An Autonomously Replicating Transforming Vector for *Sulfolobus solfataricus*

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A plasmid able to transform and to be stably maintained both in *Sulfolobus solfataricus* and in *Escherichia coli* was constructed by insertion into an *E. coli* plasmid of the autonomously replicating sequence of the virus particle SSV1 and a suitable mutant of the *hph* (hygromycin phosphotransferase) gene as the transformation marker. The vector suffered no rearrangement and/or chromosome integration, and its copy number in *Sulfolobus* was increased by exposure of the cells to mitomycin C.

Among members of the archaeal domain of life, the extreme thermophiles exhibit intriguing and unique properties at both the physiological and molecular levels (4, 5). Nevertheless, the studies of these prokaryotes have so far been focused mainly on classical biochemistry, including the purification and characterization of individual proteins and low-molecular-weight compounds, and on the molecular cloning of structural genes (3), also used for determining the evolutionary relationships within members of the same and/or different kingdoms (1, 2, 11).

In contrast, recent studies on the molecular and cell biology of prokaryotes belonging to the domain *Bacteria* have progressed remarkably even for thermophilic representatives (14, 15, 22), because of their high similarity to *Escherichia coli*, the most extensively studied model system. The applicability of the experimental approaches of the *E. coli* model can also explain the very recent rapid and full development of molecular genetic techniques, such as the use of selective genetic markers and gene transfer, for halophilic members of the domain *Archaea* (9, 10).

Appropriate manipulative strategies for the thermophilic *Archaea* are still at a very early stage of setup, because of their notable diversity compared with their bacterial counterparts (11) and even among members of the same kingdom (12).

In this respect, the genus *Sulfolobus*, whose members are sulfur-metabolizing aerobes belonging to the archaeal kingdom *Crenarchaeota* (24), seems to be the most promising candidate for developing genetic systems since it has been investigated in greater physiologic and genetic detail than its relatives. In fact, several *Sulfolobus* species have been identified in very different areas on Earth, isolated, and characterized, and more interestingly, many of them have been shown to possess extrachromosomal genetic elements such as viruses (26) and plasmids, with a broad range of hosts (25). *Sulfolobus solfataricus* has been indicated as the most versatile recipient for both viruses, such as the SSV1 particle (20), isolated from the natural *Sulfolobus shibatae* host, and plasmids (21). Moreover, it can be plated as single colonies and cultivated both autotrophically and heterotrophically on different complex or simple nutrients (7).

Here, we present the construction of a shuttle vector, pEXSs, which is based on both the SSV1 viral autonomously replicating sequence (ARS) (18) and the pGEM5Zf(−) *E. coli* plasmid sequences, and the isolation of an antibiotic resistance gene marker for *S. solfataricus*, obtained by selection of a suitable mutant of the *E. coli*-derived hygromycin phosphotransferase (*hph*) gene (6).

The *hph* mutant was sequenced in order to identify the point mutation inserted. The corresponding plasmid carrying the selective determinant was shown to efficiently transform *S. solfataricus* and to be stably maintained as an autonomously replicating plasmid in this archaeon. This plasmid possesses shuttle capability since it can be transferred from *S. solfataricus* into *E. coli* and vice versa and propagated in both prokaryotes.

**Sulfolobus growth conditions and screening for drug sensitivity.** Cells of *S. solfataricus* MT3 were kindly supplied by M. De Rosa (Istituto di Biochimica delle Macromolecole, II University of Naples), and *S. shibatae* B12 (DSM 5389) was provided by the Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany). All *Sulfolobus* cells were grown under medium, temperature, and pH conditions suggested by the DSM catalog of strains.

Different clones of *S. solfataricus* MT3 were purified by two subsequent plating cycles on Gelrite (Gellan gum; Sigma) (7) and characterized with respect to growth and nutritional requirements. Strain G0 used in this study showed high reproducibility of plating efficiency with homogeneous colony sizes and grew relatively quickly in quite a wide range of temperatures (70 to 82°C) and pH values (2.5 to 5.0), with an optimum at 75°C and pH 3.8.

Inhibition of cell growth by several antibiotics and drugs at different concentrations was investigated, and the MIC was determined by monitoring the optical density at 600 nm (OD600) of liquid cultures and/or testing the cell viability by colony formation on Gelrite plates.

Results are shown in Table 1, together with the genes known to confer resistance to their natural hosts. Hygromycin B was chosen as the selective agent for transformation because it is very stable under the *Sulfolobus* growth conditions. In fact, it was still effective against *E. coli* at a concentration of 50 μg/ml after incubation for 1 week at 75°C of a 150-μg/ml stock solution in Brock's basal medium buffered at pH 3.0. Moreover, a spontaneous resistance phenotype appeared at a very low frequency (10⁻⁹) in the *Sulfolobus* G0 population.

