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Structural Organization of pRAM4, a Cryptic Plasmid from *Prevotella ruminicola*

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A total of 530 strains of rumen bacteria were screened for the presence of plasmid DNA. The percentage of plasmid-bearing strains was found to be the highest among the *Bacteroides/Prevotella* group (9.9%), while it was less than 1% in the *Butyrivibrio* (0.2%) and *Clostridium* (0.6%) genera. A small cryptic plasmid pRAM4 from *Prevotella ruminicola* T31 was subcloned in *Escherichia coli* and completely sequenced. Two open reading frames, encoding potential polypeptides of M_r 32,322 (ORF1) and 32,122 (ORF2) with limited sequence similarity to replication initiation and mobilization proteins, respectively, could be identified within the sequence. The region upstream from ORF1 had an AT-rich (75%) region followed by four 22-bp direct repeats, a structure characteristic of replication origins. The plasmid hybridized at high stringency with plasmids from *Bacteroides/Prevotella* and *Butyrivibrio,* and with pBR322, suggesting that at least regions of the plasmid are widespread. © 1996 Academic Press, Inc.

The rumen is a strictly anaerobic ecosystem inhabited mainly by bacteria, archaea, and protozoa, with smaller numbers of fungi. Since the bacterial part of the system is predominantly responsible for the digestion of plant polysaccharides, there is substantial interest in the development of genetic manipulation methods for rumen bacteria. Conjugal transfer of the transposon Tn916 and plasmid pAMβ1 as well as of a number of natural and recombinant constructions has been demonstrated for ruminal bacteria under laboratory conditions (Hespell and Whitehead, 1991a,b; Thomson *et al.,* 1992; Flint *et al.,* 1988; Aminov *et al.,* 1994). Electrotransformation procedures also have been applied successfully to a number of rumen bacteria (Lockington *et al.,* 1988; Thomson and Flint, 1989; Thomson *et al.,* 1992; Whitehead, 1992; Cocconcelli *et al.,* 1992). However, since the efficiency and reliability of these systems remain far behind those of the well-developed *Escherichia coli* and *Bacillus subtilis* systems, there is continuing interest in the screening and characterization of plasmids from rumen bacteria, with the further aim of constructing new versatile vectors.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used were XL-1 Blue (Stratagene) and JM109 (Yanish-Perron *et al.,* 1985). The *E. coli* plasmid was pBluescript II (KS$^-$) (Stratagene), and staphylococcal plasmids were pUB110 (Jalanko *et al.,* 1981) and pC194 (Horinouchi and Weisblum, 1982). Strains of rumen bacteria, which have been isolated and characterized previously (Minato *et al.,* 1989, 1992; Arakaki *et al.,* 1994), and which were used in this work, are listed in Table 1.

Media and growth conditions. Prevotella ruminicola T31 and other anaerobic rumen bacteria were routinely maintained in RGSA medium (Atlas, 1993) with 30% clarified rumen fluid, under anaerobic conditions. *E. coli* strains were grown in LB medium. Solid me-

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dia contained 1.8% agar. Ampicillin (50 μg/ml) was added when required.

Plasmid DNA isolation. Plasmids were isolated from *E. coli* strains by a modified alkaline lysis procedure (Sambrook *et al.,* 1989) and from anaerobic rumen bacteria by a miniprep method developed for lactic acid bacteria (O'Sullivan and Klaenhammer, 1993). DNA fragments were purified from agarose gels using the GeneClean II kit (BIO 101, Inc., U.S.A.).

In vitro DNA manipulations. All routine molecular cloning procedures were as before (Sambrook *et al.,* 1989). Restriction and DNA modification enzymes were obtained from NEB (U.S.A.). Hybridization was done using a DNA labeling/detection kit from Boehringer-Mannheim (Germany), under the standard conditions recommended by the manufacturer, which are considered highly stringent (DIG nucleic acid detection kit, protocol). Nested deletions were generated using the Deletion kit supplied by Nippon Gene (Japan). Chemicals and reagents for molecular biology experiments were obtained from Boehringer-Mannheim (Germany), Sigma (U.S.A.), and Nakalai (Japan).

