JOURNAL OF BACTERIOLOGY, May 1997, p. 2976–2986 Vol. 179, No. 9
0021-9193/97/$04.00+0

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

DEBRA L. TUMBULA, TIMOTHY L. BOWEN,† AND WILLIAM B. WHITMAN*

Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605

Received 25 November 1996/Accepted 26 February 1997

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 jannaschii* (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 pHH1 (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 pNRC100 (200 kb; *Halobacterium halobium* NRC-1) is 99.5% identical to that of pHH1 (39). Only 52 bp of the A+T-rich region of pNRC100 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; *Haloferax* 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 containing the lowest concentration of puromycin tested, 1.5 μg/ml. In comparison, 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 0.023 μg of pDLT44 and 4.9 μg of Mip1 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

* Corresponding author. Phone: (706) 542-4219. Fax: (706) 542-2674. E-mail: whitman@uga.cc.uga.edu.

† Present address: New England Biolabs, Inc., Beverly, MA 01915.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Property	Reference or source
Strains		
M. maripaludis C5	Natural host strain for pURB500	57
M. maripaludis JJ		28
M. voltae PS		54
M. vannielii SB		48
M. aeolicus PL-15/H		55
E. coli XL1-Blue MRF′		Stratagene
Plasmids		
pURB500	Cryptic plasmid from *M. maripaludis* C5	59
pMEB.2	*E. coli* pUC plasmid containing methanococcal puromycin resistance (*pac*) cassette	18
Mip1	*M. voltae* integration vector with *pac* cassette	18
pUC18, pUC19	*E. coli* vectors (Amp^r)	53

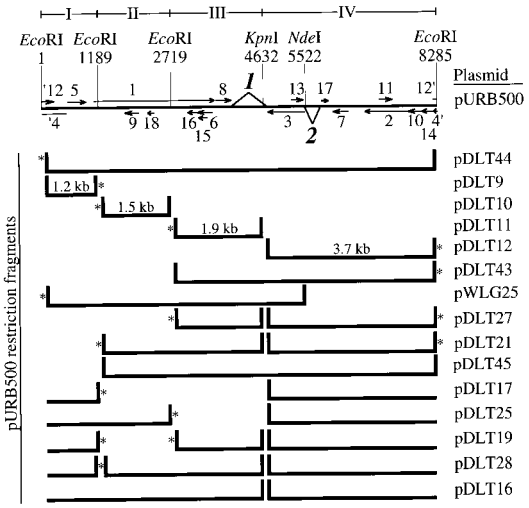


FIG. 1. Restriction map of pURB500 and other plasmids containing restriction fragments cloned in pUC. pURB500 is represented as a linear fragment on which are shown the locations of the ORFs (arrows), ORFLESS1 (*1* [see text for details]) and ORFLESS2 (*2*). Fragments I to IV represent *Eco*RI-*Kpn*I digestion products. Below, the restriction fragments of pURB500 in each plasmid constructed are diagrammed. Sequences that were adjacent to the *pac* 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.

made in the corresponding transformation or anaerobic protoplasting buffer for each experiment. Since *M. voltae* displayed some spontaneous resistance even at 5 μg 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, Melville, N.Y.) set at 2.46 kV and using a time constant of 4.6 ms.

Plasmid purification and construction. Plasmid DNA from *E. coli* was isolated by using Wizard Miniprep columns (Promega, Madison, Wis.) or Qiagen-tip 500 columns (Qiagen, Inc., Chatsworth, Calif.), or by alkaline lysis and CsCl gradient centrifugation (35). pMEB.2 and Mip1 were purified from *E. coli* AM6 (18). Other plasmids were purified from *E. coli* XL1-Blue MRF′ (Stratagene, La Jolla, Calif.). Plasmid DNA from *M. maripaludis* was isolated from 200-ml stationary phase cultures by using a modified alkaline lysis procedure. Pelleted cells (4,000 × *g*, 10 min, 4°C) were resuspended on ice in 5 ml of 50 mM Tris-chloride (pH 8.0)–10 mM EDTA–0.38 M NaCl–12% sucrose. An equal volume of 1% (wt/vol) sodium dodecyl sulfate in 0.2 M NaOH was added, and the suspension was incubated for 5 min on ice. The pH of the suspension was then neutralized with 5 ml of 3 M potassium acetate that had been brought to pH 4.8 with acetic acid. Following centrifugation (25,000 × *g*, 20 min, 4°C), plasmid DNA was precipitated from the supernatant with 0.6 volume of isopropanol and collected by centrifugation (14,000 × *g*, 15 min, 4°C). The pellet was resuspended in 1.5 ml of TE (10 mM Tris-chloride [pH 8.0], 1 mM disodium EDTA) with 10 μl of 10 mg of RNase A (DNase-free) per ml and incubated for 15 min at 37°C. Phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) extractions were followed by the addition of 1/3 volume of 7.5 M ammonium acetate, precipitation with 2 volumes of ethanol, a 70% ethanol wash, and resuspension in TE.

Restriction digests and ligations were performed according to the manufacturers' suggestions. Partial digests were performed for 10 to 30 min, substituting endonuclease diluted 1:10 in restriction buffer for full-strength endonuclease.

Three approaches were used to construct pUC vectors in *E. coli* that contained restriction fragments of pURB500 and the methanococcal puromycin resistance *pac* cassette (Fig. 1) (18). In the first method, pURB500 was digested with *Eco*RI and *Kpn*I, and the resulting restriction fragments (1.2, 1.5, 1.9, and 3.7 kb) were cloned individually into pUC18 or pUC19. The *pac* cassette was then introduced into each of these vectors at an *Eco*RI site. In the second method, the entire pURB500 plasmid was cloned into pUC18 at the *Kpn*I site, and a partial *Eco*RI digest of the resulting plasmid was ligated with the *pac* cassette. This yielded a collection of vectors containing various restriction fragments of pURB500. Each vector was interrupted at the *Kpn*I site of pURB500 by pUC18 and at an *Eco*RI site by the *pac* cassette. Finally, pWLG25 was constructed by removing the 2,974-bp *Nde*I fragment from pDLT44.

