JOURNAL OF BACTERIOLOGY, Jan. 1994, p. 419–425
0021-9193/94/$04.00+0

Vol. 176, No. 2

Analysis of the Replication Region of a Mycobacterial Plasmid, pMSC262

MINGHUI QIN, HATSUMI TANIGUCHI* AND YASUO MIZUGUCHI†

Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Iseigaoka, Yahatanishiku, Kitakyushu 807, Japan

Received 10 May 1993/Accepted 12 November 1993

We determined the nucleotide sequence of a DNA fragment which contains the replication region of pMSC262, a *Mycobacterium scrofulaceum* plasmid used to construct the *Mycobacterium-Escherichia coli* shuttle vector. The complete sequence of the fragment contained 2,504 bp with an overall G+C content of 69.8%. By deletion analysis, we found that the minimum length required for plasmid replication in *M. bovis* BCG was about 1.6 kb. Within this region, several open reading frames (ORFs) and a putative replication origin (*ori*) were identified by computer analysis. One of the ORFs, ORF2, which encodes a putative 28.9-kDa basic protein with characteristics of DNA-binding proteins, appeared to be involved in replication of the plasmid in BCG. By separation of ORF2 and the putative *ori* region, it was revealed that the relative locations of ORF2 and the putative *ori* region are likely important for replication in BCG. No DNA or amino acid homologies were found between this replication region and that of pAL5000, another mycobacterial plasmid used for vector plasmid construction. In addition, we found that this replicon did not lead to replication in *E. coli* and was compatible in BCG with pAL5000-derived vector plasmid pYUB75 (R. G. Barletta, D. D. Kim, S. B. Snapper, B. R. Bloom, and W. R. Jacobs, J., J. Gen. Microbiol. 138:23–30, 1992).

The members of the genus *Mycobacterium* are well recognized as an important class of pathogens in humans and animals. Leprosy, tuberculosis, and increasing incidences of mycobacterioses other than tuberculosis among patients with AIDS have further underscored the necessity of research aimed towards both understanding the basis of pathogenicity of mycobacteria and developing tools for the prevention of mycobacterial diseases.

Compared with that of *Escherichia coli*, the genetic knowledge of mycobacteria is sparse and the availability of genetic systems for the study of mycobacteria is limited. Recently, however, construction of shuttle vectors capable of replication in both mycobacteria and *E. coli* have been reported by several investigators (6, 7, 11, 12, 17, 18, 24–26, 29). Most of them were the hybrid plasmids (18, 24, 25, 29) generated from pAL5000 (19), a plasmid from rapidly growing *Mycobacterium fortuitum*, and an *E. coli* plasmid. Although several reports regarding the sequence, functional analysis, and host range of this plasmid have been published, the detailed mechanism of its replication in mycobacteria is still unclear.

In a previous report (5), we described another shuttle vector plasmid, pYT937, which was constructed by inserting a 2.3-kb replication region of plasmid pMSC262 from slowly growing *M. scrofulaceum* into *E. coli* plasmid pACYC177. Because the nature of mycobacterial plasmid replication and the mechanism of its controls are not understood in any detail, it is important to learn about the structure of this region and how replication is carried out in mycobacteria.

In this study, to analyze the replication mechanism of recombinant plasmid pYT937 in *M. bovis* BCG, we determined the nucleotide sequence and the minimal region required for replication and investigated the relationship between the putative *ori* region and a putative Rep protein-coding gene in the replication region of pMSC262. Also, we investigated the compatibility between our vector plasmid, pYT937, and pYUB75, a plasmid derived from pAL5000, and the host range of this 2.3-kb replicon of pMSC262 in mycobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, media, and antibiotics. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriophages M13mp18 and M13mp19 were used as DNA-sequencing vectors (28). *E. coli* strains were grown in 2YT medium or on nutrient agar (14), and mycobacterial strains were grown in 7H9 broth or on 7H10 agar or on 1% Ogawa egg slants. As antibiotics, kanamycin, tetracycline, ampicillin, and chloramphenicol were used at a final concentration of 50 μg/ml.

