

Isolation and Molecular Characterization of pMG160, a Mobilizable Cryptic Plasmid from *Rhodobacter blasticus*

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A 3.4-kb cryptic plasmid was obtained from a new isolate of *Rhodobacter blasticus*. This plasmid, designated pMG160, was mobilizable by the conjugative strain *Escherichia coli* S17.1 into *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodospseudomonas palustris*. It replicated in the latter strains but not in *Rhodospirillum rubrum*, *Rhodocyclus gelatinosus*, or *Bradyrhizobium* species. Plasmid pMG160 was stably maintained in *R. sphaeroides* for more than 100 generations in the absence of selection but showed segregational instability in *R. palustris*. Instability in *R. palustris* correlated with a decrease in plasmid copy number compared to the copy number in *R. sphaeroides*. The complete nucleotide sequence of plasmid pMG160 contained three open reading frames (ORFs). The deduced amino acid sequences encoded by ORF1 and ORF2 showed high degrees of homology to the MobS and MobL proteins that are involved in plasmid mobilization of certain plasmids. Based on homology with the Rep protein of several other plasmids, ORF3 encodes a putative *rep* gene initiator of plasmid replication. The functions of these sequences were demonstrated by deletion mapping, frameshift analysis, and analysis of point mutations. Two 6.1-kb pMG160-based *E. coli*-*R. sphaeroides* shuttle cloning vectors were constructed and designated pMG170 and pMG171. These two novel shuttle vectors were segregationally stable in *R. sphaeroides* growing under nonselective conditions.

The purple non-sulfur bacteria (PNSB) are an assemblage of phenotypically diverse species. Under anaerobic conditions, in the light all species grow photoheterotrophically when they are supplied with various organic substrates or photoautotrophically when CO₂ is the sole carbon source. Under microaerobic to aerobic conditions in the dark, many representatives can grow chemoheterotrophically, and some grow chemoautotrophically (34). PNSB provide useful tools for the study of carbon metabolism, including CO₂ fixation.

We have been conducting biochemical and genetic studies of the intermediary metabolism underlying the complex modes of growth in the PNSB (4, 23, 24). To do this, development of a versatile host-vector system is essential. Despite the fact that several strains of PNSB, including *Rhodobacter sphaeroides* (15), *Rhodobacter capsulatus* (20, 45), and *Rhodospirillum rubrum* (31), harbor one or more large endogenous plasmids, until recently (25) no cloning vector had been designed for PNSB by using endogenous plasmids. Instead, to date, only broad-host-range vectors, such as relatively large (10- to 20-kb) derivatives of plasmid RK2 (7, 8, 28), have been used. The main shortcoming of this approach is the intrinsic segregational instability of these elements under nonselective conditions in *R. sphaeroides* (9), *R. rubrum* (36), and *Rhodospseudomonas palustris* (25). Other vectors derived from the broad-host-range plasmid RSF1010 have likewise been constructed to enable manipulation of PNSB; these vectors include the cloning vectors pNH2 (21) and pDSK519 (28), the cosmid vector pJRD215

(41), and a *lacZ* fusion vector (33). Nevertheless, the RSF1010 derivative pDSK519 has similarly been demonstrated to be unstable under nonselective conditions in *R. sphaeroides* (this study) and in *R. palustris* (25).

In order to establish a versatile vector system to facilitate genetic analysis of PNSB, we screened numerous PNSB strains for endogenous plasmids that are relatively small. Isolation of the 15-kb cryptic plasmid pMG101 and subsequent construction of the stable 5.7-kb cloning vectors pMG103 and pMG105 have been described previously (25). These plasmids can replicate in their original host, *R. palustris*, and in closely related strains of *Bradyrhizobium* species but not in other PNSB. To meet the need for a stable cloning system for *R. sphaeroides*, using an identical strategy, we identified an endogenous plasmid in a closely related microorganism and constructed novel mobilizable and stable *R. sphaeroides*-*Escherichia coli* shuttle vectors.