**Construction of the plasmid vector pEXSs.** Total genomic DNA, extracted from *S. shibatae* cells as described by Guagli-
ardii et al. (8), was used as the template for the PCR amplification of a 1,700-bp region located between positions 4938 and 6617 of the SSV1 viral genome map, containing the putative ORI sequence for viral DNA replication, as suggested by Palm et al. (18). The oligonucleotides 5′-GTATGAAATTCAGAGTTGGTGC-3′ and 5′-CTAACCAGTAAAAGCTTATG-3′, both containing an EcoRI site (underlined in the sequences), were used as the 5′ and 3′ primers, respectively, for the amplification of the specific region with Pfu DNA polymerase.

The reaction was carried out at 30 cycles at 45°C annealing temperature.

Aspartate aminotransferase gene promoter and terminator sequences were chosen for the heterologous gene expression of the E. coli hygromycin phosphotransferase gene (6). PCR amplification of the DNA regions in the plasmid pLV1, provided by Maria Luisa Tutino, Naples, Italy, using Pfu DNA polymerase and the specific oligonucleotides 5′-GTATGAAATTCAGAGTTGGTGC-3′ and 5′-CTAACCAGTAAAAGCTTATG-3′, both containing an EcoRI site (underlined in the sequences), were used as the 5′ and 3′ primers, respectively, for the amplification of the specific region with Pfu DNA polymerase.

Restriction analysis of plasmids isolated from independent preparations.

Preparation of cells competent for electroporation and the electroporation conditions tested were performed according to the procedure described by Schleper et al. for S. solfataricus infection by the SSV1 viral genome (20) with identical transformation efficiency. In the optimized experiments, 10 ng of the pEXSs plasmid sublibrary extracted from 10^5 independent clones was used.

The cells were mixed with 1 ml of 2× soft Gelrite and overlaid (7) onto solid medium containing 150 g of hygromycin B per ml in polystyrene petri dishes. Plates were incubated at 75°C in a humidified chamber until the size of the colonies was about 2 mm (about 8 days). No colony growth was noted following a 15-day incubation of cells plated after electroporation with pGEM5Zf(−) DNA and a pEXSswt plasmid. This result confirms both the low frequency of the spontaneous resistance phenotype and the incapability of the wild-type hph gene to confer resistance to *Sulfolobus* cells.

Hygromycin B-resistant (HygB') clones (5 per 10 ng of the pEXSs sublibrary) were picked up, grown for several generations in selective medium (150 μg of hygromycin B per ml), and harvested from the mid-log phase of growth; both extrachromosomal and total DNA were extracted in independent preparations.

The shuttle capability was ascertained by transformation of

![FIG. 1. Plasmid map of the pEXSs shuttle vector. Unique restriction sites suitable for the insertion of foreign sequences are indicated. SoRI indicates the 1,700-bp fragment carrying the ARS of the S. shibatae SSV1 viral genome. AspAT Pr and AspAT Ter are the promoter and terminator sequences of the S. solfataricus aspartate aminotransferase gene, respectively. hph is the E. coli randomly mutagenized hygromycin phosphotransferase gene. The E. coli pGEM5Zf(−) plasmid moiety lies between the two lacZ gene fragments and comprises the sequences necessary for propagation (ORI) and transformant selection for ampicillin resistance (Amp') in E. coli.](http://jb.asm.org/)

### TABLE 1. Substances tested for the growth inhibition of *S. solfataricus* G0

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC</th>
<th>Resistance gene</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hygromycin B</td>
<td>100 μg/ml</td>
<td>hph</td>
<td>E. coli</td>
</tr>
<tr>
<td>Geneticin</td>
<td>150 μg/ml</td>
<td>hph</td>
<td>E. coli</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50 μg/ml</td>
<td>cat</td>
<td>E. coli</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>25 mM</td>
<td>Ssadh</td>
<td><em>S. solfataricus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>adh-hT</td>
<td><em>B. stearothermophilus</em></td>
</tr>
</tbody>
</table>

* Gene determinants (resistance genes) conferring resistance to their natural hosts (indicated as sources).
The present work shows that the putative SSV1 ARS is a real genetic element a good vehicle for gene transfer into Sulfolobus and that DNA-damaging agents, such as UV light or chemicals, can induce replication of the SSV1 genome and an increase of viral DNA copy number in the different Sulfolobus DNA extracts in comparison with that extracted from untreated cells. The result shows that an increase of up to 10-fold could be obtained under the best conditions of drug concentration (5 µg/ml) and exposure time (24 h) tested here.

