Molecular Analysis of pDL10 from *Acidianus ambivalens* Reveals a Family of Related Plasmids from Extremely Thermophilic and Acidophilic Archaea

Arnulf Kletzin,* Angelika Lieke,* Tim Urich,* Robert L. Charlebois1 and Christoph W. Sensen†

*Institute of Microbiology and Genetics, Darmstadt University of Technology, 64287 Darmstadt, Germany, †Department of Biology, University of Ottawa, Ontario K1N 6N5, Canada and ‡Institute for Marine Biosciences, Halifax, Nova Scotia B3H 3Z1, Canada

Manuscript received March 20, 1999
Accepted for publication May 13, 1999

ABSTRACT

The 7598-bp plasmid pDL10 from the extremely thermophilic, acidophilic, and chemolithoautotrophic Archaeon *Acidianus ambivalens* was sequenced. It contains 10 open reading frames (ORFs) organized in five putative operons. The deduced amino acid sequence of the largest ORF (909 aa) showed similarity to bacterial Rep proteins known from plagues and plasmids with rolling-circle (RC) replication. From the comparison of the amino acid sequences, a novel family of RC Rep proteins was defined. The pDL10 Rep protein shared 45–80% identical residues with homologous protein genes encoded by the Sulfolobus islandicus plasmids pRN1 and pRN2. Two DNA regions capable of forming extended stem-loop structures were also conserved in the three plasmids (48–69% sequence identity). In addition, a putative plasmid regulatory protein gene (plrA) was found, which was conserved among the three plasmids and the conjugative Sulfolobus plasmid pNOB8. A homolog of this gene was also found in the chromosome of *S. solfataricus*. Single-stranded DNA of both pDL10 strands was detected with a mung bean nuclease protection assay using PCR detection of protected fragments, giving additional evidence for an RC mechanism of replication.

The presence of a medium-sized multicopy number plasmid in the extremely thermophilic, acidophilic, and facultatively anaerobic Archaeon *Acidianus ambivalens* (formerly *Deinococcus ambivalens*) has been known since the organism was isolated by Zillig and co-workers (Zillig et al. 1985, 1986). The organism is a chemolithoautotroph growing optimally at pH 2.5 and 80°C in a mineral medium supplemented with elemental sulfur and under an atmosphere of either CO₂/H₂ or CO₂-enriched air. It had been suggested originally that the plasmid might play a role in anaerobic growth because an increase of its copy number under anaerobic growth conditions had been observed (Zillig et al. 1985, 1986). Later, the plasmid was found in several Icelandic isolates from geographically distant solfataric fields. However, its properties have not been analyzed in detail (Zillig et al. 1994).

At present, only a small number of extrachromosomal genetic elements have been found and analyzed in Crenarchaeota or in extremely thermophilic Archaea. What we do know comes mostly from the systematic screening programs conducted by Wolfram Zillig and co-workers (reviewed in Aagaard et al. 1996; Zillig et al. 1996, 1998; Aravalli and Garrett 1997; Noll and Vargas 1997). Among these are the Sulfolobus islandicus high-copy number plasmids pRN1, pRN2, and pHE7 (Zillig et al. 1994, 1996; Keeling et al. 1996, 1998) and the large conjugative plasmid pNOB8 from a Japanese *S. solfataricus* isolate (Schleper et al. 1995; Elferink et al. 1996; She et al. 1998). Only six sequences of crenarchaeotal genetic elements have been published. These include the plasmids pRN1, pRN2, and pNOB8, *S. solfataricus* insertion element, and the viruses SSV 1 and TTV 1 (Neumann et al. 1989; Neumann and Zillig 1990; Palm et al. 1991; Schleper et al. 1994; Keeling et al. 1996, 1998; She et al. 1998). In addition, the sequence and some properties are known from the plasmid pGT5 from the hyperthermophilic and sulfur-dependent euryarchaeote *Pyrococcus abyssi* (Erauso et al. 1996; Marsin and Forterre 1998). These genetic elements have been used for the development of transformation systems for Sulfolobus and *Pyrococcus* (Aagaard et al. 1996; Elferink et al. 1996; Aravalli and Garrett 1997; Noll and Vargas 1997; Cannio et al. 1998). But despite intense research in many laboratories, no stable vector/host system is yet available that allows the selection and propagation of singly transformed strains. The difficulties arise from plasmid instability in Escherichia coli or in the Sulfolobus host cells and from lack of knowledge of plasmid regions essential for replication and maintenance (Aagaard et al. 1996; Aravalli and Garrett 1997; Cannio et al. 1998).

