange System for M. mazei

Members of the methanoarchaea become regularly resistant to the purine analog 8-ADP (2.9 × 10⁻³) [10], possibly by developing spontaneous mutations in the hpt gene that
A pKS bluescript derivative was constructed carrying 800 bp of the hpt gene fused together, thereby creating an hpt deletion construct (pRS283). The nonreplicating plasmid pRS283 was transformed into M. mazei, which encodes a hypoxanthine phosphoribosyltransferase. Nevertheless, we decided to construct a Δhpt mutant by applying the markerless exchange method of Pritchett et al. [10] rather than screening for a naturally occurring hpt-deficient strain. A pKS bluescript derivative was constructed carrying 800 bp of both the 5′ and 3′ flanking chromosomal region of the M. mazei hpt gene fused together, thereby creating an hpt deletion construct (pRS283). The nonreplicating plasmid pRS283 was transformed into M. mazei [8], which will be referred to as wild type, and successful integration into the chromosome via a single homologous recombination event was confirmed by the gain of puromycin resistance (Figure S1A). Single colonies were then inoculated into liquid medium containing 20 μg/mL 8-ADP. Cells that carry the wild-type hpt gene on the chromosome are sensitive to 8-ADP unless hpt is obliterated by a pop-out event, removing the hpt gene and the plasmid backbone (Figure S1B). Unfortunately, the standard minimal medium used for M. mazei [18] cannot be used for this approach as 8-ADP had little effect on growth of the cells (Figure 1(a)). Yeast extract, which is presumably rich in purines and pyrimidines, might affect uptake of 8-ADP. Growth in media with significantly reduced yeast extract (0.5 g/L) clearly demonstrated that 20 μg/mL 8-ADP was inhibitory (Figure 1(b)). As expected, the M. mazei Δhpt grew in the presence of 8-ADP on standard and on yeast-reduced medium (Figures 1(a) and 1(b)). Single colonies of the M. mazei Δhpt mutant strain were obtained by plating on solid medium containing 8-ADP, which were subsequently tested for puromycin sensitivity and simultaneous 8-ADP resistance by streaking on the respective plates. To confirm deletion of hpt, colonies that showed the desired phenotype were subjected to Southern blot analysis (data not shown).

In a second step, the allelic exchange vector pRS345 was constructed containing the bla gene and pac-resistance cassette for selection in E. coli and M. mazei, respectively, as well as the hpt gene as counterselectable marker. To provide the hpt gene with a strong promoter, the native promoter was exchanged with promoter pmcr of M. voltae [9]. The unique ApaI site in pRS345 provided an insertion site for the mutant construct of interest.

### 3.2. Generation of a M. mazei ΔsRNA154 Chromosomal Mutant

To validate the system, we deleted the gene encoding the small RNA154 which is transcribed exclusively under nitrogen limitation and supposedly plays a central role in nitrogen stress responses [7]. A deletion construct generated by fusing the flanking regions of sRNA154 together was inserted into the ApaI site of the allelic exchange vector pRS345. The resulting plasmid (pRS632) was transformed into the M. mazei Δhpt strain followed by selection for pop-in/pop-out events as described above. Successful deletion of the sRNA154 gene was evaluated by PCR. PCR verification
Figure 4: Growth analysis of ΔsRNA_{154} versus wild type under nitrogen limitation (a) and under nitrogen sufficiency: (b) *M. mazei* strains were grown in 50 mL liquid minimal medium complemented with 150 mM methanol as carbon source under nitrogen limiting conditions (a) and with 10 mM NH\textsubscript{4} as nitrogen source (b) under a gas atmosphere of N\textsubscript{2}/CO\textsubscript{2} (80 : 20). Open circles: wild type; closed squares: *M. mazei* Δhpt; closed triangles: *M. mazei* ΔsRNA\textsubscript{154}. Standard deviations of five replicates for each strain are indicated.

Figure 5: Analysis of protein expression pattern of ΔsRNA\textsubscript{154} versus wild type (a) under nitrogen limitation and (b) under nitrogen sufficiency. The *M. mazei* strains ΔsRNA\textsubscript{154}, Δhpt, and wild type were grown in 50 mL medium under standard nitrogen-limiting conditions or under nitrogen sufficiency (see Figure 4). Cells were harvested in exponentially phase. Equal amounts of cell extracts (65 μg) were applied to a 12% SDS-PA gel, which was subsequently stained with Coomassie blue. Arrows 1–3 indicate protein bands with differential expression in *M. mazei* ΔsRNA\textsubscript{154} and wild-type strains. Marker: LMW marker GE healthcare.

with primers binding up- and downstream of sRNA\textsubscript{154} was performed yielding a PCR product of ~1,100 bp for wild type and ~940 bp for *M. mazei* ΔsRNA\textsubscript{154}. The PCR product representing the wild type was detected as expected in *M. mazei* wild type and the diploid strain with plasmid pRS632 inserted into the chromosome (Figure 2(a)). The respective amplicon for ΔsRNA\textsubscript{154} was clearly detected in the control (pRS632), in the diploid strain and was very prominent in all eight potential *M. mazei* ΔsRNA\textsubscript{154} mutants analyzed (Figure 2(b)). However, in seven out of the eight putative ΔsRNA\textsubscript{154} mutants, traces of PCR products corresponding to the product derived from sRNA\textsubscript{154} wild type were also observed. This might be explained by the fact that several archaea have been demonstrated to possess multiple genome copies, as has been recently described by Soppa and coworkers [21]. They showed that *M. acetivorans* contains up to 17 copies dependent on the growth phase [21]. This polyploidy might result in incomplete allelic exchange with some of the chromosome copies remaining wild type. Since we could only confirm one out of eight mutant candidates, it appears that this difficulty occurs more often than anticipated when generating chromosomal mutants of *M. mazei*.