**Sequence of the wild-type and mutant hygromycin phosphotransferase gene.** The coding sequence (hyg2A) of the hph gene from one of the Sulfolobus HygB'-transformed clones was determined by the dideoxy-chain termination method and compared to its wild-type counterpart. The mutant hph gene was sequenced on both strands and in duplicate by subcloning of suitable fragments and sequencing with the universal M13 primers and using the specific oligonucleotides 5'-GCAAAG TGCCGATACAA-C-3', 5'-GTTTCTGCAGCCGCTGC-3', 5'- CGATTCTTGTGGGTGCCG-3', 5'-GTCCGGCACCTGCAG CA-3', and 5'-GGGCTATATGCTCCG-3', based on the hph gene for direct sequencing of the intact sequence in the pEXs plasmid. Some silent (G348→T and G777→A) and two effective (G155→C and C156→G producing Ser52→Thr; G714→T resulting in Trp238→Cys) mutations could be located (numbers refer to the nucleotide sequence starting at the A of the ATG initiation codon); very interestingly, in addition to the nucleotide substitution inserted to construct the hybrid promoter-coding sequence, a nucleotide insertion was also located in the SsAspAT gene promoter between positions −13 and −14, upstream of the ATG start codon. The relationship between these nucleotide and/or amino acid replacements with the hypothetical thermal resistance of the mutated enzyme was not obvious. Currently under investigation are the contribution of the replacements in the coding sequence to thermal activity and stabilization of the mutant enzyme in comparison with the wild-type counterpart, as well as the possibility that the nucleotide insertion selected in the SsAspAT promoter sequence might increase hph gene transcription and, hence, enzyme levels.

**Conclusions.** Genetic elements such as viruses and plasmids could represent powerful tools, as extensively demonstrated for Bacteria and Eucarya, to elucidate the molecular genetics of the still poorly understood domain Archaea.

The present paper describes the steps necessary to construct an E. coli-S. solfataricus shuttle vector that can be selected and maintained both in S. solfataricus and in E. coli.

It had been proven that the SSV1 virus particle of S. shibatae is able to also propagate as an extrachromosomal genetic element in S. solfataricus and that DNA-damaging agents, such as UV light or chemicals, can induce replication of the SSV1 genome and an increase of viral DNA copy number in the nonintegrated form (16, 17). These features rendered this genetic element a good vehicle for gene transfer into Sulfolobus. The present work shows that the putative SSV1 ARS is a real replicon unit able to drive autonomous propagation of hybrid Sulfolobus/E. coli DNA in the shuttle vector constructed.

The construction of the shuttle vector pEXs was accompanied and hence made possible by the suitable modification of a
specific enzyme of mesophilic source by the transferring of a
gene to confer an easily selectable phenotype after its
random mutagenesis. Further tailoring of the 6.2-kb pEXs shuttle vector is under way, with the replacement of the hph
gene with other genetic markers, the insertion of different archaeal promoter and terminator sequences for gene regulation
studies, and the expression of foreign mesophilic genes for
thermal adaptation of the encoded proteins of interest.

Moreover, since the *S. solfataricus* genome sequencing
project is in progress, the gene transfer system described here
could be an effective strategy to express at reasonable levels potential coding regions as yet unknown or merely recogniz-
able only by sequence similarity in order to identify and char-
acterize genome products. The presence of a polycloning site
in the newly constructed vector seems to be quite appropriate
for these purposes.

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REFERENCES

able archaeal diversity detected in a Yellowstone National Park hot springs
Acad. Sci. USA 92:2441–2445.
84.
113. In G. di Prisco (ed.). Life under extreme conditions: biochemical ad-
aptation. Springer-Verlag, Berlin, Germany.
sequence of hygromycin B phosphotransferase gene and its expression in
*Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25:179–188.
genus *Sulfolobus*: comparison of five wild-type strains. J. Bacteriol. 171:6710–
6719.
8. Gugliardi, A., D. de Pascale, R. Cannio, V. Nobile, S. Bartolucci, and M.
Rossi. 1995. The purification, cloning, and high level expression of a glu-
taricin-like protein from the hyperthermophilic archaeon *Pyrococcus fa-
use of halobacterial shuttle vectors and further studies on Halofexus DNA
4:816–822.
mutagenesis of a defined DNA segment using a modified polymerase chain
reaction. Technique 1:11–15.
for *Thermus thermophilus* HB8: expression of a heterologous, plasmid-borne
425.
an activator required for induction of pectin lyase in *Erwinia carotovora*
characterization of an *Erwinia carotovora* subsp. *carotovora* pectin lyase gene
18. Palm, P., C. Schleper, B. Gramp, S. Yeats, P. McWilliam, W.-D. Reiter, and
W. Zillig. 1991. Complete nucleotide sequence of the virus SSV1 of the
laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring
Harbor, N.Y.
extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and of transfection with viral DNA. Proc. Natl. Acad. Sci. USA
89:7645–7649.
multicopy plasmid of the extremely thermophilic archaeon *Sulfolobus* effects
Demirgian. 1995. A chromosome integration system for stable gene transfer
23. Zillig, W. Personal communication.
1980. The *Sulfolobus*(“Caldivirgella”) group: taxonomy on the basis of the
zendorfer, and J. K. Kristjansson. 1994. Screening for *Sulfolobales*, their
16:669–668.
of thermophilic and hyperthermophilic *Archaea*. FEMS Microbiol. Rev. 18:
225–236.