DNA sequence analysis. The dideoxy chain termination method of DNA sequencing was carried out on double-stranded DNA templates with the *Taq* Dye Primer and Dye Terminator Cycle Sequencing kits (Applied Biosystem) on a Perkin–Elmer PCR apparatus. Sequencing reactions were analyzed on an automatic sequencer (373A DNA sequencer; Applied Biosystems). Sequencing primers were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. The sequence of pRAM4 was determined completely on both strands.

Computer-assisted analyses were performed using Blast v.1.4 (Altschul *et al.,* 1990) and MPsrch (Sturrock and Collins, 1993) software and using on-line GenBank, EMBL, and SwissProt databases. Sequence alignments and manipulations were performed using GENETYX, DNAsis, GeneWorks, MacTagSearch, and OLIGO software.

TABLE 1

PLASMIDS DETECTED IN RUMEN BACTERIA

Bacterial genera	Number of isolates	Number of isolates with plasmid(s)
Bacteroides spp. and *Prevotella* spp.	161	16 (9.9%)
Butyrivibrio spp.	154	3 (0.2%)
Lactobacillus spp.	49	0
Eubacterium spp.	34	0
Selenomonas spp.	31	0
Clostridium spp.	18	1 (0.6%)
Ruminobacter amylophilus	14	0
Fusobacterium spp.	13	0
Streptococcus spp.	11	0
Propionibacterium spp.	8	0
Fibrobacter succinogenes	6	0
Ruminococcus spp.	6	0
Ruminococcus albus	4	0
Ruminococcus flavefaciens	1	0
Peptostreptococcus spp.	5	0
Bifidobacterium spp.	4	0
Megasphaera spp.	3	0
Succinivibrio spp.	3	0
Veillonella spp.	1	0
Total	530	20 (3.8%)

RESULTS

Screening of Rumen Bacteria for Plasmid DNA

Plasmids of different molecular sizes ranging from 3 to 40 kb were found in 20 rumen bacteria belonging to the genera *Clostridium, Bacteroides, Prevotella,* and *Butyrivibrio* (Table 1). The percentage of plasmid bearing strains may be underestimated because the plasmid isolation protocol was not optimized for each isolate.

Cloning and Sequence Analysis of pRAM4 from P. ruminicola

A 3.1-kb cryptic plasmid pRAM4, isolated from *P. ruminicola* strain T31, was cloned in *E. coli* using vector pBluescript II (KS) and mapped (Fig. 1). This plasmid was chosen because of the consistently high plasmid yield

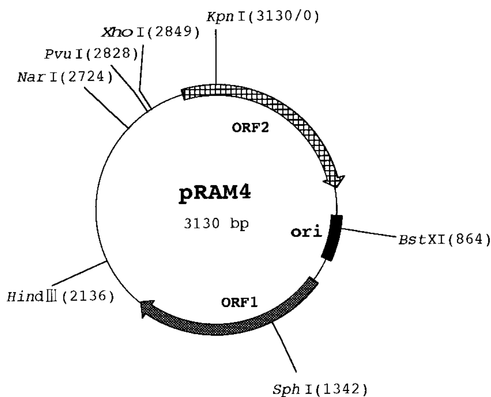

FIG. 1. Physical and functional map of pRAM4 derived from sequence analysis. The *Kpn*I site was arbitrarily taken as 0. The regions marked ORF1 and ORF2 exhibit sequence similarity with replication proteins and a mobilization protein, respectively. The region marked ''ori'' encompasses the AT-rich area followed by the putative IHF-binding site, four direct repeats, and an inverted repeat.

obtained from the original host. The complete nucleotide sequence of the 3130-bp plasmid was determined and it is shown in Fig. 2. It has an overall GC content of 49.17%. The sequence reveals two open reading frames (ORFs),[2] encoding potential polypeptides with molecular sizes of 32,322 (ORF1) and 32,122 (ORF2). Analysis of the region upstream of ORF1 revealed an AT-rich (75%) region followed by a putative site for binding of the integration host factor (IHF) and four 22-bp direct repeats (iterons) (Fig. 2). Taken together, these features are characteristic of replication origins (Kornberg and Baker, 1992), although this region does not show sequence similarity with known origin regions. Several putative promoter sequences upstream of ORF1 and ORF2 were found to be within potential hairpin–loop structures.