Cloning and sequencing of DNA. Standard procedures were used for cleavage, ligation, and transformation of DNA (20). To determine the nucleotide sequence of the replication region derived from mycobacterial plasmid pMSC262, pYT935 was employed instead of pYT937 (5; Fig. 1). This plasmid contains a 2.5-kb *Hin*cII-*Pst*I fragment of the replication region of pMSC262 and is more convenient than a 2.3-kb *Hin*dIII-*Pst*I fragment of pYT937 when the commercially available digestion kit is used. The 2.5-kb *Pst*I-*Hin*cII fragment was excised from pYT935 and cloned into M13mp18 and M13mp19 phage vectors. By using restriction endonuclease sites and an exonuclease III-mung bean nuclease digestion kit (Takara Shuzo Co. Ltd., Kyoto, Japan), sets of deletion derivatives were constructed. Furthermore, as spontaneous deletion and rearrangements occurred around the *Sph*I site, we obtained deletion fragments from the *Pvu*II site, by using the kit described above, and also smaller fragments (<0.5 kb) by using *Nar*I (nucleotide positions [np] 29, 270, and 1144), *Hin*dIII (np 210), *Sph*I (np 617), *Pvu*I (np 878 and 1486), and *Pvu*II (np 1231) restriction endonucleases in the *Pvu*II-*Hin*dIII region (Fig. 2). We determined the nucleotide sequences of these fragments on both strands by the dideoxynucleotide chain termination method

* Corresponding author. Phone: 093-691-7242. Fax: 093-602-4799.
† Present address: Public Health Laboratory of Chiba Prefecture, Nitonacho, Chu-O-Ku, Chiba 260, Japan.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) or genotype	Source and/or reference
Strains		
E. coli K-12		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ (*lac-proAB*)/F′ (*traD36 proAB*$^+$ *lacZ*ΔM15)	20
JG112	*polA rpsL thy met*	T. Miki; 13
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	20
Mycobacteria		
M. bovis BCG S10	Efficient plasmid transformation mutant	5
M. fortuitum ATCC 6841		Laboratory collection
M. phlei		Laboratory collection
Plasmids		
pACYC177	Km^r Ap^r; *E. coli* plasmid vector	4
pACYC184	Cm^r Tc^r; *E. coli* plasmid vector	4
pYT72	Km^r Ap^s; 11.3-kb *Bgl*II fragment of pMSC262 in pACYC177	5
pYT923	Km^r Ap^s; 4.3-kb *Pst*I-*Bgl*II fragment of pMSC262 in pACYC177	5
pYT935	Km^r Ap^s; 2.5-kb *Pst*I-*Hin*cII fragment of pMSC262 in pACYC177	5
pYT937	Km^r Ap^r; 2.3-kb *Pst*I-*Hin*dIII fragment of pMSC262 in pACYC177	5
pCL10	Km^r Ap^r; 1.8-kb *Nhe*I-*Sph*I fragment of pMSC262 in pACYC177lacZ	This study
pCL100	Km^r Ap^s; *Pvu*II-*Hin*cII fragment of pYT935 in pCL10	This study
pYT935PH	Km^r Ap^s; *Pvu*II-*Hin*cII fragment of pYT935 recloned in pYT935	This study
pMT933	Cm^r Tc^s; 2.5-kb *Pst*I-*Hin*cII fragment of pMSC262 in pACYC184	This study
pYUB75	Km^r; *Mycobacterium-E. coli* shuttle vector containing replication region of pAL5000	W. R. Jacobs, Jr.; 2