In this paper we describe the isolation, mobilization function, replication range, stability, and sequence of the 3.4-kb cryptic plasmid pMG160 and construction of novel genetic tools for manipulation of *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Culture conditions. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (38). PNSB were cultivated aerobically at 30°C in van Niel's complex medium (42). *Bradyrhizobium* species were grown aerobically at 30°C in 805 medium (25). When appropriate, media were supplemented with the following antibiotics. For *E. coli*, ampicillin and kanamycin were each used at a final concentration of 50 µg ml⁻¹, and tetracycline was used at a final concentration of 25 µg ml⁻¹; for *R. palustris*, kanamycin was used at a final concentration of 200 µg ml⁻¹; and for other PNSB and *Bradyrhizobium* species, kanamycin was used at a final concentration of 50 µg ml⁻¹ and tetracycline was used at a final concentration of 10 µg ml⁻¹.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source ^a
Strains		
<i>E. coli</i> S17.1	RF4 2-Tc::Mu-Km::Tn7 <i>pro res mod</i> ⁺	9
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1Δ(lac-proAB)/F</i> [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZΔM15</i>]	Takara
<i>R. blasticus</i> TCRI-14	Natural <i>R. sphaeroides</i> isolate harboring plasmid pMG160	This study
<i>R. sphaeroides</i> ATCC 17023	Type strain	ATCC
<i>R. capsulatus</i> ATCC 33303		ATCC
<i>R. capsulatus</i> ATCC 23782		ATCC
<i>R. rubrum</i> ATCC 11170	Type strain	ATCC
<i>R. gelatinosus</i> ATCC 17011	Type strain	ATCC
<i>R. palustris</i> ATCC 17001	Type strain	ATCC
<i>R. palustris</i> No. 7	Alcohol-assimilating PNSB	17
<i>Bradyrhizobium</i> sp. strain USDA 4362	Phototrophic <i>Bradyrhizobium</i> species	USDA
<i>Bradyrhizobium</i> sp. strain USDA 4377	Phototrophic <i>Bradyrhizobium</i> species	USDA
<i>B. japonicum</i> ATCC 10324	Type strain	ATCC
Plasmids		
pHSG298	Km ^r , <i>α-lac</i> multicloning site, M13 <i>ori</i>	Takara
pHSG298X	Km ^r , <i>α-lac</i> multicloning site, M13 <i>ori</i> , additional <i>XhoI</i> site compared to pHSG298	25
pHSG299	Km ^r , <i>α-lac</i> multicloning site (reverse direction of pHSG298X and pHSG298), M13 <i>ori</i>	Takara
pRK415	Tet ^r , <i>α-lac</i> multicloning site, mobilizable (<i>oriT</i>), broad-host-range plasmid RK2 derivative	28
pDK519	Km ^r , <i>α-lac</i> multicloning site, broad-host-range plasmid RSF1010 derivative	28
pBBR1MCS2	Km ^r , <i>α-lac</i> multicloning site, broad-host-range	30
pMG103	Km ^r , <i>α-lac</i> multicloning site, <i>E. coli-R. palustris</i> cloning shuttle vector	25
pMG160	3.4-kb natural plasmid (<i>mobS</i> ⁺ <i>mobL</i> ⁺ <i>repA</i> ⁺) of <i>R. sphaeroides</i> TCRI-14	This study
pMG162	Km ^r , pMG160 linearized with <i>NaeI</i> and ligated to <i>HincII</i> -linearized pHSG298	This study
pMG162-1	Km ^r , pMG162 derivative, deletion of nucleotides 3210–3431	This study
pMG162-2	Km ^r , pMG162 derivative, deletion of nucleotides 2974–3431	This study
pMG162-3	Km ^r , pMG162 derivative, deletion of nucleotides 1–41 (the 4th base of the <i>NaeI</i> site is the coordinate origin)	This study
pMG162-4	Km ^r , pMG162 derivative, deletion of nucleotides 1–81	This study
pMG162-5	Km ^r , pMG162 derivative, deletion of nucleotides 1–121	This study
pMG162-6	Km ^r , pMG162 derivative, deletion of nucleotides 1–196	This study
pMG162-7	Km ^r , pMG162 derivative, deletion of nucleotides 1–1165	This study
pMG162-8	Km ^r , pMG162 derivative, deletion of nucleotides 1–1950	This study
pMG162-9	Km ^r , pMG162 derivative, deletion of nucleotides 1–2082	This study
pMG162-10	Km ^r , pMG162 derivative, deletion of nucleotides 1–2227	This study
pMG164	Km ^r , pMG162 derivative, deletion of nucleotides 3210–1950	This study
pMG162-11	Km ^r , pMG162 derivative, 2-bp frameshift at position 161	This study
pMG162-12	Km ^r , pMG162 derivative, 4-bp frameshift at position 992	This study
pMG162-13	Km ^r , pMG162 derivative, 2-bp frameshift at position 2218	This study
pMG162-14	Km ^r , pMG162 derivative, point mutation at positions 117 (G→A) and 119 (G→A)	This study
pMG170	Km ^r , 6.1-kb, <i>α-lac</i> multicloning site, pHSG298X ligated to pMG160	This study
pMG171	Km ^r , 6.1-kb, <i>α-lac</i> multicloning site, pHSG299X ligated to pMG160	This study
pMG170- <i>pr</i>	Km ^r , pMG170 with a 2.6-kb <i>BamHI-KpnI</i> fragment encoding <i>R. sphaeroides prr</i> genes	This study
pRK415- <i>pr</i>	Km ^r , pRK415 with a 2.6 kb <i>BamHI-KpnI</i> fragment encoding <i>R. sphaeroides prr</i> genes	This study
pBBR1MCS2- <i>pr</i>	Km ^r , pBBR1MCS2 with a 2.6-kb <i>BamHI-KpnI</i> fragment encoding <i>R. sphaeroides prr</i> genes	This study
pMG170- <i>pckA</i>	Km ^r , pMG170 with a 2.3-kb <i>BamHI-PstI</i> fragment encoding <i>R. palustris pckA</i> gene	This study
pBBR1MCS2- <i>pckA</i>	Km ^r , pBBR1MCS2 with a 2.6-kb <i>BamHI-KpnI</i> fragment encoding <i>R. palustris</i> No. 7 <i>pckA</i> gene	This study

^a ATCC, American Type Culture Collection; USDA, U. S. Department of Agriculture.

DNA techniques. Plasmid DNA was isolated by the alkaline lysis procedure (38). Restriction endonucleases, the Klenow fragment, and T4 ligase were obtained from Takara (Kyoto, Japan) and were used according to the manufacturer's instructions. *E. coli* strains were transformed by the CaCl₂ method (38). PNSB and *Bradyrhizobium* species were transformed by conjugation from *E. coli* S17.1 (9) or by electroporation (25). Restriction fragments were isolated, when required, from agarose (1%, wt/vol) gels with a GENECLEAN III kit (Bio 101, Vista, Calif.) used as recommended by the manufacturer.

Isolation of an endogenous plasmid from PNSB. PNSB were isolated from enrichment cultures under anaerobic light conditions with bubbling nitrogen by using a *Rhodospirillaceae* enrichment medium (2) containing succinate (0.05%), malate (0.05%), and acetate (0.05%) as carbon sources. Isolates were grown aerobically, and plasmid DNA was isolated as described above.

DNA sequencing. To determine the complete nucleotide sequence of plasmid pMG160, a series of pMG162 deletion derivatives were constructed by using a Kilo-sequence deletion kit (Takara) as recommended by the manufacturer. DNA was sequenced on both strands by using an automated 373A DNA sequencer (Applied Biosystems/Perkin-Elmer, Foster City, Calif.). DNA sequence data were analyzed by using the INHERIT (Applied Biosystems/Perkin-Elmer) and Genetyx (Software Development, Tokyo, Japan) programs.

Isolation and analysis of 16S rDNA. Full-length 16S ribosomal DNA (rDNA) of *Rhodobacter blasticus* strain TCRI-14 was amplified by PCR, as previously described (25). The DNA sequence of the amplified 16S rDNA was determined as described above. The rDNA sequences of strain TCRI-14 and reference strains covering a total of 1,229 nucleotide positions were aligned by using the CLUSTAL V program (19). A phylogeny was reconstructed by the neighbor-joining method (37) from *K_{nuc}* values (29). Variable domains and unidentified base positions were not considered in the analysis.

Segregational plasmid stability. Stability in *R. sphaeroides* and in *R. palustris* of pMG160 derivatives and other broad-host-range plasmids was estimated as previously described (25).

Construction of *E. coli-R. sphaeroides* shuttle cloning vectors. Plasmid pMG170 was constructed by ligating pMG160 linearized by using *NaeI* with pHSG298X linearized by using *XhoI*. The *XhoI* site of plasmid pHSG298X is located between the replication origin and the kanamycin resistance gene (25). The *XhoI* cohesive ends were blunted by using the Klenow fragment. The ligation event created two *XhoI* sites. Similarly, plasmid pMG171 was constructed by ligating the 4.2-kb *ApaLI-ClaI* fragment from pMG170 containing the pMG160 replication origin to the 1.8-kb *ApaLI-ClaI* fragment from pHSG299 (25) con-

taining the same polylinker in the β -galactosidase (*lacZ*) gene as pHSG298X but in the opposite orientation.