Comparative Analysis of ORF1 and ORF2

The putative protein product of ORF1 had local amino acid sequence similarity with replication proteins from pSC101 from *E. coli* (Armstrong *et al.,* 1984), pPS10 from *Pseudomonas syringae* (Nieto *et al.,* 1992), pRO1600 from *Pseudomonas aeruginosa* (Jansons *et al.,* 1994), pSL2 from *Lactococcus lactis* (Jahns *et al.,* 1991), pChyo from *Campylobacter hyointestinalis* (Waterman *et al.,* 1993), and with the mobilization protein B of the *E. coli* bacteriocinogenic plasmid CloDF13 (Nijkamp *et al.,* 1986). The translated product of ORF2 had limited amino acid sequence similarity with the mobilization protein of a *Bacteroides* insertion element NBU1 (Li *et al.,* 1993).

Hybridization of pRAM4 Probe with Plasmids from Rumen Bacteria

To determine whether pRAM4 is related to plasmids of other ruminal bacteria, we hybridized labeled pRAM4 DNA with the plasmids extracted from *Bacteroides, Prevotella, Butyrivibrio,* and *Clostridium* spp. As controls, we tested the staphylococcal plasmids, pUB110 and pC194, which represent two classes of molecules replicating by a rolling-circle mechanism (Gruss and Ehrlich, 1989) and a theta-replicating ColE1 derivative, pBR322. The results (Fig. 3) clearly demonstrated that under the high-stringency Southern hybridization conditions hybridization was detected with plasmids from *Bacteroides, Prevotella,* and *Butyrivibrio* species and with pBR322. No signals were detected with the clostridial or staphylococcal plasmids (data not shown).

DISCUSSION

Plasmids are relatively abundant in rumen bacteria. It has been shown that from 41 to 100% of *Selenomonas* isolates contained one or more plasmid bands and that up to 28% of the *Butyrivibrio fibrisolvens* isolates carried plasmids (Hazlewood and Teather, 1988). Small cryptic plasmids have been found in 6 of 7 strains of *R. albus* and in all 3 strains of *R. flavefaciens* examined (Kelly and Asmundson, 1986; Asmundson and Kelly, 1987). At the same time, using a number of different plas-

[2] Abbreviations used: ORF, open reading frame; IHF, integration host factor.

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CATAAGGACG AAGGACACCC TCGCTCTAAA TACATCGAGG GAGATACCAA TTTCGAAGTT AATTACCACA TCCATGTCCT  80
 H  K  D   E  G  H  P   R  S  K   Y  I  E   G  D  T  N   F  E  V   N  Y  H   I  H  V  L
CTACTACTGC CAGGATCCTG AGACTGGTAA GGCCATCAGG CTTCCAAGGT CGTTCTTCAC CGAACGCCAG GACTTCCTGG 160
 Y  Y  C   Q  D  P   E  T  G  K   A  I  R   L  P  R   S  F  F  T   E  R  Q   D  F  L
CACAAGCAAC AGGTCTTGAA CGTGGAAACC CTGCTAAAGA AACCCGCAGC CAAAGACGTT CAGCCCTGCA GCAGCGCATC 240
 A  Q  A  T   G  L  E   R  G  N   P  A  K  E   T  R  S   Q  R  R   S  A  L  Q   Q  R  I
GAGGCTCAGG AACAGCGCAT CGAGCAGCTG CAGAAGGTCA TCGATCAGAA GGACAAAGAG AGGGATAAGG CCATCGAGGA 320
 E  A  Q   E  Q  R  I   E  Q  L   Q  K  V   I  D  Q  K   D  K  E   R  D  K   A  I  E  D
TGCTAAAGCG AGTATCTGGC AGACGGCTAA GAGGATCTTT GGCTCCGACA AGACCATCAA CGGCCTGAAA GCTACTATAA 400
 A  K  A   S  I  W   Q  T  A  K   R  I  F   G  S  D   K  T  I  N   G  L  K   A  T  I
AAGCTCAAAA AGACAAAATA GAGGCCCTAC AAGCCCAAAT CAAGGTCGAA AGACAGAATC ACAAGGCAGA ACTGGAGAAA 480
 K  A  Q  K   D  K  I   E  A  L   Q  A  Q  I   K  V  E   R  Q  N   H  K  A  E   L  E  K
ACCCGCCAAA ACGCCTCAAA ACCCCTCAAA AACGTTTTGA GCAAGATTGC TGCAGCCTTG GAGTACTGGC CATCAAAGCT 560
 T  R  Q   N  A  S  K   P  L  K   N  V  L   S  K  I  A   A  A  L   E  Y  W   P  S  K  L
CACCGAGGAC GGTGTTCTCG CCAAAGTCCG TGACCTCAAA GAGTCCGAAC GTTCCTGGAG ACACACCGCC TTGGACGCAA 640
 T  E  D   G  V  L   A  K  V  R   D  L  K   E  S  E   R  S  W  R   H  T  A   L  D  A
AAGAGCAGCT CAAAGCCCAG AACCAACCTT CTCAAGACCA CCAGCTTCGT CGTTGAACTC GCCCACCACT CCTGCAGGAC 720
 K  E  Q  L   K  A  Q   N  Q  P   S  Q  D  H   Q  L  R   R  ORF2
TTTCGTCCCG CATGGAGCGG GAGCGCCCCC CAGGAGCGAC CCCCTAAACA ACAAAAAAAA ATCCCAAATC GGCCATCAGG 800

