Characterization of pURB500 from the Archaeon *Methanococcus maripaludis* and Construction of a Shuttle Vector

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The complete sequence of the 8,285-bp plasmid pURB500 from *Methanococcus maripaludis* C5 was determined. Sequence analysis identified 18 open reading frames as well as two regions of potential iterons and complex secondary structures. The shuttle vector, pDLT44, for *M. maripaludis* JJ was constructed from the entire pURB500 plasmid and pMEB.2, an *Escherichia coli* vector containing a methanococcal puromycin-resistance marker (P. Gernhardt, O. Possot, M. Foglino, L. Sibold, and A. Klein, Mol. Gen. Genet. 221:273–279, 1990). By using polyethylene glycol transformation, *M. maripaludis* JJ was transformed at a frequency of 3.3 × 10^7 transformants per μg of pDLT44. The shuttle vector was stable in *E. coli* under ampicillin selection but was maintained at a lower copy number than pMEB.2. Based on the inability of various restriction fragments of pURB500 to support maintenance in *M. maripaludis* JJ, multiple regions of pURB500 were required. pDLT44 did not replicate in *Methanococcus voltae*.

Methanogenic archaea produce methane as their main end product and are abundant in a variety of anaerobic habitats (for a review, see reference 56). In general, molecular and physiological studies of methanogens have been performed without the aid of the genetic tools commonly available for other microbial groups (42). The development of a puromycin-selectable marker for mesophilic methanococci (18) has led to the recent use of chromosomal integration vectors in this group (5, 6, 26). Additionally, only one methanogen species, *Methanococcus maripaludis*, has so far been transformed at frequencies approaching those routinely obtained for *Escherichia coli* (51).

Currently, there is little information on the replication of plasmids in methanogens. The complete sequences of a number of methanogen plasmids, including the related pairs of plasmids pME2001 (4.4 kb) and pME2200 (6.2 kb) from *Methanobacterium thermoautotrophicum* Marburg and ZH3 (7, 49) and pFV1 (13.5 kb) and pFZ1 (11.0 kb) from *Methanobacterium formicicum* THF and Z-245 (40), have been described. Also, two extrachromosomal elements in *Methanococcus januschii* (16.6 and 58.4 kb) were sequenced along with the chromosome (11). However, the minimal replication regions have not been defined for any methanogen plasmid. Origins of replication are better understood in the halophilic archaea. The origin of pH1 (143 kb; *Halobacterium salinarium* NRC817) was minimized to a 2.9-kb fragment containing one complete and one truncated open reading frame (ORF), separated by 350 bp of A+T-rich sequence (45). The origin region of pNRC101 (200 kb; *Halobacterium halobium* NRC-1) is 99.5% identical to that of pH1 (39). Only 52 bp of the A+T-rich region of pNRC101 is necessary for replication. The poor stability of this replicon in *H. halobium* further suggests that it lacks partitioning functions (39). The 3.4-kb minimal origin of pHK2 (10.5 kb; *Halofexa* strain Aa2.2) contains a large ORF and also requires a region with two smaller ORFs, four inverted repeats, and an A+T-rich region (23). With regard to replication forms, a rolling-circle mechanism has been identified in plasmids of the pGRB1 plasmid family of halobacteria (2) and in plasmids from *Pyrococcus* and *Sulfolobus* species (16, 30). Finally, while there has been progress in converting endogenous plasmids from other archaea into shuttle vectors (1, 14), currently there are no shuttle vectors for any methanogens.