Sequencing and analysis. Approximately 30% of the total sequence of pURB500 was obtained by primer walking in the subclones of pURB500 in pUC18 and pUC19. The remainder was obtained from nested deletions of the subclones constructed with the Erase-a-Base system (Promega). The regions of pURB500 spanning the *Eco*RI or *Kpn*I sites were sequenced in subclones where these restriction sites were intact. Template DNA for sequencing was isolated with Wizard Miniprep columns (Promega) plus a further purification by ethanol precipitation with ammonium acetate. Sequencing of the double-stranded template was performed with a PRISM Dye Terminator Cycle Sequencing kit and a model 373A DNA Sequencer (Applied Biosystems Division of Perkin-Elmer, Foster City, Calif.).

Most of the analysis of the sequence data was performed with GCG software (Genetics Computer Group, Madison, Wis.). FRAMES was used to locate open reading frames, and REPEAT identified exact repeats. STEMLOOP with the default settings identified 994 potential stem-loop structures in pURB500, given a maximum loop size of 77 bp. The stem-loop results were sorted by number of bonds (G:C, A:T, and G:T bonds have three, two, and one, respectively). Protein motifs were identified with MOTIF, using the PROSITE database (4). GAP was used to align sequences and to determine homology values.

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

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), pFV1 (40), pFZ1 (40), pNRC100 (39), pHH1 (45), pHK2 (23), pHV2 (*Haloferax volcanii* DS2 [13]), pGRB1 (*Halobacterium* sp. GRB [19]), pHGN1 (*Halobacterium* sp. GN101 [20]), pHSB1 (*Halobacterium* sp. SB3 [2]), pHSB2 (*Halobacterium* sp. SB3 [2]), pGT5 (*Pyrococcus abyssi* GE5 [16]), or pRN1 (*Sulfolobus 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

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 *Weeksella* integrase was 24%. Even though the overall homology was low, it suggests a function of ORF2 in DNA binding or recombination. No homologies were identified between the 18 ORFs of pURB500 and the ORFs of the archaeal plasmids listed above (<32% amino acid sequence identity).

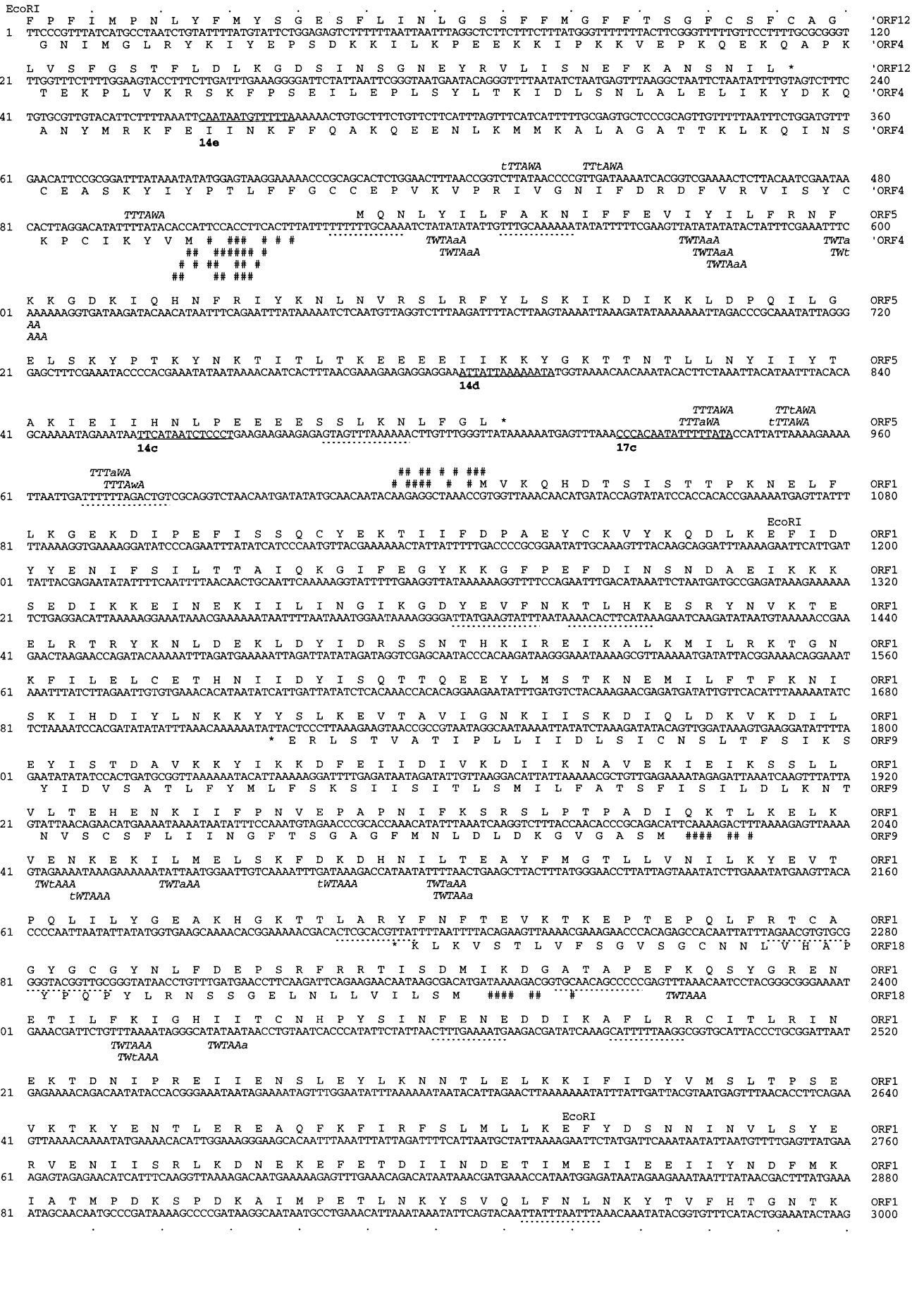
A search of the ORFs against the PROSITE database of protein motifs identified two possible motifs. ORF1 contained an ATP/GTP binding site motif (amino acids 385 to 392) that is also found in bacterial and viral proteins involved in genome replication, such as DnaA replication initiation proteins (32). ORF9 contained a lipoprotein lipid attachment motif (21) at its amino terminus (amino acids 18 to 28). This motif has also been identified in the archaeon *Natronobacterium pharaonis* in a blue copper protein (36).