CGACGCTTTA GTACTTAGTT TATTTAAGAT TTTAAGTACA GATTTAAGCG CGCGCGCGTG AGGCCAACAA GCTGGTTACC 880
            AT-rich region                                                  IHF
AACAACTTAC AGCGTTTTCA AGGGCTTATT TTCCCGACTT TTTGCGGTA TTTTCCCCGA CTTTTTTTGC GGTATTTTCC 960
                                      DR1                  DR2
CGACTTTTTT GCGGTATTTT CCCGACTTTT TTGCGGTAAA AAACCCCTTT TAACTATTTT TCCGACTTTT TGTTTTGCAA 1040
 DR3                  DR4                                         IR1         -35
AGTCGGTAAA ATATATTATC TTTGCGGCCG TAAACCAAAA AAGTCAACGG CATATGGCGT ACCCCAACGA GAAGGAAAAC 1120
  IR2      -10
CTTCCCTCAA TTATCCAAGA CCACTACATC ACAGAACGCA GGAGTAAGTA CACTGCGATG ATGAACAAGT GCTATTATAG 1200
                                           SD               ORF1 M   M  N  K   C  Y  Y  R
ACTGATGGAG AAGTTTGAAG CCCGCTATCA GGCAATTAAA GACCAGGTAT CACCCAACCT CTTCGGTGAG CATGATCGGT 1280
 L  M  E   K  F  E   A  R  Y  Q   A  I  K   D  Q  V   S  P  N  L   F  G  E   H  D  R
TTACCCTCAG TCCTCTCCTG AACTACGATA TCACCAATTC GGAGTTCGGC ATCGACAAAG AGCATGCCTA CACCGTCAAG 1360
 F  T  L  S   P  L  L   N  Y  D   I  T  N  S   E  F  G   I  D  K   E  H  A  Y   T  V  K
AGGGCGCTGC GTGCCATCAT GGGTGAGCCG TTTGTTATCA GTGGTTCAAT GAAAGGCAAT GCAGCTGTGG AACTGATAGC 1440
 R  A  L   R  A  I  M   G  E  P   F  V  I   S  G  S  M   K  G  N   A  A  V   E  L  I  A
CCCCCATCAG TATGTCAAAT ACGATCCAGA CAAAGAGGTT TTCAATATCC AGCTATCCGA GCTGATTGTC AATCACTATC 1520
 P  I  S   Y  V  K   Y  D  P  D   K  E  V   F  N  I   Q  L  S  E   L  I  V   N  H  Y
TGGAGCTGAA TGTCTATGAC CGATACAGTC CACTTATTGC CCGTTCCTTT GATGGTCAGT ACACTCCCAG GTTCTATGAG 1600
 L  E  L  N   V  Y  D   R  Y  S   P  L  I  A   R  S  F   D  G  Q   Y  T  P  R   F  Y  E
TGGGCCTGTC GATGGAGAAA ACAGGGCTTC TATGATATCG ACTATGACAA CATCAGGACG GAGTTCTGGC TGAATGCCTA 1680
 W  A  C   R  W  R  K   Q  G  F   Y  D  I   D  Y  D  N   I  R  T   E  F  W   L  N  A  Y
TACCGATGAC AAAGGCAAGA AACACAAGGC AAAATATCGT GATAATGGTG ATATCATGAA AAAGATTATC ATGCCCTCTC 1760
 T  D  D   K  G  K   K  H  K  A   K  Y  R   D  N  G   D  I  M  K   K  I  I   M  P  S
TGGATGAAAT CAGACAGGCG GTCCAAAACG GGACATGTGA TTTCTGGCTT CGTTTGGAGG AACTGCCAGA CAACCCACAG 1840
 L  D  E  I   R  Q  A   V  Q  N   G  T  C  D   F  W  L   R  L  E   E  L  P  D   N  P  Q
GTAGGCAGAC GTGGACGACC ATCCAAGCCA ACCCGGTTCC CGCTGCTGGA TGGAGTTCGA GGAAGCCAAT GCCGTTGAAC 1920
 V  G  R   R  G  R  P   S  K  P   T  R  F   P  L  L  D   G  V  R   G  S  Q   R  S  R
TCAACGACAA TGGAACTCCG AAACAGGGCT ATCTTTTCCC AGATGAAGCG GCTATCAACA AGACCTTAGA TGATATCCAG 2000