We describe here the sequence of a methanococcal plasmid and the development of a replicating shuttle vector for *M. maripaludis* JJ and *E. coli*. This is the first report of a plasmid which replicates independently in a methanogen and which can also be manipulated in *E. coli*. The vector is derived from pURB500, a negatively supercoiled (12), low-copy-number, cryptic plasmid from *M. maripaludis* C5 (57, 59). For selection, the shuttle vector contains the puromycin resistance-conferring pac cassette (18). Besides providing a shuttle vector for the methanogens, these studies are a step toward using pURB500 as a model for plasmid replication in the archaea.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. Sources of strains are given in reference 31. Methanococci were grown on H_2-CO_2 (80:20, vol/vol) in complex medium (McC) at 37°C as previously described (57) except that the vitamin solution was omitted. Growth was measured by absorbance at 600 nm. Growth on solid medium (29, 50) and preparation of the puromycin solution (51) have been described elsewhere. Inhibitory concentrations of puromycin for *Methanococcus vannielii* and *Methanococcus aeolicus* were determined in 5-ml cultures inoculated with approximately 10^6 cells. After 8 days of incubation, no growth was observed in McC without puromycin allowed overnight growth of these cultures. Selection for puromycin-resistant cells was performed at 2.5 μg/ml for all methanococci except *Methanococcus voltae*, for which 5 μg/ml was used. *M. vannielii*, *M. aeolicus*, and *M. maripaludis* JJ and C5 exhibited no spontaneous puromycin resistance at 2.5 μg of puromycin per ml. Due to low plating efficiencies, selection for puromycin-resistant cells of strain C5 and *M. aeolicus* was performed in liquid medium. *E. coli* was grown in LB medium at half the NaCl concentration (35).

Transformation methods. Attempts were made to transform *Methanococcus* species by the polyethylene glycol (PEG) method (51). Transformations with *M. maripaludis* transformation buffer (TB) (51) used 0.025 μg of pDLT44 and 4.9 μg of Mpl plasmid. For *M. voltae* and *M. vannielii*, the NaCl concentration of the TB was decreased to 80 mM. Transformations with *M. voltae* anaerobic protoplasting buffer (APB) (43) used 1 μg of each plasmid, and the incubation time of cells with plasmid DNA and PEG was reduced to 15 min. PEG solutions were...
made in the corresponding transformation or anaerobic protoplasting buffer for each experiment. Since M. voltae displayed some spontaneous resistance even at 5 g of puromycin per ml, transformation frequencies were determined from the difference between cultures that had been transformed with and without vector DNA. E. coli was transformed by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, M. Maripaludis, Calif.). Plasmid DNA from E. coli (Stratagene, La Jolla, Calif.) was isolated from 200-ml stationary phase cultures by using a modified alkaline lysis procedure. Pelleted cells (4,000 g) of puromycin per ml, transformation frequencies were determined from the locations of the ORFs (arrows), ORFLES51 (I [see text for details]) and ORFLES52 (II). Fragments I to IV represent EcoRI-KpnI digestion products. Below, the restriction fragments of pURB500 in each plasmid constructed are diagrammed. Sequences that were adjacent to the puc cassette are marked with asterisks. Other interruptions in the pURB500 sequence were due to the puc vector. Except for pDLT44, pWLG25, and pDLT12, all pDLT vectors were constructed from pUC18. pDLT12 was constructed from pUC19.

![Restriction map of pURB500 and other plasmids containing restriction fragments cloned in pUC](Image)

**FIG. 1.** Restriction map of pURB500 and other plasmids containing restriction fragments cloned in pUC. pURB500 is represented as a linear fragment on which are shown the locations of the ORFs (arrows), ORFLES51 (I [see text for details]) and ORFLES52 (II). Fragments I to IV represent EcoRI-KpnI digestion products. Below, the restriction fragments of pURB500 in each plasmid constructed are diagrammed. Sequences that were adjacent to the puc cassette are marked with asterisks. Other interruptions in the pURB500 sequence were due to the puc vector. Except for pDLT44, pWLG25, and pDLT12, all pDLT vectors were constructed from pUC18. pDLT12 was constructed from pUC19.

### RESULTS

**Sequence analysis of pURB500.** The complete sequence of pURB500 is shown schematically in Fig. 1 and in detail in Fig. 2. The 8,285-bp plasmid had a G+C content of 27.24 mol%.

The overall nucleotide sequence of pURB500 was not related (<40% nucleotide identity) to the sequenced regions of the M. jannaschii extrachromosomal elements (11), pME2001 (7, 37), pME2200 (49), pI20 (40), pNRC100 (39), pH11 (45), pH2 (23), pH2V (Halofexus volcanii DS2 [12]), pGB1 (Halobacterium sp. GRB [19]), pHGNI (Halobacterium sp. GN101 [20]), pHSB1 (Halobacterium sp. SB3 [2]), pHSB2 (Halobacterium sp. SB3 [2]), pGT5 (Pyrococcus abyssi GE5 [16]), or pRN1 (Sulfobolus islandicus REN1H1 [30]). Therefore, pURB500 appeared to be unrelated to previously sequenced archaeal plasmids.