In the archaea, common origin sequences among the different plasmids have not been identified. Studies of eukaryal replication origins have also failed to identify common sequence elements aside from a consensus sequence (WTTTA YRTTTWB) in the autonomously replicating sequences of *Saccharomyces cerevisiae* (reviewed in reference 22). Due to the high A+T content of pURB500, this sequence was very common. Therefore, it is unlikely to be a replication origin.

Direct and inverted sequence repeats are common features of origins of replication in bacterial plasmids (34). These sequence elements frequently represent binding sites for multimeric proteins or form secondary structures. pURB500 contained two relatively large regions devoid of ORFs and which had a striking number of direct and imperfect inverted repeats. These noncoding regions were designated ORFLESS1 (bp 3957 to 4742) and ORFLESS2 (bp 5502 to 5874). They contained half of the 30 strongest potential stem-loop structures and half of the direct repeats greater than 13 bp in the plasmid. The repeat structures in these two regions are represented diagrammatically in Fig. 3. While the overall complexity of these regions was striking, three detailed features were especially noteworthy. First, ORFLESS1 contained the largest repeat of pURB500, a 41-bp repeat flanking the unique *Kpn*I site (Fig. 3 and 4, group I). Second, a 13-bp sequence, ATGTTC GATTTTT, was repeated four times in ORFLESS1 (Fig. 3 and 4, group II). This sequence was not found elsewhere in pURB500. Similar multiple repeated sequences associated with the replication origin, or iterons, have been identified in some well-studied bacterial plasmids (34). Iterons have also been identified in archaeal replicons (38, 39). Third, near position 5900 in ORFLESS2, a pair of direct repeats contained a 16-bp palindrome, TACACGATATCGTGTA (Fig. 3 and 4, group III). These two repeats overlapped with the beginning of ORF17 and also formed a potential stem-loop structure. A third copy of this repeat was also present in ORFLESS1 (Fig. 3).

Construction of a replicating shuttle vector. Strain JJ is the type strain of *M. maripaludis* and is relatively well characterized (28, 57, 58, 60, 61). Because strain JJ has only approximately 65% DNA-DNA hybridization with strain C5 (31), it was not obvious that pURB500 would support replication in strain JJ. 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 *Eco*RI-*Kpn*I digestion

FIG. 2. Sequence of the pURB500 plasmid from *M. maripaludis* C5. Position 1 is in the middle of the *Eco*RI site that is interrupted in pDLT44. The forward nucleotide strand is shown. ORFs are numbered by decreasing size. Translated sequences of the 18 ORFs that would be transcribed in the forward or the reverse direction of this strand are listed above or below the nucleotide sequence, respectively. Stop positions are indicated with asterisks. ORF4 and ORF12 are interrupted in pDLT44, and the resulting peptide fragments are labeled ORF4′, ′ORF4 and ORF12′, ′ORF12. Possible internal start codons of each ORF that are in the same reading frame are not indicated. Potential RBSs for the ORFs are indicated by number signs (#) at the positions that can pair with the 3′ end of the 16S rRNA sequence 3′UCCUCCACUAGGU..., including G:U and G:T pairings (10). The RBS consensus sequence is 5′RGGRGGTGRTYYR (3′YRRAYCACCYCCY for RBSs in the opposite strand). Only RBSs within 20 bp upstream of the start codon, 6 bp into the ORF, and with at least seven matches to the consensus sequence are indicated. Potential transcriptional box A signals are indicated by the 5′*TTTAWA* consensus sequence (3′*TWTAAA* for the opposite strand), 25 to 100 bp upstream of the ORF with which they are associated. Mismatches with the box A sequence are indicated by lowercase letters. Only box A sites with ≤1 mismatch are shown. Direct repeats and potential stem-loops in the ORFLESS regions (see below) are indicated in Fig. 3 and 4. The remaining direct repeats greater than 13 bp are underlined and identified by the number of bases in the repeat plus a letter designating the repeat pair. Fifteen of the 30 strongest potential stem-loops are outside the ORFLESS regions and are dot-underlined.


EcoRI
14e
14d
14c
17c
TTTAWA
TTtAWA
TTTaWA
tTTAWA
TWTAaA
TWTa
TWt
TWTAAA
TWTAAa
TWtAAA
TWTAAa
EcoRI
ORF1
ORF4
ORF5
ORF9
ORF12
ORF18