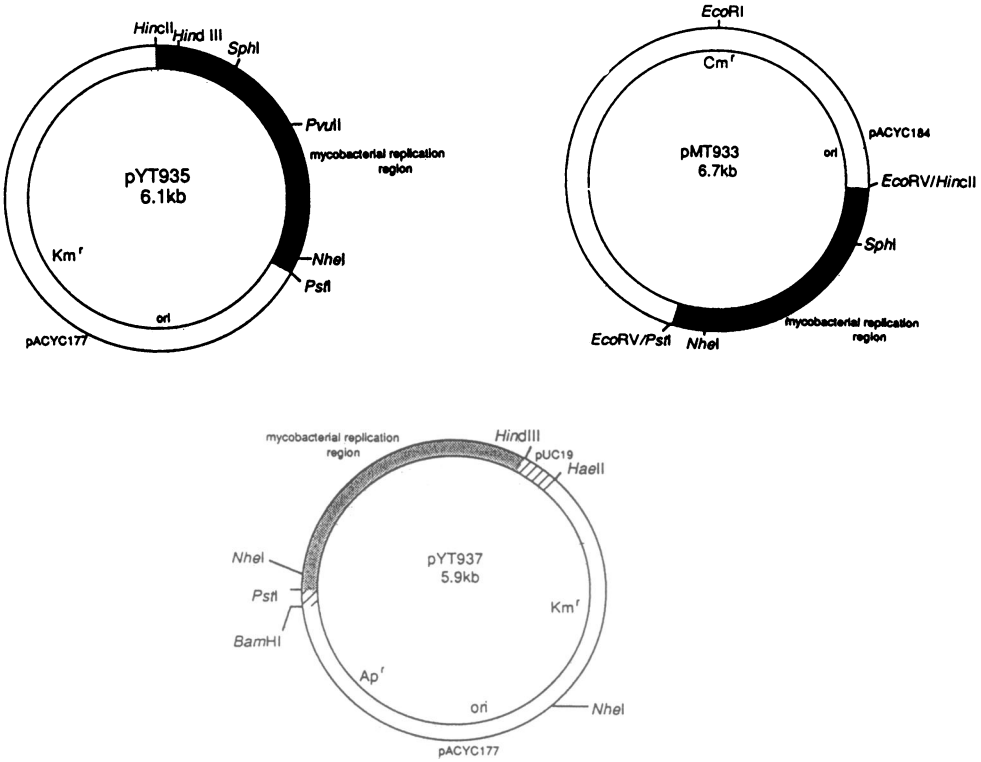

FIG. 1. Structures of *Mycobacterium-E. coli* shuttle vectors pYT937, pYT935, and pMT933. The black sections show a 2.3-kb fragment or a 2.5-kb fragment of pMSC262 whose sequence has been determined. The white and cross-hatched sections denote *E. coli* vectors pACYC177, pACYC184, and pUC19. The designation ori indicates a replication origin of pACYC177 or pACYC184.

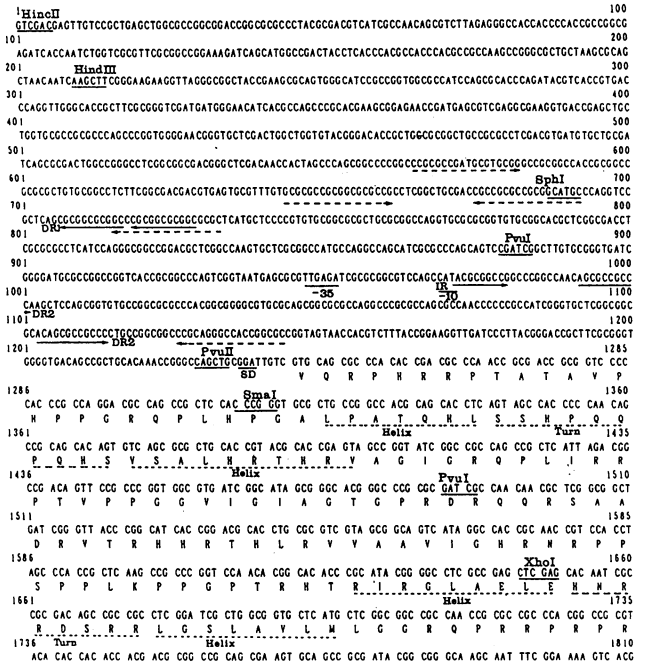

FIG. 2. Complete nucleotide sequence of the mycobacterial *Hin*cII-*Pst*I replication region. Inverted (IR) and direct (DR) repeats are indicated by arrows. Broken arrows show the 18-bp repeat sequences. ORF2 is indicated as a deduced amino acid sequence below the DNA sequence. Broken underlines within ORF2 show the helix-turn-helix motifs, and SD indicates the Shine-Dalgarno-like sequence.

(21). The chemical reaction was performed with a Chemical Robot DSP-240AX apparatus (Seiko Instruments Inc., Tokyo, Japan) with a fluorescent-dye primer *Taq* DNA polymerase sequencing kit which contains 7-deaza-dGTP (Applied Biosystems Inc., Foster, Calif.). Gel electrophoresis and analysis of raw data were done with an ABI 373A DNA Sequencer (Applied Biosystems Inc.). The sequence data obtained were analyzed by the DNASIS program (Hitachi Software Engineering Co. Ltd., Yokohama, Japan).