Plasmid copy number determination. Plasmid copy numbers were determined as previously described (25). The 2.6-kb *Bam*HI-*Kpn*I fragment containing the *prxA*, *prrB*, and *prrC* genes from *R. sphaeroides* (12, 13) was amplified by PCR by using *R. sphaeroides* chromosomal DNA as the template and oligonucleotide primers P1 (ATATGGATCCAGGCTCTCGCCCTTGGCCAG) and P2 (CTC TGGTACCAAGGTCGGACGCGGCATCCG). The PCR product was subsequently cloned into the *Bam*HI-*Kpn*I sites of plasmids pMG170, pRK415 (28), and pBBR1MCS2 (30). The resulting plasmids were designated pMG170-*prr*, pRK415-*prr*, and pBBR1MCS2-*prr*, respectively. Each chimeric plasmid was used to transform *R. sphaeroides*. Southern hybridization of total DNA from recombinant *R. sphaeroides* strains harboring plasmid pMG170-*prr*, pRK415-*prr*, or pBBR1MCS2-*prr* was performed by using as a probe the same 2.6-kb *Bam*HI-*Kpn*I fragment containing the *R. sphaeroides prxA*, *prrB*, and *prrC* genes that was used in construction of the plasmids. The probe was labeled with the DIG system (Boehringer, Mannheim, Germany). Chromosomal DNA was digested with *Bam*HI, *Kpn*I, and *Sma*I. Signal detection was performed with a Fujifilm LAS-1000 image analyzer system (Fuji, Tokyo, Japan).

The *prr* probe hybridized to a 3.0-kb *Bam*HI-*Sma*I fragment originating from the chromosome and to a 2.6-kb *Bam*HI-*Kpn*I fragment originating from plasmids pMG170-*prr*, pRK415-*prr*, and pBBR1MCS2-*prr*. Since only one copy of the *prr* gene is present in each genome, plasmid copy numbers were calculated by determining the ratio of plasmid-derived hybridization signals to chromosome-derived hybridization signals by using the Fujifilm Image Gauge program (version 3.1; Fuji).

In a similar manner, the copy numbers of plasmids pMG170 and pBBR1MCS2 in *R. palustris* were estimated by using the *R. palustris pckA* gene (25). The 2.2-kb *Bam*HI-*Pst*I fragment containing the phosphoenolpyruvate carboxykinase (*pckA*) gene from *R. palustris* No. 7 (24) was amplified by PCR performed with *R. palustris* No. 7 chromosomal DNA as the template and primers P3 (ATATG GATCCCGCGCGAAGCCGACTGGC) and P4 (CTCTCTGCAGGCGCA GATGAATCA), and it was subcloned into the *Bam*HI-*Pst*I sites of plasmid pMG170. The resulting plasmid was designated pMG170-*pckA* and was used to transform *R. palustris* No. 7. Likewise, the 2.2-kb *Bam*HI-*Spe*I fragment containing the *R. palustris* No. 7 *pckA* gene was amplified by PCR performed with *R. palustris* No. 7 chromosomal DNA as the template and primers P3 and P5 (CTCTACTAGTGCGCAGATGAATCA). The resulting PCR product was subcloned into the *Bam*HI-*Spe*I sites of plasmid pBBR1MCS2. The resulting plasmid was designated pBBR1MCS2-*pckA* and was used to transform *R. palustris* No. 7.

Southern hybridization of total DNA from *R. palustris* No. 7 harboring plasmid pMG160-*pckA* restricted with *Sma*I, *Bam*HI, and *Pst*I and Southern hybridization of total DNA from *R. palustris* No. 7 harboring plasmid pBBR1MCS2-*pckA* restricted with *Sma*I, *Bam*HI, *Pst*I, and *Spe*I were performed by using as a probe the same 2.2-kb *Bam*HI-*Pst*I fragment containing the *R. palustris* No. 7 *pckA* gene labeled with the DIG system (Boehringer). Hybridization of this probe resulted in detection of two fragments in each sample. One of these fragments corresponded to the 2.5-kb *Sma*I-*Pst*I fragment of the chromosomally encoded *pckA* gene. The second fragment corresponded either to the internal 2.2-kb *Bam*HI-*Pst*I fragment originating from plasmid pMG160-*pckA* or to the internal 2.2-kb *Bam*HI-*Spe*I fragment originating from plasmid pBBR1MCS2. Hybridization signals were detected with a Fujifilm LAS-1000 image analyzer system (Fuji). As described above, plasmid copy numbers were calculated by determining the ratio of the two hybridization signals from each sample by using the Fujifilm Image Gauge program (version 3.1; Fuji).

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers for the sequences described in this paper are AB082959 (plasmid pMG160), AB017799 (strain TCRI-14 rDNA), AB082960 (plasmid pMG170), and AB082961 (plasmid pMG171).

RESULTS

Isolation of plasmid pMG160 and main morphological characteristics of its host strain. The screening method which was previously employed to isolate novel PNSB strains is biased towards isolation of *R. palustris* that can utilize alcohol (25). As a result, with the aim of isolating cryptic plasmids that replicate in other genera of the PNSB group, we repeated the screening procedure using enrichment media based on succinate, malate, and acetate as carbon sources and on bubbling

nitrogen gas during anaerobic cultivation. In particular, this approach takes advantage of the nitrogen fixation properties of PNSB. We isolated various PNSB strains by searching for small plasmids (<20 kb). Approximately 100 strains were isolated from sewage, rice fields, and other places. One of these strains, TCRI-14, harbored an endogenous plasmid that was approximately 3.4-kb long. This plasmid was designated pMG160.

Strain TCRI-14 is a gram-negative, nonsporulating rod with rounded ends. The cells divide by binary fission. Cultures are red to brown-red under anaerobic conditions in the light and pale pink to white under aerobic conditions in the dark. The in vivo spectrum of cell suspensions grown anaerobically in the light had absorption maxima at 375, 590, 805, and 860 nm, which is characteristic of the presence of bacteriochlorophyll *a*. Likewise, the spectrum had absorption maxima at 450, 480, and 510 nm, demonstrating that carotenoids of the normal spheroidene series were present (34). These results indicate that strain TCRI-14 is a member of the genus *Rhodobacter*.