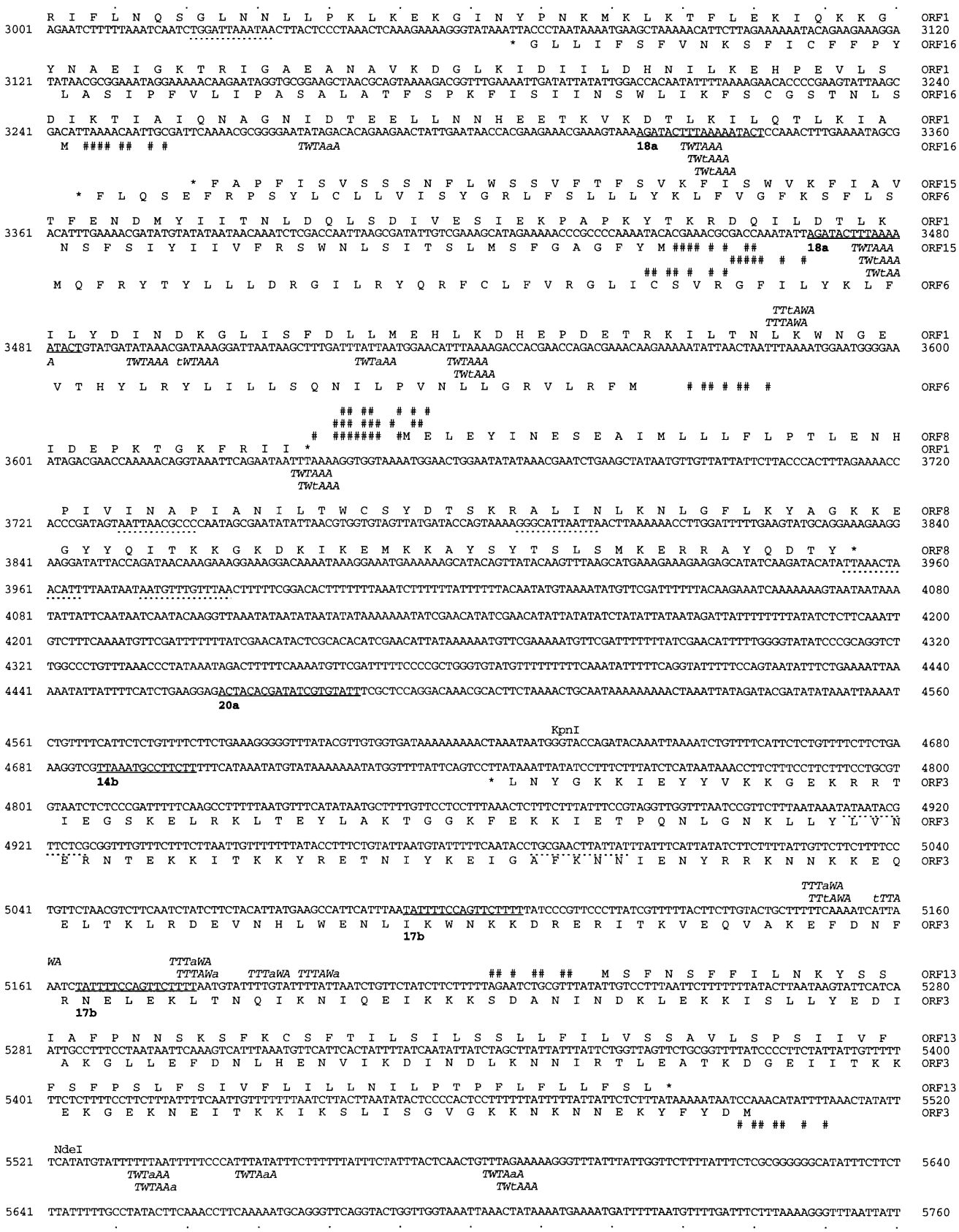


FIG. 2—*Continued.*

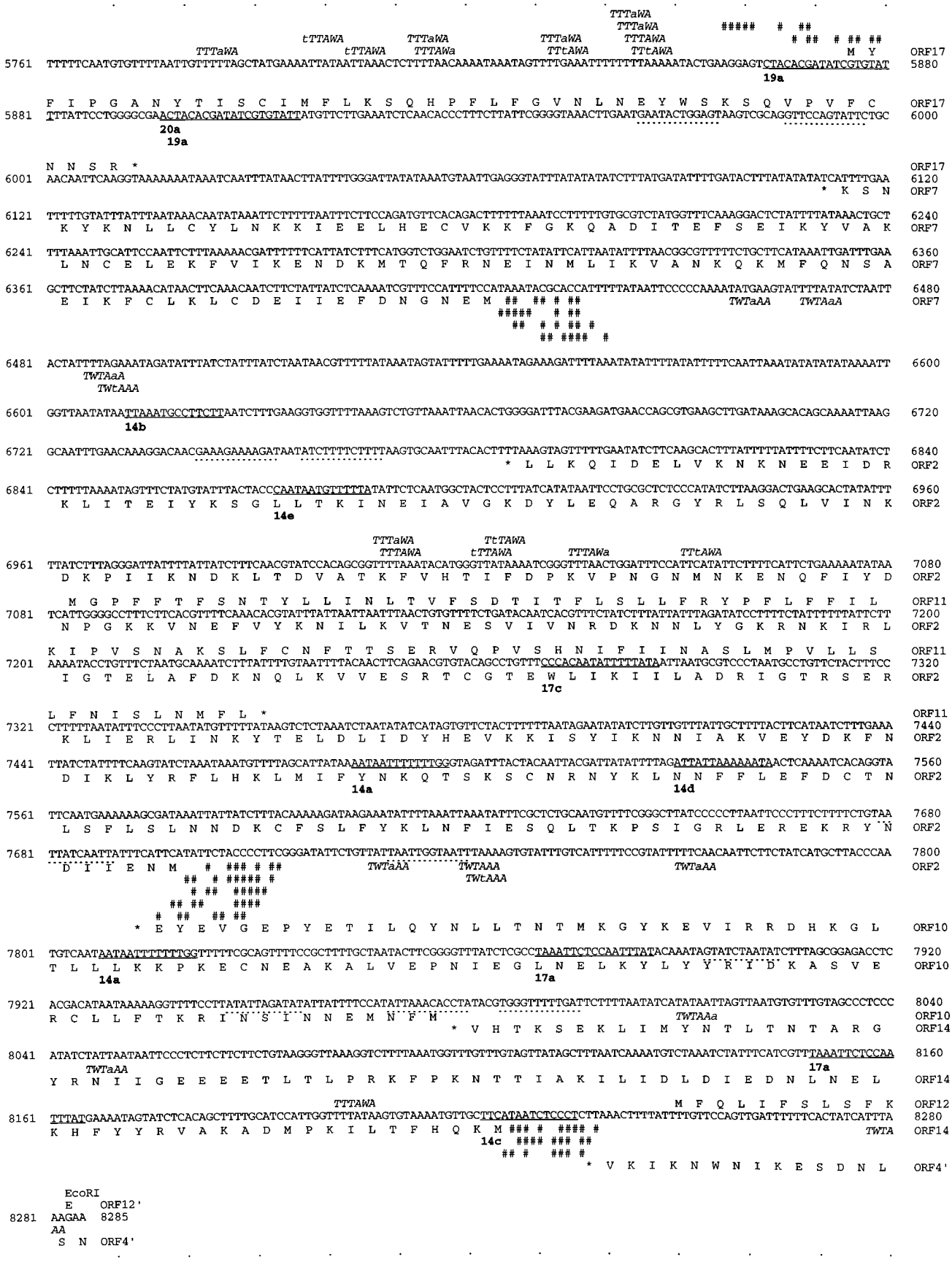


FIG. 2—*Continued.*

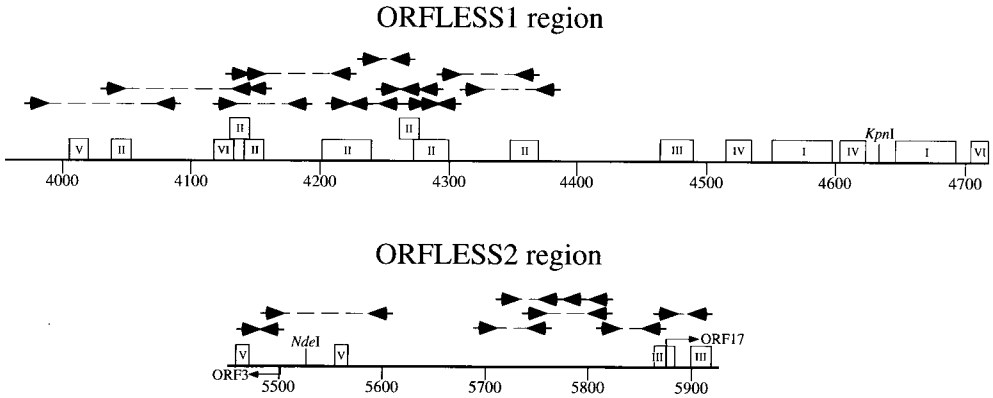


FIG. 3. ORFLESS1 and ORFLESS2, two regions of pURB500 which lacked ORFs and contained potential stem-loop structures (arrows) and direct repeats (boxes). Opposing arrows that can pair together to form potential stem-loops are connected by a dashed line. Shown are potential stem-loops with at least 21 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).

(pDLT9-12 [Fig. 1]). 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.

```
I
4551 AAATTAAAATCTGTTTTCATTCTCTGTTTTCTTCTGAAAGG 4591
4644 AAATTAAAATCTGTTTTCATTCTCTGTTTTCTTCTGAAAGG 4684

II
4039        ATGTTCGATTTTTt 4052
4131                       aTATCGAACATAT 4143
4140                       aTATCGAACATAT 4152
4204 TTTcAAAATGTTCGATTTTTtTATCGAACATA 4235
4258    aAAAATGTTCGA 4269
4269    aAAAATGTTCGATTTTTtTATCGAACAT 4296
4351 TTTcAAAATGTTCGATTTTT 4370

III
4465 ACTACACGATATCGTGTATTT 4485
5863  CTACACGATATCGTGTATTT 5882
5897 ACTACACGATATCGTGTATT 5916

IV
4518 ATAAAAAAAAACTAAAT 4534
4611 ATAAAAAAAAACTAAAT 4627

V
4010 TCTTTTTTATTTTT 4023
5462  CTTTTTTATTTTT 5474
5555 TCTTTTTTATTT 5566

VI
4122 TATAAAAAAATAT 4134
4715 TATAAAAAAATAT 4727
```

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 these 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.

To construct a shuttle vector, pURB500 and pMEB.2 were partially digested with *Eco*RI. 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/μg 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/μg (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 *Eco*RI site as in pDLT44, suggesting that the other two *Eco*RI 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