The mechanism of replication of the 3445-bp *Pyrococcus abyssi* plasmid pGT5 has been studied in some detail (Erauso et al. 1996; Marsin and Forterre 1998). It was found that the plasmid replicates via a rolling-circle (RC) mechanism known from many bacterial plasmids and bacteriophages (reviewed in Ilyina and Koonin 1992; Khan 1997; Rasooly and Rasooly 1997). Single-
stranded DNA was detected with non-denaturing Southern hybridization experiments. The Rep protein encoded by one of the two open reading frames (ORFs) had a strand nicking and closing activity (Erauso et al. 1996; Marsin and Forterre 1998). It was suggested that pRN1 might also replicate via a RC mechanism (Keeling et al. 1996). However, no sequence similarity was found between pGT5 and the crenarchaeotal plasmids.

Here, we present the features of the A. ambivalens plasmid pDL10 and preliminary evidence for a RC mechanism of replication. From comparison to the distantly related S. islandicus plasmids pRN1 and pRN2 and to other RC plasmids and phages, we discuss the nature of the putative double-strand and single-strand origins and the phylogenetic relationship of a large plasmid-encoded Rep protein. The results allowed us to define a novel class of Rep proteins. Furthermore, conserved regions in these plasmids were found, which might be essential for replication and maintenance in the host cells.

**MATERIALS AND METHODS**

Organism and growth conditions, DNA and RNA preparations: A. (formerly Desulfurolobus) ambivalens DSM 3772 was grown aerobically and anaerobically as described (German Collection of Microorganisms, Braunschweig, Germany; Zillig et al. 1985, 1986; Fuchs et al. 1996). Total genomic DNA was prepared by the CTAB method combined with CsCl purification (Ellington 1998). A small amount of native pDL10 plasmid DNA was prepared with the QIAGEN plasmid mini kit (QIAGEN, Hilden, Germany). Total RNA was prepared from growing cultures of A. ambivalens by acidic phenol/guanidinium extraction (RNA-PURE-kit; Peqlab, Erlangen, Germany), followed by further purification over either RNeasy spin columns (QIAGEN, Hilden, Germany) or Qiagen Tip 20 columns according to a recommendation by the manufacturer, respectively. A Bacillus subtilis strain harboring the Staphylococcus aureus plasmid pE194 was obtained from the German Collection of Microorganisms (DSM 4554; Braunschweig, Germany).

Cloning and sequencing of pDL10: The purified plasmid was inserted with the unique HindII restriction site into pBlueScript II KS" using standard protocols (construct pDL10-H6; Stratagene, La Jolla, CA; Sambróo k et al. 1989). Subclones were constructed by deleting different parts of the original construct with various restriction enzymes in several steps. The sequence of the entire plasmid was determined on both strands by sequencing the subclones with the help of universal and specifically synthesized primers. All sequencing was done on a LICOR automatic sequencer (MWG, Ebersberg, Germany). The uniqueness of the HindII restriction site located in copG was checked by PCR amplification of the copG gene with specific primers (Figure 1, primer no. 5, GAG GGC GCC CGC GAT GAA GAA AAA GAG TCT ACA G; primer no. 6, TCT AAG CGC TGA TAA TGA TTA CCT GTT CCT TC, obtained from Interactiva, Ulm, Germany), cloning of the 236-bp product into the pCR-script vector (Stratagene), and sequencing. The pDL10 sequence was submitted to the EMBL database (accession no. AJ225333). The database entry is numbered from the unique Smal site. The sequencing of the S. solfataricus genome has been described elsewhere (Charlesbois et al. 1998; Sensen et al. 1998).