pURB500 contained numerous ORFs (Fig. 1). To judge the potential biological significance of the ORFs, the following criteria were used. ORFs greater than 249 bp were considered to be potentially significant. This criterion identified 14 ORFs. Four smaller ORFs were also judged to be potentially significant because their G+C contents were greater than 32 mol% (data not shown). This criterion was based on the observations that intergenic regions in methanococci are very A+T rich and ORFs are enriched in G+C content (reviewed in reference 42). Although some of the ORFs of pURB500 were small, the

| Strains or plasmid | Property | Reference or source |
|--------------------|----------|---------------------|
| Strains M. maripaludis CS | Natural host strain for pURB500 | 57 |
| M. maripaludis JJ | | 28 |
| M. voltae PS | | 54 |
| M. vannelli SB | | 48 |
| M. aeolicus PL-15/H | | 55 |
| E. coli XL1-Blue MRF | | Stratagene |

| Plasmids | | |
|-----------------|----------|---------------------|
| pURB500 | Cryptic plasmid from M. maripaludis CS | 59 |
| pMEB2 | E. coli PUC plasmid containing methanococcal puromycin resistance (pac) cassette | 18 |
| Mip1 | | 18 |
| pUC18, pUC19 | E. coli vectors (Amp') | 53 |

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**Nucleotide sequence accession number.** The nucleotide sequence of pURB500 has been deposited in GenBank under accession no. U47023.

**Restriction map of pURB500 and other plasmids containing restriction fragments cloned in pUC.** pURB500 is represented as a linear fragment on which are shown the locations of the ORFs (arrows), ORFLES51 (I [see text for details]) and ORFLES52 (II). Fragments I to IV represent EcoRI-KpnI digestion products. Below, the restriction fragments of pURB500 in each plasmid constructed are diagrammed. Sequences that were adjacent to the puc cassette are marked with asterisks. Other interruptions in the pURB500 sequence were due to the puc vector. Except for pDLT44, pWLG25, and pDLT12, all pDLT vectors were constructed from pUC18. pDLT12 was constructed from pUC19.

**RESULTS**

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association of small proteins with regulation of plasmid replication has been observed in some well-studied systems (34).

The start codons of the ORFs of pURB500 were either ATG, GTG, or TTG. The utilization of start codons other than ATG is not unusual in the methanogens (10). ORF1 in particular began with GTG. The next potential start codon, ATG, was located 609 bp downstream of the GTG codon, effectively reducing the size of ORF1 by 23%. The absence of stop codons (TAA, TAG, or TGA) in this 609 bp is significant given the low mole percent G+C content of ORF1. Except for ORF13, all of the ORFs also contained potential downstream start codons. The sequence was examined for features associated with expression. Potential ribosome binding sites (RBSs) homologous to the 3' end of the archaeal 16S rRNA (10) were found near the start codons for all of the ORFs except ORF5, -10, -11, and -12 (Fig. 2). Sequences corresponding to box A of the transcriptional promoter of methanogens, TTTAWA (42, 62), were also upstream of all of the ORFs (Fig. 2). However, due to the low mole percent G+C content of pURB500, these sequences were so common throughout the plasmid that they were not considered diagnostic of transcribed regions. Some of the ORFs appeared to form three polycistronic operons (Fig. 2). ORF1 and ORF8 were separated by only 11 bp and may also be associated with ORF5. ORF4, -14, -10 and -2 were closely spaced or overlapped. Also, ORF6, -15, and -16 overlapped. Stem-loop structures resembling transcription terminators were also found downstream of these potential polycistronic operons (Fig. 2). Potential stem-loop structures were also associated with the 5' or 3' ends of other ORFs, which suggested the presence of additional transcriptional terminators or other regulatory sequences (Fig. 2). Such stem-loops were at the 3' ends of ORF5, -10, -14, and -18, although these stem-loops also partially overlapped the ORFs. Stem-loops were also present near the 5' ends of ORF1, -2, -4, -5, -10, -17, and -18.