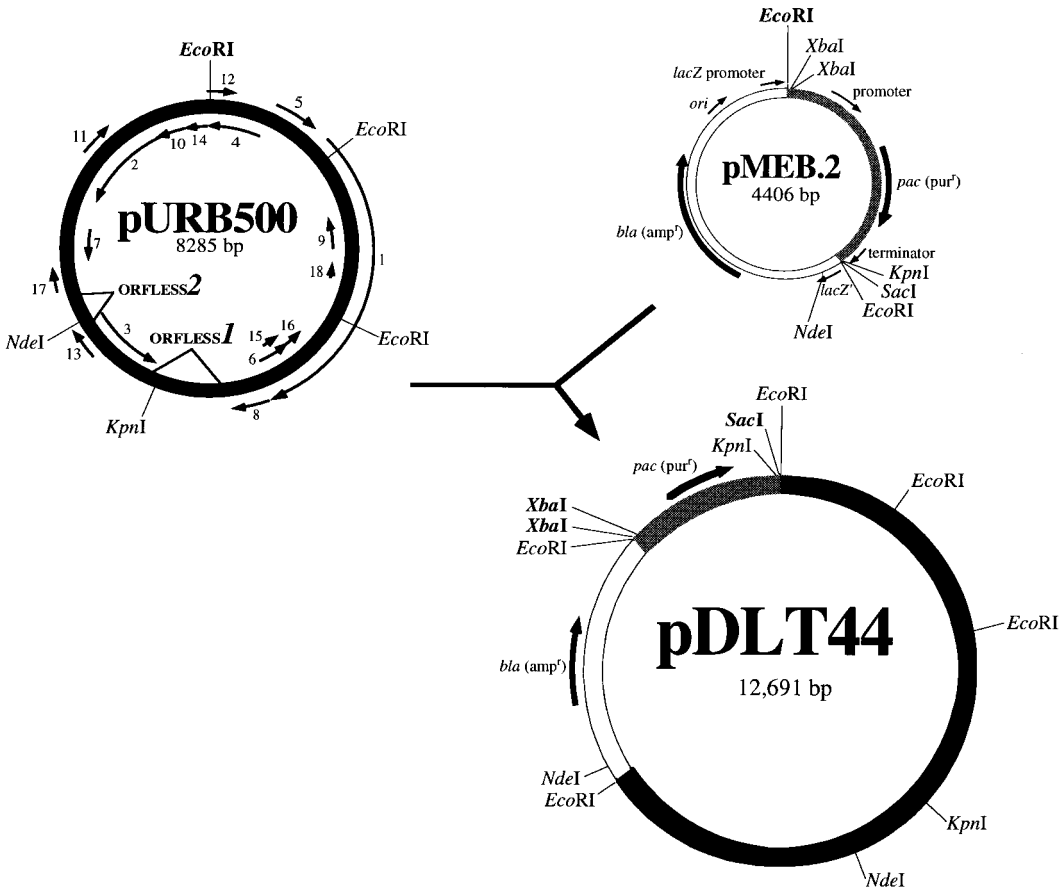


FIG. 5. Construction of the replicating shuttle vector pDLT44. pURB500 and pMEB.2 were partially digested with *Eco*RI, ligated, and transformed into *M. maripaludis* JJ. pDLT44 was isolated from a single puromycin-resistant colony. The locations of the ORFs on pURB500 are indicated by their respective numbers. In boldface are the *Eco*RI sites of pURB500 and pMEB.2 that were interrupted in constructing pDLT44 and the *Sac*I and *Xba*I sites suitable for cloning.

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 *Kpn*I site and the *Eco*RI 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 *Nde*I 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 *Kpn*I 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-

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

Plasmid[a]	Proportion of ampicillin-resistant colonies[b]
pDLT44	$<7.4 \times 10^{-8}$
pDLT3	1.2
pDLT2	9.4×10^{-1}
pDLT7	1.0
pDLT12	8.2×10^{-2}
pDLT43	1.8×10^{-6}
pDLT27	6.3×10^{-5}
pDLT21	3.0×10^{-7}
pDLT45	5.2×10^{-7}
pDLT17	1.5×10^{-4}
pDLT25	2.1×10^{-7}
pDLT19	2.8×10^{-7}
pDLT28	1.1×10^{-6}
pDLT16	1.2×10^{-6}
pMEB.2	1.4
pUC18	1.1
None	$<1.3 \times 10^{-7}$

[a] 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.

[b] 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 1/1,000 into LB broth without ampicillin and grown overnight. This culture was diluted 1/1,000 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.

tive cells after 10 h, presumably when the ampicillin was depleted. Similarly, following transfer into medium without ampicillin, the proportion of pDLT44-containing cells decreased 3 to 4 orders of magnitude in an overnight culture (data not shown).

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 pDLT2, -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 instability was also associated with specific sites or combinations of regions. The *Kpn*I site was important, and interruption of the plasmid at this site increased the stability (pDLT44 versus pDLT16). In contrast, interruption of the plasmid at the *Eco*RI site between fragments I and II had no effect on stability (pDLT28 versus pDLT16). Although they had no effect on stability by themselves, fragments I, II, and III lowered the stability when present in combination with fragment IV. Evidence for the importance of fragment I came from the comparisons of pDLT44 versus pDLT45, pDLT27 versus pDLT19, and pDLT12 versus pDLT17. Evidence for the importance of fragment III came from the comparison of pDLT17 versus pDLT25. Fragment II did not appear to be important when the *Kpn*I site was intact (pDLT43 versus pDLT45). However, when this site was interrupted, fragment II affected stability (pDLT17 versus pDLT25 and pDLT27 versus pDLT21). Therefore, the instability 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 (<89 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; $<10^{-6}$ 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 (<89 transformants/μg; $<1.1 \times 10^{-8}$ transformants/CFU) or APB (<20 transformants/μg; $<4.0 \times 10^{-7}$ 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 ColE1, 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 interruption of the ORFLESS1 region at the *Kpn*I 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 pFV1 and pFZ1 of *Methanobacterium thermoformicicum* (40) and pRN1 of *S. islandicus* (30) and in the SSV1 virus of *Sulfolobus shibatae* (32). The absence of significant sequence similarity between ORF1 and these replicons 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 *Eco*RI digestion of pURB500, were interrupted in the only *Eco*RI 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 ORFLESS regions. In plasmids such as pSC101 and the IncQ and IncW plasmid families of the gram-negative bacteria (34, 52) and in the archaeal replicons pNRC100 (39), pHH1 (45), and pGT5 (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]), pGT5 (*Pyrococcus abyssi* [16]), and pHK2 (*Haloferax* 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 pGT5 (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 methanococcus 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, *Sau*3AI 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 *Sau*3AI (59). Similarly, sequencing of *M. maripaludis* JJ chromosomal DNA fragments has revealed close to one *Sau*3AI 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 *Pst*I-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 some physiological studies (57, 58, 60, 61) sets the stage for future studies on this strictly anaerobic archaeon.

ACKNOWLEDGMENTS

We thank Albrecht Klein and Odile Possot for providing plasmids pMEB.2 and Mip1 and Warren L. Gardner for providing pWLG25. We also thank Zohar Mann for technical assistance with the Erase-a-Base system.

This work was supported by grant DE-FG05-94ER20158 from the Department of Energy. D. Tumbula was also supported in part by grant BIR-9413235 from the National Science Foundation.