Southern analysis: Digoxigenin-labeled, strand-specific RNA probes were synthesized from pDL10-H6 using T3 (Stratagene) or T7 RNA-polymerases with the DIG RNA-labeling kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). For a denaturing and a non-denaturing Southern, total genomic DNA, undigested and digested with appropriate restriction enzymes, was separated on 1% agarose gels and blotted to a nylon membrane (Pall Filtron, Dreieich, Germany), omitting the denaturing and neutralizing steps of the standard protocol prior to the transfer (Sambróo k et al. 1989). For control, the same DNA was separated and transferred under standard denaturing conditions. The hybridizing strand-specific RNA probes were detected according to the manufacturer's instructions (Roche Diagnostics). The hybridizing strand-specific RNA probes were detected according to the manufacturer's instructions (Roche Diagnostics).

PCR detection of single-stranded DNA: For the detection of small amounts of single-stranded plasmid DNA the following protocol was developed: a combination of PCR with digestions using the restriction enzyme MseI (New England Biolabs, Beverly, MA) and the single strand-specific nuclease such as mung bean nuclease (MBN; Stratagene) or S1 nuclease (Roche Diagnostics). A total of 2 μg of genomic A. ambivalens DNA was digested with 10 units of the restriction enzyme MseI in a reaction volume of 50 μl in the buffer supplied by the manufacturer (recognition site: TTAA). A total of 400 ng of the MseI-digested DNA was incubated with MBN or nuclease S1 in a total volume of 300 μl in the appropriate buffer followed by phenol/ chloroform extraction and ethanol precipitation. The four fractions containing undigested DNA, MseI-digested DNA (1 μl of a 1:10 dilution each), MseI/MBN, or MseI/S1-digested DNA (1 μl undiluted) were subjected to PCR amplification with DAP DNA polymerase (Eurogentech, Seraing, Belgium) and several primer pairs (see below and Figure 1), including the copG-specific primers described above. The amplification reaction was performed in a volume of 50 μl on a Robocycler over 42 cycles (Stratagene). The amplified fragments were analyzed by electrophoresis on agarose gels (copG, 236 bp; DL10, 501 bp; rep, 2766 bp; SL10, 5735 bp). The amplification of PCR products after MseI digestion but not after MseI/MBN double digestion was indicative of the presence of single-stranded DNA. On the basis of these experiments, an MBN protection assay was developed to determine the strand specificity. A single PCR cycle was performed with 400 ng of the MseI-digested DNA (denaturation, 90 sec at 94°C; annealing with either of the copG and SL10-primers, 90 sec at 56°C; and a primer extension step with DAP DNA polymerase, 15 min at 70°C). After phenol/ chloroform extraction and ethanol precipitation, the samples were digested with MBN or S1 as described above. The protected double-stranded DNA was detected with PCR using the copG or the rep primers. The sequences of the primers were as follows: SL10-1 (primer 1, Figure 1), AGAAGC TAGCCG AAGTAA GGTAAG TAA, SL10-2 (2), AATTAG GCCGCC ACGTCA CAC GAG CAGAGGGG; rep-N (3), GAACT AGATTA CGAGG CAATTG CAGA AAGCTA AATTAC; rep-C (4), TTGTTT AAGCCG TCCCGG GCAAGG AGATTG CCCCAG TG; DL10-P1 (7), ATCTCT CCTCCT AGAACA CGG; DL10-P2 (8), CTGTTT ACCTAG GCGCGT GAG. The kilobase ladder was from Stratagene and the φX174/Hif1 small nucleic acid marker was from MBI Fermentas (Vilnius, Lithuania).