Proteins homologous to the pURB500 ORFs were not identified in TFASTA and BLAST searches of the GenBank and EMBL databases (<25% amino acid sequence identity). Also, a search against the potential coding regions of the M. jannaschii genome (11) revealed homologous sequences only between ORF2 and M. jannaschii ORF MJ0367 (39% identity). MJ0367 has 30% identity with an integrase identified in the bacterium Weeksella zoohelcum (9). This integrase is homologous to a diverse family of integrases, resolvases, and recombinases. The sequence identity between ORF2 and the archaeal plasmids listed above (Weeksella pac, Naschii, 32% amino acid sequence identity). A search of the ORFs against the PROSITE database of proteins homologous to a diverse family of integrases, resolvases, and recombinases was not obvious that pURB500 would support replication in strain JI. To test this point, the pac cassette containing the puromycin marker was introduced into pURB500. This goal was achieved by transforming strain C5 with a series of suicide vectors constructed from E. coli pUC vectors. These vectors contained the pac cassette (18) plus each of the four fragments of pURB500 formed by a complete EcoRI-KpnI digestion.
FIG. 2—Continued.
Puromycin-resistant transformants were expected to result from homologous recombination between the suicide vectors and the endogenous pURB500 plasmid. Two of these suicide vectors, pDLT11 and pDLT12, yielded puromycin-resistant transformants in strain C5, and the formation of cointegrate plasmids was verified by restriction digests of plasmid preparations from the transformants (data not shown). The cointegrate plasmids also transformed strain JJ to puromycin resistance, and their presence was again verified by restriction mapping. Therefore, pURB500 was able to support plasmid replication in strain JJ.

However, when the cointegrate plasmids were electroporated into *E. coli*, only small plasmids containing the pUC vector, *pac*, and the original cloned fragments of pURB500 were obtained, apparently as products of homologous recombination within the cointegrate plasmids. Presumably, these small plasmids were also produced in *M. maripaludis* because faint bands corresponding to these recombination products could be detected in restriction digests of plasmid preparations from *M. maripaludis* (data not shown). These small plasmids no longer transformed *M. maripaludis*. Therefore, the cointegrate plasmids were not suitable shuttle vectors due to their limited stability.

To construct a shuttle vector, pURB500 and pMEB.2 were partially digested with EcoRI. After ligation, transformation into *M. maripaludis* JJ, and selection for puromycin resistance, pDLT44 was isolated (Fig. 5). This vector contained pMEB.2 and the entire pURB500 plasmid interrupted next to nucleotide position 1, apparently disrupting ORF4 and ORF12. pDLT44 purified from strain JJ transformed *E. coli* at high frequencies (4 × 10^8 transformants/mg of DNA), using electroporation. This frequency was comparable to that obtained with pDLT44 purified from *E. coli* (data not shown). Using the PEG method (51), pDLT44 purified from *E. coli* transformed strain JJ at a frequency of 3.3 × 10^7 transformants/mg (average of three experiments). This transformation frequency was at least 10-fold higher than that obtained with the integration vector pKAS102 (46, 51). This difference may reflect the absence of a requirement for integration of pDLT44. The pDLT44 shuttle vector also contains *Sac*I and *Xba*I sites in nonessential regions derived from pMEB.2 (Fig. 5).

Transformation of *M. maripaludis* JJ with the ligation mixture yielded another vector, pDLT42, which also contained the entire pURB500 sequence and the *pac* cassette but which lacked the *E. coli* pUC vector sequences from pMEB.2. The pURB500 sequence in pDLT42 was also interrupted at the same EcoRI site as in pDLT44, suggesting that the other two EcoRI sites of pURB500 could not be interrupted without affecting replication in *M. maripaludis*.

Regions of pDLT44 essential for replication. To identify the origin of replication or other essential regions of pURB500, the ability of various cloned restriction fragments to transform *M. maripaludis* JJ was tested. As judged by Southern hybridization, pURB500 does not contain regions of large sequence identity with strain JJ genomic DNA (59). Thus, transformants would be expected to contain a self-replicating plasmid. Because replication origins of many small plasmids are limited to a region of 2 kb or less, a small region sufficient for replication could be identified in this manner.

Remarkably, a relatively large region of pURB500 appeared to be required for replication since none of the other plasmid constructions tested transformed *M. maripaludis* JJ. Of all of

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**FIG. 3.** ORFLESS1 and ORFLESS2, two regions of pURB500 which lacked ORFs and contained potential stem-loop structures (arrows) and direct repeats (boxes). Opposing arrows that can pair together to form potential stem-loops are connected by a dashed line. Shown are potential stem-loops with at least 21 bonds (ORFLESS1) or at least 18 bonds (ORFLESS2). Direct repeats at least 13 bp in length are grouped (I to VI) by related sequences (Fig. 4).