The mutant depicted in lane 11 (Figure 2(b)) showing the ΔsRNA\textsubscript{154} PCR product was further examined for plasmid removal. PCR analyses using chromosomal DNA from *M. mazei* wild type, the Δhpt mutant, the diploid strain, and ΔsRNA\textsubscript{154} as well as pRS632 as positive control clearly demonstrated the presence of the *bla* and *pac* genes.
exclusively in the diploid strain and the plasmid control, whereas for \( \Delta sRNA_{154} \), both genes were not detectable. As a second line of evidence, Northern blot analyses were performed with total RNA derived from the wild type, \( \Delta hpt \) and \( \Delta sRNA_{154} \) strains grown under nitrogen limitation and using a radioactively labelled oligonucleotide probe against \( sRNA_{154} \). Consistent with the previous data, Northern blot analyses clearly demonstrated that \( sRNA_{154} \) with a size of 130 nucleotides (nct) is present in the wild type and \( \Delta hpt \) strains under nitrogen limitation but is not detectable in the \( sRNA_{154} \) deletion strain, further confirming successful markerless allelic exchange (Figure 3). By generating this \( \Delta hpt\Delta sRNA_{154} \) mutant, which will be referred to as \( \Delta sRNA_{154} \) strain, we have effectively established the markerless exchange system in \( M. mazei \).

3.3. Characterization of the \( \Delta sRNA_{154} \) Mutant Strain. To analyze the functional role of \( sRNA_{154} \) in nitrogen metabolism, we characterized the \( M. mazei \) \( \Delta sRNA_{154} \) mutant growing under conditions of nitrogen limitation, in which the \( sRNA \) is strongly expressed. Growth analyses demonstrated reduced growth of \( M. mazei \) \( \Delta sRNA_{154} \) with a growth rate of \( \mu = 0.02 \text{~h}^{-1} \) compared to \( \mu = 0.03 \text{~h}^{-1} \) obtained for the wild type (Figure 4(a)). Nevertheless, \( sRNA_{154} \) did not reach the same final cell densities as the wild type. Negative effects on nitrogen fixation due to the absence of the \( hpt \) gene were excluded by analysing growth behaviour of the parental strain (\( M. mazei \) \( \Delta hpt \)) (Figure 4(a)). As expected, no different growth phenotype of these three \( M. mazei \) strains was observed under nitrogen sufficiency as under this condition the \( sRNA_{154} \) is not transcribed (Figure 4(b)).

Characterizing the protein expression patterns of \( \Delta sRNA_{154} \) under nitrogen limitation and nitrogen sufficiency by one-dimensional SDS-PAGE clearly demonstrated differences in the protein patterns only under nitrogen depletion (Figure 5). At least three different proteins were differentially synthesized under nitrogen limitation in the absence of \( sRNA_{154} \) in comparison to the wild type (Figure 5(a)). Two proteins (1 and 2) with the molecular mass of approximately 66 and 40 kDa were exclusively or significantly more strongly expressed in the mutant, whereas a 35 kDa protein (3) was present in the wild type but appears to be absent in the mutant. These findings indicate that \( sRNA_{154} \) controls the protein expression either directly or indirectly and again strongly support a prominent function of the \( sRNA_{154} \) in nitrogen regulation.

The \( \Delta sRNA_{154} \) mutant represents the first chromosomal deletion mutant of a small RNA in \( M. mazei \). As it is only transcribed under nitrogen fixing conditions, presumably under the control of the global nitrogen regulator NrpRI [7], we suggest that \( sRNA_{154} \) plays a central role in regulation of nitrogen metabolism. The differences in the cytoplasmic protein patterns that result in reduced growth of \( sRNA_{154} \) under nitrogen fixing conditions argue for a prominent role of \( sRNA_{154} \) in regulation of nitrogen fixation. Posttranscriptional regulation by \( sRNA_{154} \) would add another level of regulation of nitrogen metabolism in \( M. mazei \) possibly resulting in tighter control or fine tuning of translation of the target mRNAs.

4. Conclusion

By generating a \( \Delta hpt \) strain and a plasmid for allelic replacements, we successfully applied the markerless exchange system to \( M. mazei \). The method was further optimized by using medium with reduced yeast extract, thereby enhancing the toxic effect of 8-ADP during counterselection. Generation of \( \Delta sRNA_{154} \) revealed the role of \( sRNA_{154} \) in nitrogen metabolism as demonstrated by reduced growth as well as differential synthesis of at least three proteins under nitrogen fixing conditions in the absence of \( sRNA_{154} \).

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