EMBL accession nos. are AJ225333 for pDL10 and Y18868 for S. solfataricus.

RESULTS AND DISCUSSION

Sequence analysis of pDL10: The circular multicopy double-stranded plasmid pDL10 from A. ambivalens (Zillig et al. 1985, 1986; Fuchs et al. 1996) was isolated
Plasmid pDL10 from A. ambivalens

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein or region</th>
<th>Plasmid pair</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pDL10-pRN1</td>
</tr>
<tr>
<td>Rep protein</td>
<td>45/66</td>
</tr>
<tr>
<td>CopG protein</td>
<td>30/32</td>
</tr>
<tr>
<td>sso DNA</td>
<td>60</td>
</tr>
<tr>
<td>dso DNA</td>
<td>55</td>
</tr>
</tbody>
</table>

Pairs with significantly higher similarity are in boldface.

**Figure 1.**— Genetic maps of the plasmid pDL10 from the thermoacidophilic archaean A. ambivalens and of the S. islandicus plasmids pRN1 and pRN2. The putative protein-encoding genes are indicated by shaded arrows, and the numbers indicate their length in amino acid residues: rep, replication initiator protein; copG, copy number control protein; plrA, plasmid regulatory protein; P, palindromes; ?D, possible stem-loop structures. pDL10 was ligated with the unique HindIII site into pBluescript IIKS+ vector. pRN1 and pRN2 (Figures 1 and 2). Similarity was also found to an ORF from the genome sequence of Mycobacterium tuberculosis (Figure 2). We concluded that this ORF encoded the initiator protein of plasmid replication (Rep protein). Immediately preceding the rep gene, an ORF encoding a 72-aa protein was found, whose deduced amino acid sequence shared 23–30% identical residues with copy number control proteins from RC plasmids (CopG; Table 1 and Figures 1 and 2). Similar copG genes had been recognized in pRN1 and pRN2 (Figures 1 and 2; Keeling et al. 1996, 1998). Another ORF encoded a 71-aa basic protein (calculated isoelectric point of 10.6), which showed a high degree of similarity to proteins from the plasmids pRN1, pRN2, and pNOB8 (ORF90b; 49–80% identical residues; Table 2, Figures 1 and 2; Keeling et al. 1996, 1998; She et al. 1998). The basic character of the five proteins implied a DNA-binding function, and their conservation suggested an essential role in plasmid maintenance, regulation of gene expression, or copy number control. The gene was termed plrA, encoding a putative plasmid regulatory protein (Figures 1 and 2). Interestingly, a homolog was found in the S. solfataricus genome (accession no. Y18868; Table 2, Figures 1 and 2). The deduced amino acid sequence of the chromosomal plrA gene shared 38–44% identical residues with the plasmid homologs. The gene was located on the chromosome adjacent to a second gene (plrB) encoding another short and basic protein with no similarity to known proteins. The two ORFs overlapped by 4 bp. There was a consensus promoter motif upstream of plrA, suggesting that plrAB forms an operon. The genes flanking plrAB did not have any recognizable similarity to known genes (data not shown). The function of plrA from 0.5 g (wet weight) of anaerobically grown cells by the alkaline lysis method and purification over QIAGEN Tip 20 columns. The plasmid was ligated with its unique HindIII restriction site into the pBluescript IIKS+ vector (pDL10-H6). Its complete nucleotide sequence was determined on both strands. With PCR using primers flanking the HindIII site, the copG gene was amplified (see Figure 1 and below). With subsequent cloning and sequencing of the product, it was confirmed that the HindIII site present in pDL10 was unique.