**FIG. 4.** Direct repeat groups of the ORFLESS1 and ORFLESS2 regions. Shown are exact repeats 13 bp or longer. Two 12-bp repeats were also included in the groups. Repeats were grouped (I to VI) if they shared at least 11 bp of identical sequence. Mismatches within each group are shown in lowercase.
the plasmids listed in Fig. 1 (except pDLT16 and pDLT45, which were not tested), only pDLT44 transformed *M. maripaludis*. Because pDLT9-12 did not transform, either multiple regions were essential for replication or a single essential region was interrupted at one of the restriction sites. These sites were not interrupted in pWLG25, which also failed to transform. Therefore, multiple regions of pURB500 were required for replication. This conclusion was also supported by the inability of pDLT28 to transform. pDLT28 contained the complete pURB500 but was interrupted at the unique KpnI site and the EcoRI site at position 1189. Because it failed to transform, either or both of these sites must be in regions essential for replication. However, both sites were intact in pWLG25, which failed to transform. Therefore, some region to the right side of the NdeI site was also necessary. This region contained ORF2, -7, -10, -11, -14, and -17, possibly the promoter of ORF3, and most of ORFLESS2. Because pDLT43 failed to transform, some sequence in the first 2.7 kb of pURB500 was also required. This plasmid was missing ORF1, -4, -5, -9, -12, and -18. Also, different combinations of both the upstream and downstream regions of pURB500 were not sufficient (pDLT27, -21, -17, -25, and -19), although these vectors were also interrupted at the KpnI site. In summary, these plasmids indicated that small sequences of pURB500 did not support replication.

**Stability of *E. coli* plasmids containing pURB500 sequences.** Sequences with a high mole percent A+T content frequently cause cloning problems or copy number reductions in high-copy-number vectors of *E. coli* (15). It has been proposed that A+T-rich sequences are recognized by *E. coli* as transcriptional signals and that binding of RNA polymerase interferes with the replication machinery. Alternatively, expression of particular sequences of pURB500 may have generated products which are either toxic in *E. coli* or inhibitory to plasmid replication. Plasmid minipreps from *E. coli* of pDLT44 gave approximately 50-fold-lower yields in comparison to other pUC derived-plasmids containing the pac cassette and fragments of pURB500 (data not shown). After repeated subculturing in *E. coli*, sequence rearrangements or deletions of pDLT44 were not observed by restriction mapping (data not shown), and pDLT44 still successfully transformed *M. maripaludis* JJ.

pDLT44-containing *E. coli* grew poorly in the presence of ampicillin. When the fraction of pDLT44-containing cells in a culture was monitored by following plasmid-encoded ampicillin resistance, cultures were rapidly overgrown by clones that lacked the plasmid (data not shown). Only 10% of the cells resuspended from a single colony were ampicillin resistant when directly replated. This fraction increased to 30% during the initial 10 h of growth in the presence of ampicillin but then dropped to a few percent after 20 h. In contrast, the relative frequency of cells containing the parent pMEB.2 plasmid was 80% and did not change under the same conditions (data not shown). The decrease in the proportion of pDLT44-containing cells appeared to be largely due to growth of ampicillin-sensi-

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**FIG. 5. Construction of the replicating shuttle vector pDLT44.** pURB500 and pMEB.2 were partially digested with EcoRI, ligated, and transformed into *M. maripaludis* JJ. pDLT44 was isolated from a single puromycin-resistant colony. The locations of the ORFs on pURB500 are indicated by their respective numbers. In boldface are the EcoRI sites of pURB500 and pMEB.2 that were interrupted in constructing pDLT44 and the SacI and XbaI sites suitable for cloning.
I site was intact (pDLT43 versus pDLT45). However, when and pDLT12 versus pDLT17. Evidence for the importance of (pDLT28 versus pDLT16). Although they had no effect on Eco restriction maps for some of the plasmids are shown in Fig. 1. pDLT3, -2, and -7 contained the same restriction fragments of pURB500 as pDLT9, -10, and -11, respectively, but did not contain the pac cassette.