AGATGGCTCA TCACCTGCAC TGCGAAGGGG AACACCCTCG ATAAGAAACT TCCTGAGAAG ATCCTCAACC AGATTCGTGT 2080

CAGGGCATTC AACACCATTA ACCCAGAACC TGACCTTCCC CGTCAGGTGT TGGTGAAGCT TCAGGATATC TACTGTCGCC 2160

TGCAGAAGAA ACCGAATGCC AGGTGGGGTG AAGTGGTGAT GGTTATCAAG ACGGCTTTAA GAGAAGACTT TGGAATCGTT 2240

GTTTGATATT GGCGTATAAG GGCGGGCTAA CCAATCTCCG CTGGCTCCGA TTGAAGGCTC TATCGAGCAG CCCTCTTACC 2320

GCCTACGGCT CCAAGGTACT ATCCGTTTCG GTGCTCAGTC GCCGCACCGA GCCTTTGCTA TGTCGGCTTG CGCACAGTAA 2400

CATTTTTGGG GTATATTCTG CGACTCTGAG TAACCTTGCA TGAGATTCCG AGTAACAAAA AAGGCCTAAA AAATGCGACT 2480

GCAGCAACGG CAAACTCATC CTTTACAAGT TACATCAAAG CCCCCTCCAG TTAAAGATAT ATAAATCTCC AGATCGACCT 2560

GTACGACTAT AGCGGAAAAT GCCTATCTTT GCAGTTAGTT TTTCGGGAGA AAAACGCACA CGTGCACCCT TGTTAAAAAA 2640
                                                                              -35
ATTAACGCTT CGCGCTATTT TTTCACCAAA AGTGCTGTGT GCCAGGAGAG GGGTTGAACC CCCTCCGTGG ACCTCCCCCG 2720
 IR3            -10 IR4
GCTGGCGCCC GATGCGTCAA ACTAACAAGT CCGACGATGG CAACAAATAG CAAAAAAGGC GCGTGCTCCG CCAACATTCA 2800