The plasmid consisted of 7598 bp of DNA with a G+C content of 37%. It contained 10 ORFs between 60 and 909 amino acid residues in size spanning 75.3% of the total plasmid (Figure 1). The largest ORF, designated rep, encoded a protein similar in size and sequence to proteins encoded by the S. islandicus plasmids pRN1 and pRN2 (45–72% identical residues; Table 1, Figures 1 and 2; Keeling et al. 1996, 1998). This conserved hypothetical protein contained a nucleotide-binding domain (P loop; for review, see Saraste et al. 1990). It shared 21–26% identical residues with replication initiator proteins of bacterial RC-replicating phages and plasmids, including the satellite phage P4 from E. coli, the temperate phage Sfi21 from Streptococcus thermophilus, the Lactococcus delbrueckii plasmid pWS58, and the actinophage Phi C-31 (see below and Figure 1). Similarity was also found to an ORF from the genome sequence of the thermoacidophilic archaean A. ambivalens (accession no. Y18868; Table 2, Figures 1 and 2; Keeling et al. 1996, 1998). This conserved hypothetical protein contained a nucleotide-binding domain (P loop; for review, see Saraste et al. 1990). It shared 21–26% identical residues with replication initiator proteins of bacterial RC-replicating phages and plasmids, including the satellite phage P4 from E. coli, the temperate phage Sfi21 from Streptococcus thermophilus, the Lactococcus delbrueckii plasmid pWS58, and the actinophage Phi C-31 (see below and Figure 1).
Figure 2.—Multiple amino acid alignment of the conserved parts of the Rep (A), CopG (B), and PlrA proteins (C) highlighted with BOXSHADE (http://ulrec3.unil.ch/software/BOX_form.html/). Black shading was assigned when at least half of the sequences share an amino acid. (A) Partial alignment of the Rep proteins: S.® 21, Streptococcus thermophilus temperate phage S.® 21 (total length 382 aa; accession no. AF004379); P4, bacteriophage P4 primase (777 aa; X51522); PhC31, P9 from the actinophage Phi-C31 (AJ006589); Mtu, Mycobacterium tuberculosis hypothetical genomic protein MTCY336.22 (471 aa; Z95586). The motifs 1 and 3 denote the conserved Rep protein motifs identified by Ilyina and Koonin (1992); motif 2 ("HUHUUU") was not found in the sequences shown. (B) CopG protein alignment: pLS1, S. agalactiae plasmid pLS1 (M29725); pWV01, Lactococcus lactis plasmid pWV01 (L08862). A large number of similar CopG amino acid sequences from plasmids of gram-positive bacteria were identified in database searches but have been excluded from the alignment. (C) Alignment of the conserved putative regulatory proteins PlrA from pDL10, pRN1, pRN2, pNOB8, and S. solfataricus. The length of the amino acid sequences is not known. At present, we can only speculate that it might encode a regulatory protein.

Comparison of pDL10 with the S. islandicus plasmids pRN1 and pRN2: Four regions with significant sequence similarity were identified in the two pRN plasmids and pDL10: the rep gene, the plrA gene, an ~280-nt noncoding DNA region containing the putative single-strand origin (sso), and another 520–660-nt noncoding sequence containing long palindromes, the putative double-strand origin (dsso; see below and Table 1, Figures 1 and 3). Outside of these regions the nucleotide sequence similarity was <44%, which is below the level of randomness in A+T-rich sequences. The Rep proteins of the three plasmids shared 45–72% identical pairwise comparison of the amino acid sequences of the putative PlrA proteins

<table>
<thead>
<tr>
<th>PlrA source</th>
<th>pDL10</th>
<th>pRN1</th>
<th>pRN2</th>
<th>pNOB8</th>
<th>S. solfataricus</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDL10 (73 aa)</td>
<td>—</td>
<td>58</td>
<td>58</td>
<td>49</td>
<td>44</td>
</tr>
<tr>
<td>pRN1 (80 aa)</td>
<td>74</td>
<td>—</td>
<td>80</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>pRN2 (81 aa)</td>
<td>73</td>
<td>90</td>
<td>—</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>pNOB8 (80 aa)</td>
<td>56</td>
<td>57</td>
<td>52</td>
<td>—</td>
<td>38</td>
</tr>
<tr>
<td>S. solfataricus, c (76 aa)</td>
<td>59</td>
<td>47</td>
<td>49</td>
<td>47</td>
<td>—</td>
</tr>
</tbody>
</table>