In order to confirm the phylogenetic position of strain TCRI-14, we determined the sequence of 1,439 nucleotides of the rDNA of this strain. The resulting sequence was compared with known 16S rDNA sequences, as described previously (25) (Fig. 1). Strain TCRI-14 formed a tight phylogenetic cluster with *R. blasticus*, *R. sphaeroides*, and *R. capsulatus* and was less closely related to other members of the PNSB or to *Bradyrhizobium* and *Rhizobium* species. A bootstrap analysis confirmed the monophyly of the strain TCRI-14 cluster in 100% of the trees generated (14). These results confirm that strain TCRI-14 belongs to the genus *Rhodobacter* and that it is phylogenetically closely related to *R. sphaeroides* and *R. capsulatus*. The near identity (98%) of its 16S rDNA sequence with that of *R. blasticus* ATCC 33485 (GenBank accession number D16429) indicates that strain TCRI-14 is a strain of *R. blasticus* (synonyms, *Rhodopseudomonas blastica* and *Rhodobacter blastica* [27, 35]).

Replication range, mobilization, and stability of plasmid pMG160. Plasmid pMG160 is relatively small compared to known endogenous plasmids originating from bacteria belonging to the PNSB group. A restriction map of plasmid pMG160 was generated, and the shuttle vector pMG162 was constructed by linearizing pMG160 plasmid DNA with *Nae*I and ligating it to *Hinc*II-linearized and blunt-ended plasmid pHSG298 DNA. Plasmid pHSG298 does not replicate in PNSB. The ligation mixture was used to transform *E. coli*. PNSB strains were transformed both by electroporation and by conjugation with *E. coli* S17.1. Plasmid pMG162 could replicate in *R. sphaeroides* ATCC 17023, in *R. capsulatus* ATCC 33303 and ATCC 23782, and in *R. palustris* No. 7 (17) and ATCC 17001. On the other hand, no kanamycin-resistant transformants or transconjugants were recovered from *R. rubrum* ATCC 11170, *R. gelatinosus* ATCC 17011, *Bradyrhizobium japonicum* ATCC 10324, or phototrophic *Bradyrhizobium* strains USDA 4362 and USDA 4377. Transformability of all of these strains except *R. gelatinosus* ATCC 17011 (which we did not previously test) with a kanamycin resistance-conferring replicative plasmid has been reported elsewhere (9, 25, 36), corroborating the view that pMG162 does not replicate in these organisms. In addition, while plasmid pMG162 was shown to be mobilizable by the conjugative strain *E. coli* S17.1, no mobilization was observed from *E. coli* strain JM109, demonstrating that the *tra*

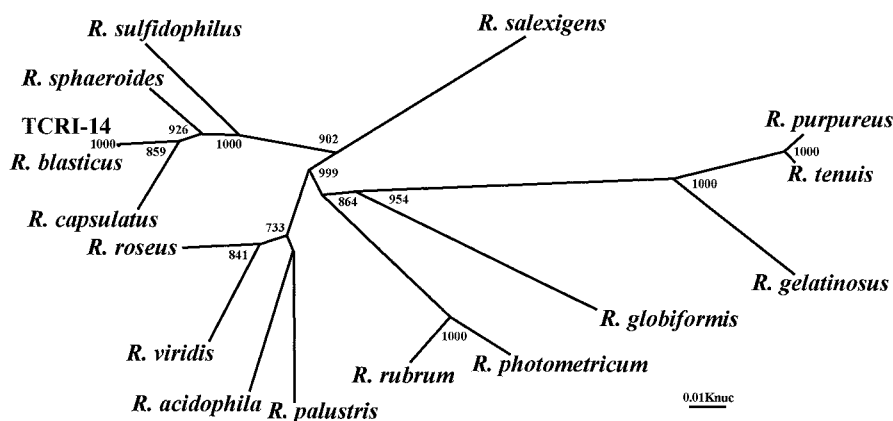


FIG. 1. Unrooted distance matrix tree showing phylogenetic relationships of selected strains of proteobacteria based on 16S rDNA sequences. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1,000 replicates. The numbers at the nodes indicate bootstrap percentages based on 1,000 replicates. The accession numbers for the sequences used are as follows: strain TCRI-14, AB017799; *Rhodobacter sphaeroides*, D16425; *Rhodobacter capsulatus*, D16428; *Rhodobacter blasticus*, D16429; *Rhodopseudomonas palustris*, D25312; *Rhododiplanes roseus*, D25313; *Rhodopseudomonas acidophila*, M34128; *Rhodopseudomonas viridis*, D25314; *Rhodospirillum photometricum*, D30777; *Rhodophila globiformis*, D86513; *Rhodobacter sulfidophilus*, D16430; *Rhodospirillum rubrum*, D30778; *Rhodospirillum salexigens*, M59070; *Rhodocyclus tenuis*, D16208; *Rhodocyclus purpureus*, M34132; and *Rhodocyclus gelatinosus*, D16214.

gene products present in *E. coli* S17.1 are necessary for the mobilization process to occur.

In order to assess the stability of plasmid pMG160, *R. sphaeroides* and *R. palustris* cultures containing pMG162 were grown under nonselective conditions. The broad-host-range plasmids pDK519 (28), pRK415 (28), and pBBR1MCS2 (30) in *R. sphaeroides* and in *R. palustris* were used as controls for segregational instability. Plasmid pMG103 in *R. palustris* was used as a positive control for plasmid stability (25). We observed that pMG162 was stably maintained in *R. sphaeroides* for more than 150 generations but was unstable (0% maintenance) in *R. palustris* after 30 generations (Fig. 2). Although pBBR1MCS2 showed moderate stability in *R. sphaeroides* (56% maintenance after 100 generations), pBBR1MCS2 was unstable in *R. palustris* and all the other broad-host-range plasmids tested in this study were unstable in both organisms (Fig. 2). On the other hand, as previously observed, pMG103 was segregationally stable in *R. palustris* for more than 100 generations (25). Nevertheless, pMG162 in *R. palustris* and all the broad-host-range

plasmids discussed above were stably maintained in both hosts when selective pressure (kanamycin or tetracycline challenge) was applied. The observed differences in stability could perhaps be ascribed to differences in copy number and in host factors in these strains, since interactions between host- and plasmid-encoded factors intervene in the plasmid replication process (43).

DNA sequence of 3.4-kb plasmid pMG160. The complete nucleotide sequence (3.4 kb) of pMG160 was determined in order to define the plasmid-partitioning, replication, and mobilization functions. The G+C content of plasmid pMG160 was 67.0%, which is similar to the values previously reported for the genomes of *R. sphaeroides* (68.4 to 69.9%) and *R. capsulatus* (65.5 to 66.8%) (22). Computer analysis revealed the presence of three open reading frames (ORFs), ORF1, ORF2, and ORF3.