Subclones of pURB500 were investigated to determine if particular regions of pURB500 were responsible for the instability. In contrast to pDLT44, plasmids pUC18, pMEB.2, and pUC18, -2, -3, and -7 were maintained in cultures without measurable loss (Table 2). Thus, the pac cassette and small fragments of pURB500 were stable. Plasmids containing larger portions of pURB500 were lost, although more slowly than pDLT44. For example, fragment IV (Fig. 1) in pDLT12 caused some instability. The addition of upstream regions to pDLT12 increased the instability (pDLT43, -27, -21, -45, -17, -25, -19, -28, and -16). However, the stability was not strictly related to the size. For instance, pDLT28 and pDLT16 contained the entire pURB500 sequence but were more stable than pDLT44 (Table 2). In addition, the stabilities of pDLT43, -45, -28, and -16, were similar, but their sizes differed greatly. Instead, the addition of upstream regions to pDLT12 resulted from contributions of multiple regions of pURB500.

Host range of pDLT44 in the methanococci. Because no replicating shuttle vectors are currently available for any methanogen, the possibility of transforming other species with pDLT44 was of interest. Therefore, three additional species of methanococci were tested as possible hosts. For prokaryotes, disruption of the cell wall is often necessary for successful transformation (24). Similarly, protoplast or spheroplast formation appears to be required for high transformation frequencies in the methanococci (44, 51). Unlike M. maripaludis, M. voltae did not form spheroplasts when resuspended in the TB developed for M. maripaludis (data not shown). Although M. voltae formed spheroplasts in TB when the NaCl concentration was lowered from 0.38 M to 0.08 M, transformation with pDLT44 by the PEG method (51) was not detected (<5 transformants/μg; <10⁻⁷ transformants/CFU). With this method, the Mip1 integration vector (18) transformed M. voltae (94 transformants/μg; 8.8 × 10⁻⁴ transformants/CFU). Therefore, pDLT44 did not appear to replicate in M. voltae. An APB developed for M. voltae (43) was also tested. Using the APB with the PEG method failed to yield transformants with either pDLT44 or Mip1 (<40 transformants/μg; <10⁻⁷ transformants/CFU). For M. vannielii, both TB with 0.08 M NaCl and APB caused about 5% of the cells to form spheroplasts. PEG transformation with pDLT44 yielded no puromycin-resistant colonies using either TB with 0.08 M NaCl (<5 transformants/μg; <1.1 × 10⁻⁸ transformants/CFU) or APB (<20 transformants/μg; <4.0 × 10⁻⁷ transformants/CFU). Finally, M. aeolicus formed spheroplasts in APB. However, in contrast to M. voltae and M. vannielii, cells of M. aeolicus recovered poorly following PEG transformation, and no puromycin-resistant colonies were obtained (<2 transformants/μg; <10⁻⁴ transformants/CFU). However, due to the absence of positive control DNA, it cannot be concluded that pURB500 will not replicate in either M. vannielii or M. aeolicus. Also, to our knowledge, the use of the pac cassette in these two species has not been previously tested. In any case, pDLT44 could not be transformed into these other methanococcal species in these initial experiments.

**DISCUSSION**

In bacteria, several features of plasmids and chromosomes are important for replication. Many replication origins contain multiple direct repeats, or iterons, which serve as binding sites for replication initiation proteins. Bramhill and Kornberg (8) have proposed that these direct repeats constitute regions where the DNA strands unwind prior to the initiation of replication. Also, in plasmids such as CoIE1, RNA transcripts of the origin possess complex secondary structures and are involved in replication control (34). By analogy, either one or both of the ORFLESS regions may be the origin of replication since they encode multiple direct repeats and potential stem-loops. This interpretation is supported by the observation that the ORFLESS1 region at the KpnI site yielded vectors which failed to replicate in M. maripaludis. However, an intact ORFLESS1 region was not in itself sufficient for replication because plasmids containing deletions elsewhere also failed to replicate.