REFERENCES

1. **Aagaard, C., I. Leviev, R. N. Aravalli, P. Forterre, D. Prieur, and R. A. Garrett.** 1996. General vectors for archaeal hyperthermophiles: strategies based on a mobile intron and a plasmid. FEMS Microbiol. Rev. **18:**93–104.
2. **Akhmanova, A. S., V. K. Kagramanova, and A. S. Mankin.** 1993. Heterogeneity of small plasmids from halophilic archaea. J. Bacteriol. **175:**1081–1086.
3. **Argyle, J. L., D. L. Tumbula, and J. A. Leigh.** 1996. Neomycin resistance as a selectable marker in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **62:**4233–4237.
4. **Bairoch, A., and P. Bucher.** 1994. PROSITE: recent developments. Nucleic Acids Res. **22:**3583–3589.
5. **Berghöfer, Y., K. Agha-Amiri, and A. Klein.** 1994. Selenium is involved in the negative regulation of the expression of selenium-free [NiFe] hydrogenases in *Methanococcus voltae*. Mol. Gen. Genet. **242:**369–373.
6. **Blank, C. E., P. S. Kessler, and J. A. Leigh.** 1995. Genetics in methanogens: transposon insertion mutagenesis of a *Methanococcus maripaludis nifH* gene. J. Bacteriol. **177:**5773–5777.
7. **Bokranz, M., A. Klein, and L. Meile.** 1990. Complete nucleotide sequence of plasmid pME2001 of *Methanobacterium thermoautotrophicum* (Marburg). Nucleic Acids Res. **18:**363.
8. **Bramhill, D., and A. Kornberg.** 1988. A model for initiation at origins of DNA replication. Cell **54:**915–918.
9. **Brassard, S., H. Paquet, and P. H. Roy.** 1995. A transposon-like sequence adjacent to the *Acc*I restriction-modification operon. Gene **157:**69–72.
10. **Brown, J. W., C. J. Daniels, and J. N. Reeve.** 1989. Gene structure, organization, and expression in archaebacteria. Crit. Rev. Microbiol. **16:**287–338.
11. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J.-F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, W. F. Kirkness, K. B. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H.-P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. G. Venter.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058–1073.
12. **Charbonnier, F., and P. Forterre.** 1994. Comparison of plasmid DNA topology among mesophilic and thermophilic eubacteria and archaebacteria. J. Bacteriol. **176:**1251–1259.
13. **Charlebois, R. L., W. L. Lam, S. W. Cline, and W. F. Doolittle.** 1987. Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. Proc. Natl. Acad. Sci. USA **84:**8530–8534.
14. **DasSarma, S.** 1995. Natural plasmids and plasmid vectors of halophiles, p. 241–250. *In* F. T. Robb, K. R. Sowers, S. DasSarma, A. R. Place, H. J. Schreier, and E. M. Fleischmann (ed.), Archaea: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
15. **Dillard, J. P., and J. Yother.** 1991. Analysis of *Streptococcus pneumoniae*