ORF1 (positions 136 to 438) consists of 303 nucleotides, corresponding to a gene product 100 amino acids long with a predicted molecular weight of 10,636. The deduced amino acid sequence shows low but significant levels of similarity to the sequences of the *mobS* gene product of plasmid pTF1 from *Thiobacillus ferrooxidans* (31.2%) (11) (accession no. X52699) and the *mobC* gene product of the broad-host-range plasmid RSF1010 (27.9%) (40) (accession no. M28829). ORF1 was therefore designated the *mobS* gene. Upstream of the pMG160 *mobS* gene, there is a 38-bp sequence (positions 89 to 126) homologous to the minimal 38-bp transfer origin (*oriT*) of plasmids pTF1 (sequence similarity, 67.6%) (11) and RSF1010 (60.0%) (3). The 38-bp pMG160 DNA has a palindromic sequence, as observed in the pTF1 and RSF1010 *oriT* sequences (11). Furthermore, there is significant conservation in pMG160, pTF1, and RSF1010 of the sequences in the immediate vicinity of the specific nick sites reported for pTF1 and RSF1010 by Scherzinger et al. (39) (Fig. 3).

ORF2 (positions 431 to 1,855) has an 8-bp overlap with the 3' end of the putative *mobS* gene. It is transcribed in the same direction, and it comprises 1,425 nucleotides corresponding to

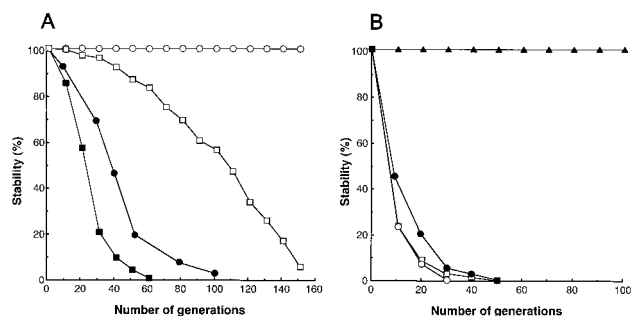


FIG. 2. Segregational stability in *R. sphaeroides* of plasmids pMG162, pBBR1MCS2, pDK519, and pRK415. The stabilities of plasmid pMG162 (○) and the broad-host-range plasmids pBBR1MCS2 (□), pDK519 (●), and pRK415 (■) in *R. sphaeroides* (A) and in *R. palustris* (B) under nonselective conditions were determined. Plasmid pMG103 (▲) was used as a stable plasmid control in *R. palustris* (25).

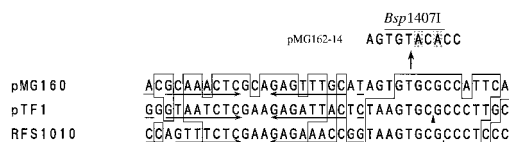


FIG. 3. Comparison of the putative *oriT* sequence of pMG160 with the 38-bp minimal *oriT* sequences of pTF1 and RFS1010. The putative *oriT* sequence of pMG160 was aligned with the 38-bp minimal *oriT* region of pTF1 and RFS1010. Nucleotides that were identical in at least two of the three sequences are enclosed in boxes. The possible palindromic sequence is indicated by the converging arrows. The arrowheads indicate the specific nick sites of the plasmids. The 2-bp point mutation in the putative *oriT* sequence in plasmid pMG162-14 is indicated above the sequence alignment. The nucleotides that were replaced are indicated by shading. A line above the mutated sequence indicates the location of the newly created *Bsp1407I* site.

474 amino acids with an estimated molecular weight of 51,209. The deduced amino acid sequence indicates that there are low but significant levels of similarity along the length of the protein to the sequences of the *mobL* gene product of plasmid pTF1 from *T. ferrooxidans* (27.8%) (11) (accession no. X52699) and the *mobA* (or *repB*) gene product of the broad-host-range plasmid RFS1010 (22.4%) (40) (accession no. M28829). In addition, approximately 200 amino acids at the amino terminus of the ORF2 product also showed significant levels of similarity to the amino terminus of the *traA* gene product of plasmid pMRC01 from *Lactococcus lactis* (33.3%) (10) (accession no. AE001272), to the *nes* gene products of plasmid pSK41 (32.0%) (1) (accession no. AF051917) and plasmid pGO1 (32.0%) (5) (accession no. U50629) from *Staphylococcus aureus*, to the *traA* gene product of plasmid pNGR234a from *Rhizobium* sp. strain NGR234 (30.3%) (16) (accession no. P55418), and to the *orfI* gene product of plasmid pIP501 from *Streptococcus agalactiae* (30.2%) (44) (accession no. L39769). All these proteins are putative *oriT* nickases that facilitate strand transfer of conjugative plasmids to recipient cells, and all the corresponding genes are neighbors of the *oriT* sequences in the corresponding plasmids. ORF2 was designated the *mobL* gene. However, for the three Mob proteins and the five *oriT* nickases, specific conserved regions were not observed (data not shown). Downstream of the putative *mobL* gene, there is an imperfect inverted repeat (positions 1,877 to 1,907) which has a GC-rich stem-loop structure.

ORF3 is located downstream of the putative *mobL* gene (positions 2,136 to 3,155). It is transcribed in the same direction as the *mob* genes, and it consists of 1,020 nucleotides, corresponding to 339 amino acids with a predicted molecular weight of 37,588. The deduced amino acid sequence exhibits low but significant levels of similarity to the sequences of the *rep* gene products of plasmid pMO1 from *Francisella tularensis* (23.6%) (accession no. AF055345), plasmid pEA29 from *Erwinia amylovora* (23.2%) (32) (accession no. AF264948), plasmid pFA3 from *Neisseria gonorrhoeae* (20.5%) (18) (accession no. M31727), and plasmid pYC from *Yersinia pestis* (18.1%) (accession no. AF152 923). ORF3 was therefore designated the *repA* gene. However, comparative amino acid sequence alignment revealed that there are no specific conserved regions in the five Rep proteins (data not shown). Upstream of the pMG160 *repA* gene, there are three 16-bp perfect direct re-

peats (GGGAGAAATGGGGCCG; positions 2,031 to 2,088), which are separated by 5-bp spacers (CAAAA). Downstream of the pMG160 *repA* gene, we observed an imperfect inverted repeat (positions 3,345 to 3,378) constituting a GC-rich stem-loop structure.

Localization of the replication, stability, and mobilization regions. A series of deletion mutants, frameshift mutants, and point mutants of plasmid pMG162 were constructed (Fig. 4) in an attempt to identify the replication, stability, and mobilization regions of pMG160. These plasmids were used to transform *R. sphaeroides* by electroporation as a way to determine the functionality of the replication regions. The mobilization properties of the mutants were estimated by conjugation by using the conjugative strain *E. coli* S17.1. Plasmid pHSG298, which contains an M13 replication origin, was not able to propagate in *R. sphaeroides* and thus was used in these experiments as a negative control. Plasmid pMG162 was used as a positive control for plasmid replication.