Upper right triangle, percentage identity; lower left triangle, percentage similarity; c, chromosomal PlrA protein. The lengths of the amino acid sequences are given in parentheses. Pairs with significantly higher similarity are in boldface.
aa residues, the PlrA proteins 58–80% (Table 2). The similarity between the Rep proteins of pDL10 and pRN2 was 65–67%, Table 1). A different result was obtained for the PlrA protein, which was significantly more related between pRN1 and pRN2 (90% similarity vs. 73–74%; Table 2).

**Rep proteins and RC replication:** The Rep proteins of pDL10, pRN1, and pRN2 and p4, Sfi21, pWS58, Phi-C31, and M. tuberculosis vary considerably in size (382–979 aa). However, they share a conserved region of ~300 aa residues flanking an A/GTP-binding motif (P loop; Figure 2; Saraste et al. 1990). Although the overall degree of conservation is low, 82 out of 295 positions are conserved in the alignment (Figure 2). No sequence similarity was found to RepB or RepC proteins from other RC-replicating plasmids from bacteria and from the hyperthermophilic archaeon P. abyssi.

All Rep proteins from RC replication plasmids and phages fall into at least five different families, and almost all of them have three sequence motifs in common: motif 1 (UKxUTG; U represents an aa residue with a bulky and hydrophobic side chain), motif 2, the so-called “HUHUUU motif,” and motif 3, which contains one or two active site tyrosine residues (see Figure 2; Ilyina and Koonin 1992; Khan 1997). The most prominent motif 2 can be found in a vast number of bacterial and archaeal Rep proteins, including the Rep proteins from the small halobacterial plasmid pGRD and relatives (Akhmanova et al. 1993), the P. abyssi plasmid pGT5 (Ernuso et al. 1996), and the very small cryptic plasmid pRQ7 from the extremely thermophilic bacterium Thermotoga sp. RQ7 (Yu and Noll 1997). However, the HUHUUU motif was not found in the sequences of the putative Rep proteins from pDL10, pRN1, and pRN2 or from p4, Sfi21, pWS58, and Phi-C31. In contrast, motifs 1 and 3 were identified in all of these sequences (Figure 2). We concluded that the Rep proteins from these plasmids and phages are phylogenetically related and belong to a hitherto unrecognized class of Rep proteins.

Whereas the putative Rep proteins of pDL10, pRN1, and pRN2 are more closely related to those of RC bacteriophages, all three archaeal plasmids share several features known from bacterial RC plasmids. The replication cycle of RC plasmids is initiated by the Rep protein, which introduces a nick at the double-strand origin (dso), generating a 3'-OH terminus (Helinski et al. 1996; Khan 1997; Marsin and Forterre 1998). Melting of the DNA is facilitated by a region of high A+T content adjacent to an inverted repeat, capable of forming a stable cruciform, which enables the active site tyrosine residue of the Rep protein to attack the nicking target (for review, see Helinski et al. 1996; Khan 1997; Rasooly and Rasooly 1997). The plasmid pDL10 contains two perfect 26-mer palindromes in one of the noncoding regions flanking an imperfect palindrome (Figure 1). All three palindromes are able to form a stem-loop or a cruciform required by RC plasmids for the initiation of the leading-strand synthesis. Similar palindromes were found in pRN1 and pRN2 (48–60% identity; Figure 1). We concluded that this DNA region could be the DSO site of the three plasmids, in structural similarity to RC plasmids.

In addition, a putative sso adjacent to the stop codon of the rep gene of pDL10, pRN1, and pRN2 was identified (Table 1 and Figures 1–3). The sso consists of a structurally conserved 150-bp inverted repeat flanked by two regions with high sequence similarity (61–69% sequence identity; Figure 3). The inverted repeats in each of the three plasmids have little sequence similarity. However, all of them are capable of forming an extended stem-loop structure in structural similarity to the single-strand origin SSO of bacterial RC plasmids (SSO, Figure 3; Khan 1997; Kramer et al. 1997, 1998). We concluded that this region contains the SSO of the three plasmids, where host-specific RNA polymerases initiate the replication of the lagging strand by synthesizing a short RNA primer from a specific palindromic site.