GGCCGCCAAG GCCAACTGTC TGCAGCACGA TCGACGTGAC GAGAACTCCT CGAGAGTCCC GTCCTATGTC AACCACAATC 2880

TTAGCGGCCA TAACCACACC ATCTTCGAAG ATGATATGAT TAAGGGGCGA GCCTCGATTG TGCCTCTGGT GAAGAGAGCC 2960

GAGCTGCTCT ACACCGAGAA GACTGGCCAG CGATGCCAGA AGTCCTAACT CCGTTCCGTG AGGACTGTCT TTCATTGCCT 3040
                SD          ORF2 M  P  E   V  L  T   P  F  R   E  D  C  L   S  L  P
GGCCATGGCG ATATCACAGA TGACCAGATA AAGGCCTATC TGCGTAACGT CGAAGAGAAA TACAAGATTA AACCTCTCGG 3120
 G  H  G   D  I  T  D   D  Q  I   K  A  Y   L  R  N  V   E  E  K   Y  K  I   K  P  L  G
AGCTTGGTAC  3130
 A  W  Y
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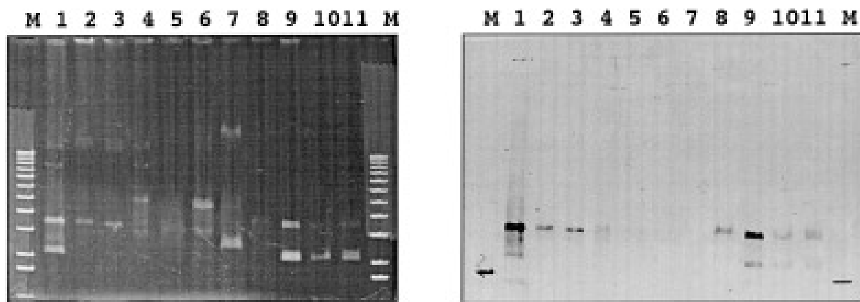

FIG. 3. Hybridization of pRAM4 to plasmids from rumen bacteria. Lane M, 1-kb DNA ladder containing the pBR322-derived 1636-bp band (BRL). Lanes 1–11, plasmids from rumen bacteria: *P. ruminicola* T31 (lane 1), *P. ruminicola* T2 (lane 2), *Butyrivibrio* sp. T10 (lane 3), *Bacteroides* sp. T3 (lane 4), *P. ruminicola* T26 (lane 5), *P. ruminicola* To124 (lane 6), *Butyrivibrio* sp. T67 (lane 7), *Bacteroides* sp. PF20-16 (lane 8), *P. ruminicola* PF20-3 (lane 9), *Bacteroides* sp. PF20-20 (lane 10), and *Bacteroides* sp. PF20-19 (lane 11).

mid isolation techniques, White and Champion (1986) were unable to demonstrate the presence of plasmid DNA in 25 *Ruminococcus* strains, including at least 3 strains reported by Kelly and Asmundson (1986) to contain plasmids. We could not find any plasmids in our 31 strains of selenomonads and 11 strains of ruminococci, and only 3 plasmid-bearing strains were observed among 154 strains of *Butyrivibrio.* Only in the *Bacteroides/Prevotella* group were there up to 10% of isolates which contained plasmids.

The plasmid pRAM4 from *P. ruminicola* had no sequence similarity with plasmids previously characterized in rumen bacteria belonging to *Selenomonas* and *Butyrivibrio* spp. (Attwood and Brooker, 1992; Hefford *et al.,* 1993; Zhang and Brooker, 1993). The putative origin of replication also does not display any significant identity with DNA sequences listed in the databases. However, replication origins do not necessarily have similarity at the DNA sequence level. Some similarity was demonstrated between ORF1 and plasmid-encoded replication proteins from several bacterial plasmids and between ORF2 and the mobilization protein of a nonreplicative *Bacteroides* insertion element NBU1 (Li *et al.,* 1993). Therefore, it is postulated that ORF1 is a plasmid replication protein and that the ORF2 locus may be involved in mobilization. Functional studies are required to confirm this hypothesis.

The pRAM4 probe hybridized with plasmids from *Bacteroides, Prevotella,* and *Butyrivibrio* species. Since a large probe was used for hybridization, it is not possible to say which particular regions are related in these plasmids. Based on our finding of similarity to replication proteins and on the hybridization of the probe to pBR322 (Fig. 3), we speculate that this similarity may be to regions from the origins of replication and/or encoding replication proteins.

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FIG. 2. Complete nucleotide sequence of pRAM4. The sequence starts at the last base of the recognition sequence of the unique *Kpn*I site. The amino acid sequences of putative ORFs are shown under the nucleotide sequence. The putative promoter regions are shown in boldface and the putative ribosome-binding sites are indicated by bold lines. Direct and inverted repeats are shown by arrows and the AT-rich region is underlined. The putative IHF-binding site, calculated using MacTagSearch Program (Goodrich *et al.,* 1990), is marked by dashed line. The GenBank Accession No. of the sequence is U30294.

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