Plasmid pMG162-1, containing a 3,210-bp fragment (positions 1 to 3,210) but lacking the region downstream of the *repA* gene that includes a GC-rich stem-loop structure, can replicate in *R. sphaeroides* like the original plasmid, pMG162. Moreover, it is also mobilizable from *E. coli* S17.1 into *R. sphaeroides*. On the other hand, plasmid pMG162-2, containing a 2,974-bp fragment (positions 1 to 2,974) that constitutes a deletion mutation in the *repA* carboxy terminus, was not able to replicate in *R. sphaeroides*. A series of additional deletion plasmids were constructed (Fig. 4). Plasmids pMG162-3 to pMG162-8, all of which included an intact *repA* gene and the three-direct-repeat structure, could replicate in *R. sphaeroides*. However, plasmid pMG162-9, which contains a 1,350-bp fragment (positions 2,082 to 3,431) with an intact *repA* gene but lacks the three-direct-repeat structure, as well as plasmid pMG162-10, which contains a 1,205-bp fragment (positions 2,227 to 3,431) comprising *repA* gene sequences except the amino terminus, could not replicate in *R. sphaeroides*. In addition, a frameshift mutation created by insertion of 2 bp into the *AccI* site in the *repA* sequence in plasmid pMG162 prevented replication in *R. sphaeroides*. The resulting frameshift plasmid, pMG162-13, was constructed by digestion of pMG162 with *AccI*, followed by blunting with the Klenow fragment and ligation. The data obtained suggest that the *repA* gene product and its upstream region, including the three-direct-repeat structure, are essential for pMG160 replication. Moreover, the observation that plasmid pMG164 containing a 1,261-bp fragment (positions 1,950 to 3,210) replicates in *R. sphaeroides* but pMG162-10 does not replicate in *R. sphaeroides* precisely mapped the replication origin and the minimum region necessary for plasmid replication. In these experiments, all the plasmids that were able to replicate in *R. sphaeroides* also showed segregational stability (100% maintenance) under nonselective conditions. This observation suggests that the replication and stability functions of this plasmid are linked.

Although plasmid pMG162-3, which contains a 3,391-bp fragment (positions 41 to 3,431) but lacks 40 bp from the *NaeI* site upstream of the putative *oriT* sequence, can be mobilized from the conjugative strain *E. coli* S17.1 into *R. sphaeroides*, plasmid pMG162-4, which contains a 3,351-bp fragment (positions 81 to 3,431) with a similar (from the *NaeI* site) but longer 80-bp deletion, did not exhibit any mobilization. Like-

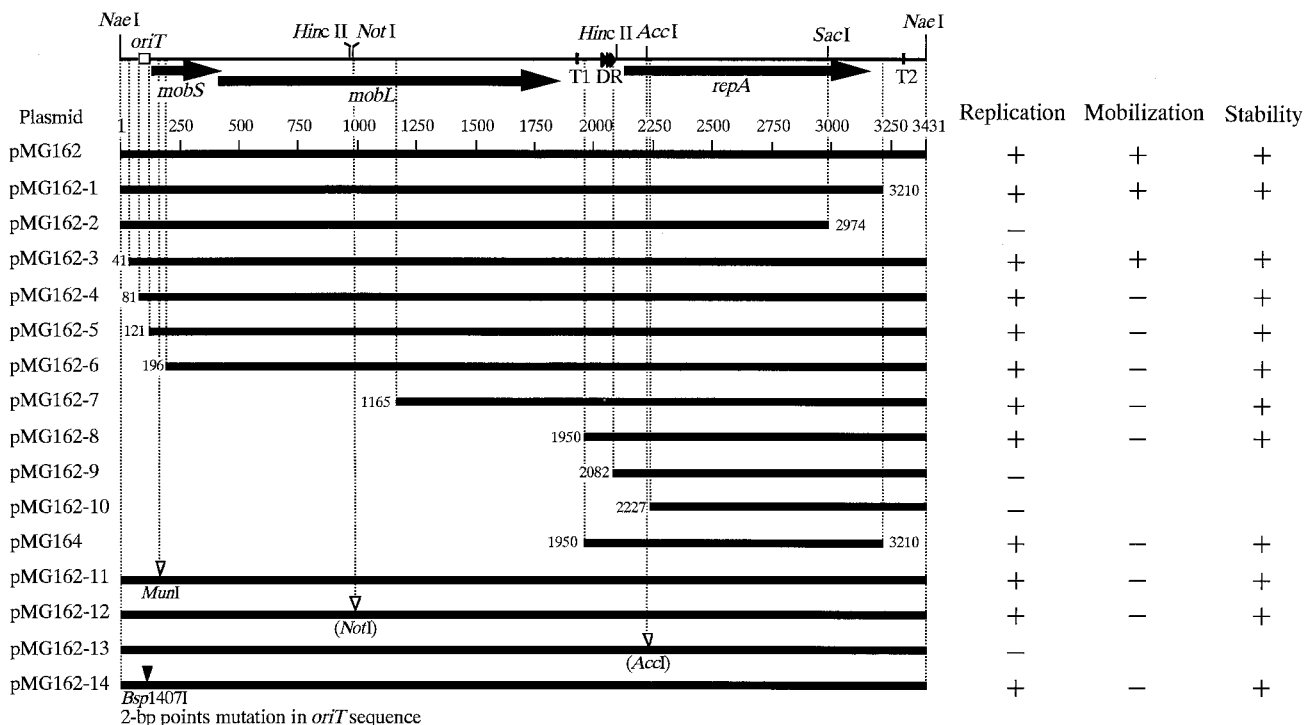


FIG. 4. Deletion mapping and gene disruption analysis of plasmid pMG160. Deletion mapping and gene disruption analysis of plasmid pMG160 were carried out in order to identify the mobilization, stability, and replication regions. A partial restriction cleavage map of pMG160 is shown at the top. The arrows indicate the *mobS*, *mobL*, and *repA* genes. The solid arrowheads indicate direct repeats. The putative *oriT* sequence is indicated by an open box. Two GC-rich stem-loop structures (T1 and T2) are indicated by vertical lines. The scale is graduated (in base pairs) from the left (*NaeI* site) to the right (*NaeI* site), corresponding to the nucleotide sequence of the 3,431-bp *NaeI*-*NaeI* fragment. Plasmid pMG162 derivatives comprising deletion and frameshift mutants are listed on the left. A horizontal line represents each fragment. The coordinates (in base pairs) of the deletion mutations are shown at the ends of the lines. The open arrowheads indicate the positions (in base pairs) of frameshift mutations, and the solid arrowheads indicate the positions (in base pairs) of point mutations. Construction of these plasmids is described in Table 1. Replication was considered positive when kanamycin-resistant transformants grew following electrotransformation and when the presence of episomic DNA was confirmed by alkaline lysis extraction of plasmid DNA followed by gel electrophoresis. Replication was considered negative when no transformants were obtained in three repeat experiments. Mobilization experiments were carried out as described in Materials and Methods. Plasmid mobilization was confirmed by using a scheme similar to that used to demonstrate replication. Plasmid stability in *R. sphaeroides* was evaluated as described in Materials and Methods. Stability represents 100% plasmid maintenance.