Many bacterial plasmids also contain rep genes, which encode proteins required for replication (41). Three pieces of evidence suggest that the large ORF1 of pURB500 may encode an archaeal Rep protein. (i) ORF1 contained an ATP/GTP binding site motif, which is common in replication pro-

**TABLE 2. Stability of pURB500 subclones in E. coli**

| Plasmid | Proportion of ampicillin-resistant colonies |
|---------|--------------------------------------------|
| pDLT44  | <7.4 × 10⁻⁸                             |
| pDLT3   | 1.2                                      |
| pDLT2   | 9.4 × 10⁻¹                             |
| pDLT7   | 1.0                                      |
| pDLT12  | 8.2 × 10⁻²                             |
| pDLT43  | 1.8 × 10⁻⁵                             |
| pDLT27  | 6.3 × 10⁻⁵                             |
| pDLT21  | 3.6 × 10⁻⁷                             |
| pDLT45  | 5.2 × 10⁻⁷                             |
| pDLT17  | 1.5 × 10⁻⁴                             |
| pDLT25  | 2.1 × 10⁻¹                             |
| pDLT19  | 2.8 × 10⁻⁷                             |
| pDLT28  | 1.1 × 10⁻⁶                             |
| pDLT26  | 1.2 × 10⁻⁶                             |
| pMEB.2  | 1.4                                      |
| pUC18   | 1.1                                      |
| None    | <1.5 × 10⁻⁷                             |

* Restriction maps for some of the plasmids are shown in Fig. 1. pDLT3, -2, and -7 contained the same restriction fragments of pURB500 as pDLT9, -10, and -11, respectively, but did not contain the pac cassette.

**Other notes**

- Single-colony isolates of the plasmids in E. coli were inoculated into LB broth with 50 μg of ampicillin per ml and grown overnight. The culture was then diluted 1/10,000 into LB broth without ampicillin and grown overnight. This culture was diluted 1/10,000 again into LB broth, grown overnight, and plated to determine the proportion of ampicillin-resistant colonies. These data were obtained in two separate experiments in which the values obtained for the control plasmids (pDLT44, pDLT12, pMEB.2, and pUC18) varied fourfold or less.

- The stability of pURB500 subclones was determined by inoculating E. coli cultures with pDLT44 by the PEG method (51) was not detected (<5 transformants/μg; <10⁻⁷ transformants/CFU). With this method, the Mip1 integration vector (18) transformed M. voltae (94 transformants/μg; 8.8 × 10⁻⁴ transformants/CFU). Therefore, pDLT44 did not appear to replicate in M. voltae. An APB developed for M. voltae (43) was also tested. Using the APB with the PEG method failed to yield transformants with either pDLT44 or Mip1 (<40 transformants/μg; <10⁻⁷ transformants/CFU). For M. vannielii, both TB with 0.08 M NaCl and APB caused about 5% of the cells to form spheroplasts. PEG transformation with pDLT44 yielded no puromycin-resistant colonies using either TB with 0.08 M NaCl (<5 transformants/μg; <1.1 × 10⁻⁸ transformants/CFU) or APB (<20 transformants/μg; <4.0 × 10⁻⁷ transformants/CFU). Finally, M. aeolicus formed spheroplasts in APB. However, in contrast to M. voltae and M. vannielii, cells of M. aeolicus recovered poorly following PEG transformation, and no puromycin-resistant colonies were obtained (<2 transformants/μg; <10⁻⁴ transformants/CFU). However, due to the absence of positive control DNA, it cannot be concluded that pURB500 will not replicate in either M. vannielii or M. aeolicus. Also, to our knowledge, the use of the pac cassette in these two species has not been previously tested. In any case, pDLT44 could not be transformed into these other methanococcal species in these initial experiments.

**DISCUSSION**

In bacteria, several features of plasmids and chromosomes are important for replication. Many replication origins contain multiple direct repeats, or iterons, which serve as binding sites for replication initiation proteins. Bramhill and Kornberg (8) have proposed that these direct repeats constitute regions where the DNA strands unwind prior to the initiation of replication. Also, in plasmids such as CoIE1, RNA transcripts of the origin possess complex secondary structures and are involved in replication control (34). By analogy, either one or both of the ORFLESS regions may be the origin of replication since they encode multiple direct repeats and potential stem-loops. This interpretation is supported by the observation that the ORFLESS1 region at the KpnI site yielded vectors which failed to replicate in M. maripaludis. However, an intact ORFLESS1 region was not in itself sufficient for replication because plasmids containing deletions elsewhere also failed to replicate.