The copy number of bacterial RC plasmids is controlled by the cooperation of a copy number control protein (CopG) and other regulatory factors, such as single-stranded DNA (for review, see Rasooly 1997). The copG gene is usually located immediately upstream of the rep gene. The three plasmids pDL10, pRN1, and pRN2 contain a homologous gene in a similar position. However, the degree of conservation is low (Table 1 and Figure 2).

When comparing the gene organization of pDL10, pRN1, and pRN2, conserved and variable regions can be distinguished. One of the conserved regions consisted of the copG and rep genes and the sso region immediately following the rep genes. The second region consisted of the 520–660-bp dso boxes containing two to three long palindromes followed by the plrA genes (Figure 1). The order of the two regions has been reversed in pDL10 compared to pRN1 and pRN2. We concluded that these two conserved regions should contain all the necessary information for plasmid replication and copy number control.

**Detection of single-stranded plasmid DNA:** Denaturing and nondenaturing Southern blotting and hybridization experiments were performed with strand-specific RNA probes to detect single-stranded DNA in preparations of total genomic DNA. However, no hybridization signal was observed in nondenaturing Southern analysis with either of the probes. The control hybridizations done after denaturation of the DNA gave strong specific signals corresponding to the pDL10 plasmid or its restriction fragments (Figure 4A).

A different PCR-based approach was used involving a novel MBN or nuclease S1 protection assay, both to detect single-stranded plasmid DNA and to determine...
Figure 3.—(Top) MFOLD plot of the inverted repeats of the sso region from pDL10, pRN1, and pRN2 in comparison to the SSO site of the plasmid pLS1 from the gram-positive bacterium Streptococcus agalactiae. The shaded boxes in the pLS1 sequence highlight the −10 and −35 bacterial consensus promoter boxes, and the start sites of the RNA primer (pRNA) and the plasmid DNA are marked with arrows (modified and redrawn after Khan 1997; Kramer et al. 1997). (Bottom) Nucleotide sequence alignment of the conserved sso region downstream of the stop codons (boldface) of the rep genes of pDL10, pRN1, and pRN2. Double lines denote a conserved box of high A+T content (26 out of 30 in pDL10); the base-pairing nucleotides of the stem-loop structures shown above are underlined; vertical line at position 114 denotes the mirror line of the inversion in the repeats.

The leading strand. For the detection of single-stranded DNA by PCR, total genomic DNA was first digested with the restriction enzyme MspI to degrade double-stranded DNA (recognition site TTAA; 63 cleavage sites in pDL10). This was followed by MBN or S1 digestion to degrade single-stranded DNA. The PCR amplification of pDL10 DNA with various primer pairs gave positive results with MspI-digested DNA, but no product was seen with MspI plus MBN or MspI plus S1-digested DNA even after 42 cycles (Figure 4). Four different pDL10 PCR products were amplified with MspI-digested DNA as template. The longest fragment was 5735 bp in length, covering 75% of the total plasmid length and 56 out of 63 mixtures were then subjected to MBN digestion followed by PCR amplification with various primer pairs.

plasmid pE194 was treated in the same way. pE194 plasmid DNA was only amplified from undigested template DNA, showing that background amplification in enzyme-treated DNA can be effectively prevented (Figure 4E). These results indicated the presence of single-stranded plasmid DNA in A. ambivalens and support the conclusion that pDL10 replicates via a RC mechanism.