wise, plasmid pMG162-5 bearing a 3,311-bp fragment (positions 121 to 3,431) which lacked a portion of the putative *oriT* sequence and plasmid pMG162-6 carrying a 3,236-bp fragment (positions 196 to 3,341) with a deletion in both the putative *oriT* sequence and the amino terminus of the *mobS* gene could not be mobilized. The view that both the *mobS* and *mobL* gene products are essential for pMG160 mobilization was supported by the results of frameshift mutation experiments. Using PCR, we inserted into plasmid pMG162 a 2-bp frameshift mutation that generated a new and unique *MunI* site within the *mobS* gene (positions 161 to 170; CTGTCATGGT was changed to CTGTC AATTGGT). Likewise, we created another frameshift mutation in this plasmid by inserting 4 bp into the *NotI* site of the *mobL* sequence by digestion of pMG162 with *NotI*, followed by blunting with the Klenow fragment and ligation. The mutations in the resulting plasmids, pMG162-11 and pMG162-12 (Fig. 4), respectively, eliminated the mobilization property. Moreover, using PCR, we introduced a 2-bp point mutation into the putative *oriT* sequence of pMG162 by replacing two guanines with two adenines. The latter mutation could be detected by the presence of a new and unique

Bsp1407I site (Fig. 3 and 4). As expected, the resulting pMG162-14 plasmid could not be mobilized. Plasmids pMG162-11, pMG162-12, and pMG162-14 replicated in *R. sphaeroides*, as demonstrated by the appearance of transformants following electroporation and cultivation on selective medium. These data suggest that the *mobS* and *mobL* gene products, as well as the putative *oriT* sequence, play important roles in the mobilization function of plasmid pMG160.

Construction of *E. coli*-*R. sphaeroides* shuttle cloning vectors. To develop a versatile host-vector system, we constructed the *E. coli*-*R. sphaeroides* shuttle cloning vectors pMG170 and pMG171. Both of these plasmids are 6,068 bp long and contain a kanamycin resistance marker. They were constructed as described in Materials and Methods. These shuttle vectors have a polylinker containing unique *EcoRI*, *KpnI*, *BamHI*, *XbaI*, *SalI*, *PstI*, *Sse8387I*, and *SphI* sites in the β -galactosidase (*lacZ α*) gene. By using a host such as *E. coli* JM109 plated on isopropyl- β -D-thiogalactopyranoside (IPTG)-5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) agar plates, insertional inactivation could be used to identify recombinant plasmids (Fig. 5). We verified that both pMG170 and pMG171

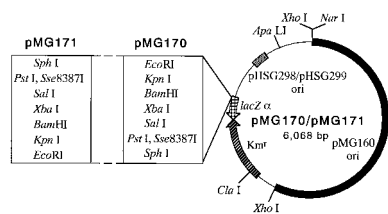


FIG. 5. Physical and genetic maps of pMG170 and pMG171. *lacZ* α , β -galactosidase α -peptide gene from pHSG298 or pHSG299; Km^r , kanamycin resistance gene; pMG160 *ori*, the 3.4-kb *NaeI-NaeI* fragment of whole pMG160 DNA including the *mobS*, *mobL*, and *repA* genes; pHSG298 *ori* and pHSG299 *ori*, replication origins of pHSG298 and pHSG299, respectively. The arrows indicate the directions of transcription. Unique restriction sites are shown. There are two *XhoI* sites in these plasmids. Compared to pMG170, in plasmid pMG171 the *lacZ* α multicloning site is inverted.

were able to replicate in *R. sphaeroides*, that they could be mobilized from *E. coli* S17.1 into *R. sphaeroides*, and that they were maintained in *R. sphaeroides* for more than 100 generations without selective pressure.

In order to estimate the copy number of pMG170 in *R. sphaeroides* and *R. palustris*, plasmid pMG170-*pr*r containing the *R. sphaeroides prr* genes and plasmid pMG170-*pckA* including the *R. palustris pckA* gene were constructed. Only single copies of both the *pr*r and *pckA* genes are present in the corresponding original genomes (25). As control plasmids we used pBBR1MCS2-*pr*r and pRK415-*pr*r for copy number estimation in *R. sphaeroides* and pBBR1MCS2-*pckA* for copy number estimation in *R. palustris*. Southern blotting of *R. sphaeroides* total DNA harboring the various plasmids was conducted by using the 2.6-kb *Bam*HI-*Kpn*I PCR product encoding the *prrABC* genes as a probe (Fig. 6). The plasmid-derived and chromosome-derived hybridization signals were determined. The corresponding ratios suggested that the number of pMG170 copies per chromosome is in the range from 18 to 23 in *R. sphaeroides*. In this species, the number of copies of the broad-host-range plasmid pBBR1MCS2 was found to be in the range from 9 to 14, while the number of copies of the RK2 derivative pRK415 was in the range from 4 to 7, suggesting that there is a strong correlation between copy number and stability. Similarly, by using the *pckA* probe, the numbers of copies of plasmids pMG170 and pBBR1MCS2 were determined in *R. palustris* to be two or three and one, respectively.