sequences cloned into *Escherichia coli*: effect of promoter strength and transcription terminators. J. Bacteriol. **173:**5105–5109.
16. **Erauso, G., S. Marsin, N. Benbouzid-Rollet, M.-F. Baucher, T. Barbeyron, Y. Zivanovic, D. Prieur, and P. Forterre.** 1996. Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: evidence for rolling-circle replication in a hyperthermophile. J. Bacteriol. **178:**3232–3237.
17. **Gardner, W. L., J.-P. Yu, and W. B. Whitman.** Unpublished data.
18. **Gernhardt, P., O. Possot, M. Foglino, L. Sibold, and A. Klein.** 1990. Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221:**273–279.
19. **Hackett, N. R., M. P. Krebs, S. DasSarma, W. Goebel, U. L. RajBhandary, and H. G. Khorana.** 1990. Nucleotide sequence of a high copy number plasmid from *Halobacterium* strain GRB. Nucleic Acids Res. **18:**3408.
20. **Hall, M. J., and N. R. Hackett.** 1989. DNA sequence of a small plasmid from *Halobacterium* strain GN101. Nucleic Acids Res. **17:**10501.
21. **Hayashi, S., and H. C. Wu.** 1990. Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22:**451–471.
22. **Held, P. G., and N. H. Heintz.** 1992. Eukaryotic replication origins. Biochim. Biophys. Acta **1130:**235–246.
23. **Holmes, M. L., F. Pfeifer, and M. L. Dyall-Smith.** 1995. Analysis of the halobacterial plasmid pHK2 minimal replicon. Gene **153:**117–121.
24. **Hopwood, D. A.** 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. **35:**237–272.
25. **Ilyina, T. V., and E. V. Koonin.** 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic Acids Res. **20:**3279–3285.
26. **Jarrell, K. F., D. P. Bayley, V. Florian, and A. Klein.** 1996. Isolation and characterization of insertional mutations in flagellin genes in the archaeon *Methanococcus voltae*. Mol. Microbiol. **20:**657–666.
27. **Jarrell, K. F., C. Julseth, B. Pearson, and J. Kuzio.** 1987. Paucity of the *Sau*3AI recognition sequence (GATC) in the genome of *Methanococcus voltae*. Mol. Gen. Genet. **208:**191–194.
28. **Jones, W. J., M. J. B. Paynter, and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135:**91–97.
29. **Jones, W. J., W. B. Whitman, R. D. Fields, and R. S. Wolfe.** 1983. Growth and plating efficiency of methanococci on agar media. Appl. Environ. Microbiol. **46:**220–226.
30. **Keeling, P. J., H.-P. Klenk, R. K. Singh, O. Feeley, C. Schleper, W. Zillig, W. F. Doolittle, and C. W. Sensen.** 1996. Complete nucleotide sequence of the *Sulfolobus islandicus* multicopy plasmid pRN1. Plasmid **35:**141–144.
31. **Keswani, J., S. Orkand, U. Premachandran, L. Mandelco, M. J. Franklin, and W. B. Whitman.** 1996. Phylogeny and taxonomy of mesophilic *Methanococcus* spp. and comparison of rRNA, DNA hybridization, and phenotypic methods. Int. J. Syst. Bacteriol. **46:**727–735.
32. **Koonin, E. V.** 1992. Archaebacterial virus SSV1 encodes a putative DnaA-like protein. Nucleic Acids Res. **20:**1143.
33. **Kornacki, J. A., C.-H. Chang, and D. H. Figurski.** 1993. *kil-kor* regulon of promiscuous plasmid RK2: structure, products, and regulation of two operons that constitute the *kilE* locus. J. Bacteriol. **175:**5078–5090.
34. **Kües, U., and U. Stahl.** 1989. Replication of plasmids in gram-negative bacteria. Microbiol. Rev. **53:**491–516.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Mattar, S., B. Scharf, S. B. H. Kent, K. Rodewald, D. Oesterhelt, and M. Engelhard.** 1994. The primary structure of halocyanin, an archaeal blue copper protein, predicts a lipid anchor for membrane fixation. J. Biol. Chem. **269:**14939–14945.
37. **Meile, L., J. Madon, and T. Leisinger.** 1988. Identification of a transcript and its promoter region on the archaebacterial plasmid pME2001. J. Bacteriol. **170:**478–481.
38. **Mojica, F. J. M., C. Ferrer, G. Juez, and F. Rodríguez-Valera.** 1995. Long stretches of short tandem repeats are present in the largest replicons of the Archaea *Haloferax mediterranei* and *Haloferax volcanii* and could be involved in replicon partitioning. Mol. Microbiol. **17:**85–93.
39. **Ng, W.-L., and S. DasSarma.** 1993. Minimal replication origin of the 200-kilobase *Halobacterium* plasmid pNRC100. J. Bacteriol. **175:**4584–4596.
40. **Nölling, J., F. J. M. van Eaden, R. I. L. Eggen, and W. M. de Vos.** 1992. Modular organization of related archaeal plasmids encoding different restriction-modification systems in *Methanobacterium thermoformicicum*. Nucleic Acids Res. **20:**6501–6507.
41. **Nordström, K.** 1993. Plasmid replication and maintenance, p. 1–38. *In* K. G. Hardy (ed.), Plasmids: a practical approach. IRL Press, Oxford, England.
42. **Palmer, J. R., and J. N. Reeve.** 1993. Structure and function of methanogen genes, p. 497–534. *In* M. Kates, D. J. Kushner, and A. T. Matheson (ed.), The biochemistry of archaea (archaebacteria). Elsevier, Amsterdam, The Netherlands.
43. **Patel, G. B., C. G. Choquet, J. H. E. Nash, and G. D. Sprott.** 1993. Formation and regeneration of *Methanococcus voltae* protoplasts. Appl. Environ. Microbiol. **59:**27–33.
44. **Patel, G. B., J. H. E. Nash, B. J. Agnew, and G. D. Sprott.** 1994. Natural and electroporation-mediated transformation of *Methanococcus voltae* protoplasts. Appl. Environ. Microbiol. **60:**903–907.
45. **Pfeifer, F., and P. Ghahraman.** 1993. Plasmid pHH1 of *Halobacterium salinarium*: characterization of the replicon region, the gas vesicle gene cluster and insertion elements. Mol. Gen. Genet. **238:**193–200.
46. **Sandbeck, K. A., and J. A. Leigh.** 1991. Recovery of an integration shuttle vector from tandem repeats in *Methanococcus maripaludis*. Appl. Environ. Microbiol. **57:**2762–2763.
47. **Sioud, M., G. Baldacci, P. Forterre, and A.-M. de Recondo.** 1988. Novobiocin induces accumulation of a single strand of plasmid pGRB-1 in the archaebacterium *Halobacterium* GRB. Nucleic Acids Res. **16:**7833–7842.
48. **Stadtman, T. C., and H. A. Barker.** 1951. Studies on the methane fermentation: a new formate-decomposing bacterium, *Methanococcus vannielii*. J. Bacteriol. **62:**269–280.
49. **Stettler, R., P. Pfister, and T. Leisinger.** 1994. Characterization of a plasmid carried by *Methanobacterium thermoautotrophicum* ZH3, a methanogen closely related to *Methanobacterium thermoautotrophicum* Marburg. Syst. Appl. Microbiol. **17:**484–491.
50. **Tumbula, D. L., T. L. Bowen, W. J. Jones, and W. B. Whitman.** 1995. Growth of methanogens on solidified media, p. 49–55. *In* F. T. Robb, K. R. Sowers, S. DasSarma, A. R. Place, H. J. Schreier, and E. M. Fleischmann (ed.), Archaea: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
51. **Tumbula, D. L., R. A. Makula, and W. B. Whitman.** 1994. Transformation of *Methanococcus maripaludis* and identification of a *Pst*I-like restriction system. FEMS Microbiol. Lett. **121:**309–314.
52. **Valentine, C. R. I., and C. I. Kado.** 1989. Molecular genetics of IncW plasmids, p. 125–163. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press, London, England.
53. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
54. **Ward, J. M., P. H. Smith, and D. R. Boone.** 1989. Emended description of strain PS (= OGC 70 = ATCC 33273 = DSM 1537), the type strain of *Methanococcus voltae*. Int. J. Syst. Bacteriol. **39:**493–494.
55. **Whitman, W. B.** 1989. Methanococcales, p. 2185–2190. *In* J. T. Staley, M. P. Bryant, and N. Pfennig (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, Md.
56. **Whitman, W. B., T. L. Bowen, and D. R. Boone.** 1991. The methanogenic bacteria, p. 719–767. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York, N.Y.
57. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. Syst. Appl. Microbiol. **7:**235–240.
58. **Whitman, W. B., S. Sohn, S. Kuk, and R. Xing.** 1987. Role of amino acids and vitamins in nutrition of mesophilic *Methanococcus* spp. Appl. Environ. Microbiol. **53:**2373–2378.
59. **Wood, A. G., W. B. Whitman, and J. Konisky.** 1985. A newly-isolated marine methanogen harbors a small cryptic plasmid. Arch. Microbiol. **142:**259–261.
60. **Xing, R. Y., and W. B. Whitman.** 1991. Characterization of enzymes of the branched-chain amino acid biosynthetic pathway in *Methanococcus* spp. J. Bacteriol. **173:**2086–2092.
61. **Yu, J.-P., J. Ladapo, and W. B. Whitman.** 1994. Pathway of glycogen metabolism in *Methanococcus maripaludis*. J. Bacteriol. **176:**325–332.
62. **Zillig, W., P. Palm, H.-P. Klenk, D. Langer, U. Hüdepohl, J. Hain, M. Lanzendörfer, and I. Holz.** 1993. Transcription in archaea, p. 367–391. *In* M. Kates, D. J. Kushner, and A. T. Matheson (ed.), The Biochemistry of archaea (archaebacteria). Elsevier, Amsterdam, The Netherlands.