A similar scheme was applied in an attempt to identify the leading strand of plasmid replication. MspI-digested total genomic DNA was subjected separately to a single PCR cycle with four different primers (topG and SL10 primers; see materials and methods). The reaction mixtures were then subjected to MBN digestion followed by PCR amplification with various primer pairs. It was expected that only in the case of strand protection
would a PCR product be obtained. Surprisingly, both strands were protected against MBN digestion with this method (Figure 4, B and C). The results might indicate that either strand can serve as a template for leading strand synthesis.

**Transcription:** The ORFs of pDL10 are organized in five clusters that might represent transcriptional units (not shown). These were assigned when closely spaced or overlapping ORFs were found and when the putative operon unit had an archaeal consensus promoter motif upstream of the ATG translational start codon of the first ORF (box A; Hain et al. 1992). We tried to detect pDL10 transcripts in Northern blotting experiments with total RNA and strand-specific digoxygenin-labeled RNA probes synthesized from the T3 and T7 promoters of pDL10-H6 (performed after Röder and Pfeifer 1996). However, no transcripts were detected with RNA from different growth phases of aerobically and anaerobically grown A. ambivalens cultures.

**Plasmid stability and anaerobic growth:** In the course of this study, we observed that the plasmid levels in A. ambivalens cultures decreased significantly over a period of several years of continuous propagation in the laboratory. A low stability would impair its possible use as a cloning vector. As a consequence, a vector system based on pDL10 and possibly the pRN plasmids would require a strong selective pressure for plasmid maintenance. The ability of the strain to grow anaerobically or to switch between aerobic and anaerobic growth conditions was not affected by the decreased plasmid levels (data not shown). These were assigned when closely spaced or overlapping ORFs were found and when the putative operon unit had an archaeal consensus promoter motif upstream of the ATG translational start codon of the first ORF (box A; Hain et al. 1992). We tried to detect pDL10 transcripts in Northern blotting experiments with total RNA and strand-specific digoxygenin-labeled RNA probes synthesized from the T3 and T7 promoters of pDL10-H6 (performed after Röder and Pfeifer 1996). However, no transcripts were detected with RNA from different growth phases of aerobically and anaerobically grown A. ambivalens cultures.

**Plasmid stability and anaerobic growth:** In the course of this study, we observed that the plasmid levels in A. ambivalens cultures decreased significantly over a period of several years of continuous propagation in the laboratory. A low stability would impair its possible use as a cloning vector. As a consequence, a vector system based on pDL10 and possibly the pRN plasmids would require a strong selective pressure for plasmid maintenance.

The ability of the strain to grow anaerobically or to switch between aerobic and anaerobic growth conditions was not affected by the decreased plasmid levels (data not shown). It had been observed previously that the copy number of pDL10 increased when A. ambivalens was grown anaerobically and at relatively high pH (Zillig et al. 1985, 1986, 1994). It was postulated therefore that pDL10 might play an important role for the growth of the organism under anaerobic conditions. However, our observations speak against this hypothesis. In addition, we did not detect any plasmid-encoded genes with similarity to genes known to be involved in the energy metabolism or in regulatory mechanisms of archaea and bacteria. We concluded that pDL10 is not directly involved in anaerobic chemolithoautotrophic growth of A. ambivalens.

**Conclusions:** The sequence analysis of pDL10 revealed several conserved genes or DNA regions, rep, pira, cop6, dso, and sso, which were also found in pRN1 and pRN2. These observations and the presence of single-stranded DNA in A. ambivalens cells indicate that the three archaeal plasmids might replicate via a RC mechanism. The comparison of related plasmids from...
extremely thermophilic Archaea allowed us to distinguish between variable and possibly disposable regions and regions that are essential for plasmid replication and maintenance in the host cell. This knowledge is important for the construction of transformation vectors and can also serve as a basis for the development of an in vitro replication system.

We thank Felicitas Pfeifer, Darmstadt, for support and encouragement, Wolfram Zillig, Martinsried, for a long and fruitful time in his laboratory and for the plasmid source, and Christa Schleper for critically reading the manuscript. This is National Research Council publication number 42294.

LITERATURE CITED


Communicating editor: F. Pfeifer