DISCUSSION

We isolated a previously undiscovered strain of *R. blasticus*, strain TCRI-14, that harbors a 3.4-kb cryptic plasmid. This plasmid, designated pMG160, encodes replication, mobilization, and stability functions. Each of the genes and the origin of transfer were precisely mapped by deletion analysis, by analysis of frameshift and point mutations, and by gene sequencing. Plasmid pMG160 is a narrow-range genetic element that replicates and is stable in *R. sphaeroides* but shows 96.5% stability after 100 generations in *R. capsulatus*. On the other hand, it is highly segregationally unstable in *R. palustris*, and it does not propagate in *R. rubrum*, *R. gelatinosus*, *B. japonicum*, or phototrophic *Bradyrhizobium* strains. The whole episome was used to construct two *E. coli-R. sphaeroides* cloning shuttle vectors,

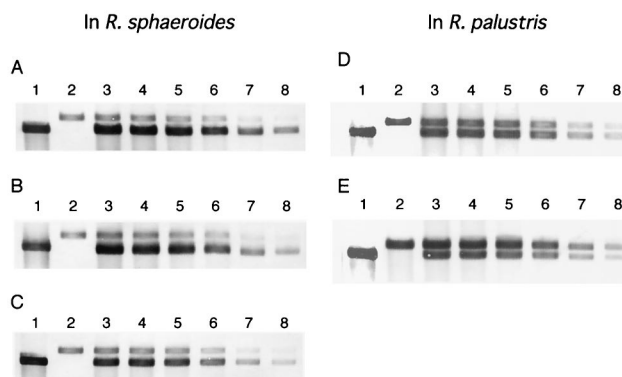


FIG. 6. Numbers of copies of plasmid pMG170 in *R. sphaeroides* and *R. palustris*. Plasmid copy numbers were determined by Southern blot hybridization analysis. Copy numbers were determined for pMG170 in *R. sphaeroides* (A) and in *R. palustris* (D), for pBBR1MCS2 in *R. sphaeroides* (B) and in *R. palustris* (E), and for pRK415 in *R. sphaeroides* (C). Total DNA from the *R. sphaeroides* wild-type and *R. sphaeroides* transformants harboring plasmid pMG170-*pr*r, pBBR1MCS2-*pr*r, or pRK415-*pr*r was digested with *Bam*HI, *Kpn*I, and *Sma*I. Similarly, total DNA from wild-type *R. palustris* and *R. palustris* transformants containing pMG170-*pckA* or pBBR1MCS2-*pckA* was digested with *Bam*HI, *Pst*I, and *Sma*I. Lanes 1, 2.6-kb PCR fragment containing the *prrA*, *prrB*, and *prrC* genes from *R. sphaeroides* (A, B, and C) and the 2.2-kb PCR fragment containing the *pckA* gene from *R. palustris* (D and E); lanes 2, *Bam*HI-*Sma*I-digested *R. sphaeroides* wild-type chromosomal DNA (A, B, and C) and *Sma*I-*Pst*I-digested *R. palustris* wild-type chromosomal DNA (D and E); lanes 3 to 8, 1, 0.75, 0.5, 0.25, 0.1, and 0.05 μ g of *Bam*HI-*Sma*I-*Kpn*I-digested total DNA, respectively, from *R. sphaeroides* harboring plasmid pMG170-*pr*r (A), pBBR1MCS2-*pr*r (B), or pRK415-*pr*r (C) and from *R. palustris* harboring plasmid pMG170-*pckA* (D) or pBBR1MCS2-*pckA* (E). In each gel, the upper band corresponds to chromosomal DNA fragments, whereas the lower bands correspond to episomal DNA fragments.

pMG170 and pMG171, that are segregationally stable in *R. sphaeroides* without selective pressure. Isolation of plasmid pMG160 thus yielded a versatile host-vector system that can be used to facilitate genetic manipulation of *Rhodobacter* species, combining stability and mobilization properties.

The sequence analysis results suggested that pMG160 is a novel plasmid belonging to a new plasmid family since there are only low levels of similarity between its *mobS*, *mobL*, and *repA* genes and those of other plasmids, including *Rhodobacter* plasmids. Alternatively, it may be worthwhile to refine our understanding of the roles that these various *mob* genes may have in plasmid activity. Given the conservation of the 38-bp *oriT* with a palindromic sequence, the mechanisms underlying the mobilization process of pMG160 appear to be akin to the mechanisms for transfer of plasmid pTF1 originating from *T. ferrooxidans* (11) and of broad-host-range plasmid RSF1010 (40).

The three ORFs encoded by pMG160 are transcribed in the same direction, and *mobS* and *mobL* are putatively cotranscribed. A putative transcription terminator consisting of a GC-rich stem-loop structure is present downstream of *mobL*. Both *mobS* and *mobL*, as well as the origin of transfer *oriT*, are necessary for mobilization to occur. On the other hand, replication and stability functions are linked. The *repA* gene comprises 1,020 bp with a putative terminator consisting of a GC-rich stem-loop structure downstream of its stop codon. The

repA gene product exhibits low but significant levels of similarity with its counterparts in a variety of plasmids. Upstream of the pMG160 *repA* gene are three typical iterons (16-bp direct repeats) necessary for pMG160 replication. Iterons have been shown to represent binding sites for Rep proteins and to play a role in plasmid copy number control. In addition, upstream of the iterons is an AT-rich region (63% A+T, coordinates 1932 to 2018). A genetic organization consisting of replicons controlled by iterons has been observed for numerous broad-host-range vectors that are stably maintained. AT-rich regions are genetic elements that are associated with strand opening and assembly of host initiation factors. These features are general features of replication via a theta mechanism (6). Moreover, plasmids pEA29 and pFA3 (whose RepA proteins exhibit low but significant levels of homology with the RepA protein of pMG160) have a similar organization in their replicons, as exemplified by the presence of a cluster of direct repeats and the presence of an AT-rich region located upstream of the iterons (18, 32). In addition, plasmid pMG160 and plasmid pEA29 lack the typical domains displayed by plasmids replicating via a rolling-circle mechanism often characterized by structural and segregational instability problems (26). These observations are consistent with a theta structure-mediated replication mechanism for both pEA29 (32) and pMG160. The numbers of copies of the pMG160 derivatives pMG170 and pMG171 are different in *R. sphaeroides* and in *R. palustris*. Differences in the numbers of copies, in addition to host factors, may account for the observed differences in stability in these two closely related organisms.

Despite the drawback that the host range is relatively narrow, because of their intrinsic properties pMG170 and pMG171 are ideal cloning vectors for genetic manipulation of *Rhodobacter* species. These two novel *E. coli*-*Rhodobacter* shuttle vectors can be mobilized from a conjugative strain. They bear the ColE1 replication origin for high-copy-number replication in *E. coli* and the pMG160 replication origin for replication in *R. sphaeroides* and *R. palustris*, and the numbers of copies in these species are 18 to 23 and 2 or 3, respectively. Moreover, these two vectors contain a polylinker that enables insertional inactivation of the *lacZ* gene. Importantly, in the absence of selective pressure they exhibit 100% segregational stability in *R. sphaeroides* and thus are ideal plasmids for fundamental research on *R. sphaeroides* and its industrial microbiology.

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