Many bacterial plasmids also contain rep genes, which encode proteins required for replication (41). Three pieces of evidence suggest that the large ORF1 of pURB500 may encode an archaeal Rep protein. (i) ORF1 contained an ATP/GTP binding site motif, which is common in replication pro-
teins of bacterial and viral genomes (32). In the archaea, this motif has also been identified in ORFs in plasmids pFY1 and pFZ1 of Methanobacterium thermoformicicum (40) and pRN1 of S. islandicus (30) and in the SSV virus of Sulfolobus shibatae (32). The absence of significant sequence similarity between ORF1 and these replications is not unexpected because Rep proteins are generally not highly conserved. (ii) pDLT43, which did not contain ORF1 as well as some other ORFs, did not replicate. (iii) pDLT42 and pDLT44, which were generated by partial EcoRI digestion of pURB500, were interrupted in the only EcoRI site that did not disrupt ORF1. However, ORF1 does not have the typical orientation of rep genes with respect to the suspected origin(s), the ORF LESS regions. In plasmids such as pSC101 and the IncO and IncW plasmid families of the gram-negative bacteria (34, 52) and in the archaeal replicons pNRC100 (39), pHH1 (45), and pGTS (16), the rep gene is transcribed away from the origin. In contrast, ORF1 is transcribed toward its suspected origin.

Conserved sequence motifs have been identified in the replication proteins of rolling-circle replicating plasmids (25). However, these motifs were not present in the 18 potential ORFs of pURB500. In the archaea, rolling-circle motifs have been identified in the small, multicopy archaeal plasmids pRN1 (S. islandicus [30]), pGTS (Pyrococcus abyssi [16]), and pHK2 (Halofexas sp. Aa2.2 [23]) and the pGRB plasmid family (Halobacterium spp. [25]). Possible replication intermediates consisting of single-stranded copies of the plasmid have also been identified for pGTS (16) and pGRB-1 (47). In contrast, the possibility that pURB500 replicates by a theta mechanism is supported by the presence of dimers in electron micrographs of the plasmid (59).

Unfortunately for the further reconstruction of pDLT44, even the relatively small size reductions that were attempted led to vectors which did not replicate in M. maripaludis JJ. In contrast, studies of other archaeal plasmids have defined much smaller regions required for replication. It is not known if the large required regions of pURB500 contain only replication functions or whether they also encode kil functions analogous to those found in the bacterial plasmid RK2 (reviewed in reference 33). In these systems, kil genes become lethal when the kil override (kor) genes are absent. Also, the sequences which contributed to the instability in E. coli were distributed throughout the plasmid. The stability results may explain the inability to recover in E. coli the cointegrate plasmids from strain C5. In the presence of even low concentrations of the more stable recombination products, clones with the complete pURB500 would be greatly selected against.

Two lines of evidence suggest that pURB500 may have originated from a methanococcal related to M. voltae. First, the G+C content of strain C5 is 33.2 ± 0.0 mol% (57) and is significantly higher than that of pURB500 (27.24 mol%). The lower G+C content of pURB500 suggests that it was recently acquired by strain C5. Among the methanococci, strains related to the M. voltae group have the lowest G+C content, 29.2 to 31.5 mol% (31, 57). Second, Sau3AI restriction sites are rare in both the M. voltae genome (27) and pURB500. pURB500 completely lacks the GATC recognition sequence, although 20 sites are expected based on its mole percent G+C content. In contrast, the chromosomal DNA of M. maripaludis C5 is well digested by Sau3AI (59). Similarly, sequencing of M. maripaludis JJ chromosomal DNA fragments has revealed close to one Sau3AI site per 323 bp, which is the frequency expected based on its mole percent G+C content (17). Surprisingly, the shuttle vector pDLT44 did not replicate in M. voltae. It is not unusual for a plasmid to have a host range restricted to a single bacterial species or strain. Alternatively, some host-specific replication functions may have been interrupted in the shuttle vector.

While pDLT44 is therefore currently usable only in M. maripaludis JJ, there are several advantages to using this strain genetically. The recent genome sequencing of a close relative, M. jannaschii (11), allows the possibility of using related sequences to quickly obtain specific genes from M. maripaludis JJ. Also, an efficient transformation procedure has been optimized for strain JJ (51). This strain also lacks restriction activities which interfere significantly with transfer of plasmid DNA between M. maripaludis and E. coli, despite the presence of a Psml-like restriction activity (51). An additional genetic marker based on neomycin resistance has recently been developed for M. maripaludis (3). The growing genetic repertoire of this species combined with well-documented growth studies and cell-physiological studies (57, 58, 60, 61) sets the stage for future studies on this strictly anaerobic archaeon.

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