Construction of deletion mutants. The sequenced deletion fragments were isolated from M13mp18 and M13mp19 recombinant phages, and the protruding ends of each fragments were converted to blunt ends by T4 DNA polymerase. The fragments were then recloned into the *Hin*cII site of plasmid vector pACYC177. These deletion mutants were first amplified in *E. coli* and then used to transform BCG S10. Transformation was carried out by the electroporation method described previously (5), with a Bio-Rad Gene Pulser and a controller (Bio-Rad Laboratories, Hercules, Calif.). For rapid detection and analysis of plasmids in transformants of mycobacteria, the method described by Baulard et al. (3) was used. Plasmid DNAs from *E. coli* were prepared by the standard alkaline lysis procedure (20).

Construction of pACYC177lacZ, pCL10, pCL100, and pYT935PH. For easy cloning, we constructed plasmid pACYC177lacZ by inserting a 0.4-kb *Hae*II fragment of pUC19 into the *Hae*II site (np 3021) of pACYC177 by partial digestion. This *Hae*II fragment contains part of the *lacZ* gene and multicloning sites. We recloned the 1.7-kb *Nhe*I-*Sph*I fragment of the replication region of pMSC262 into this pACYC177lacZ plasmid and generated recombinant plasmid pCL10. By inserting a 0.6-kb *Pvu*II-*Hin*cII fragment of the replication region of pMSC262 into the *Hin*cII sites of pCL10 and pYT935, we constructed pCL100 and pYT935PH, respectively. Both contained two copies of the *Pvu*II-*Sph*I fragment of the replication region.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D14416.

RESULTS

Cloning, sequencing, and sequence features. As shown in Fig. 1, *Mycobacterium-E. coli* shuttle vectors pYT937 and pYT935 consist of two portions: *E. coli* plasmid vector pACYC177 (and pUC19 in pYT937) and the mycobacterial replication region of pMSC262 (5). To investigate the replication mechanism of these vectors in mycobacteria, we determined the nucleotide sequence of the *Pst*I-*Hin*cII fragment of pYT935 on both strands by the dideoxynucleotide chain termination method (21). However, in some regions, DNA sequencing was very difficult. Especially around the *Sph*I site, deletions due to spontaneous rearrangements occurred frequently. Therefore, we generated several deletion fragments from *Pvu*II sites with an exonuclease III-mung bean nuclease digestion kit (Takara Shuzo Co. Ltd., Kyoto, Japan) and also small fragments (<0.5 kb) by using several restriction endonuclease sites (see Materials and Methods) in the *Pvu*II-*Hin*cII region. We then determined the nucleotide sequences of these fragments over the *Pvu*II-*Hin*cII region and confirmed the accuracy of the data by digestion with several restriction endonucleases.

As shown in Fig. 2, the replication region *Pst*I-*Hin*cII fragment of pMSC262 contained 2,504 bp. It had one endonuclease-sensitive site each for *Pst*I, *Nhe*I, *Xho*I, *Sma*I, *Pvu*II, *Sph*I, *Hin*dIII, and *Hin*cII and two sites for *Pvu*I. The overall G+C content of this region was 69.8%, which corresponds to those of pAL5000 and mycobacterial chromosomal DNAs (65 and 63.3 to 70.6%, respectively; 1, 19). The G+C content of a region from np 1010 to 1146 was as high as 87%.

No sequence homologies between the replication regions of pMSC262 and pAL5000 were found (10, 19).

We found many repeats which consisted of nucleotide sequences more than 10 bp long. For example, there were 24 inverted repeats and 5 direct repeats in the fragment stretching from the *Pst*I site to the *Hin*dIII site. Around the *Sph*I site, 18-nucleotide-long repeat sequences were found at np 565 to 582, 644 to 661, 692 to 675, 733 to 716, and 1153 to 1136. This repeat sequence-rich structure may be a reason for the difficulties in the DNA sequencing analysis described above.

Determination of the minimum replication region. To determine the minimum replication region of pMSC262, DNA fragments with deletions of various sizes (H1, H2, H3, H4, H5, P1, P2, P3, P4, and HP32 in Fig. 3) were isolated from M13 recombinant phages and recloned into *E. coli* plasmid vector pACYC177. These recombinant plasmids were used to trans-

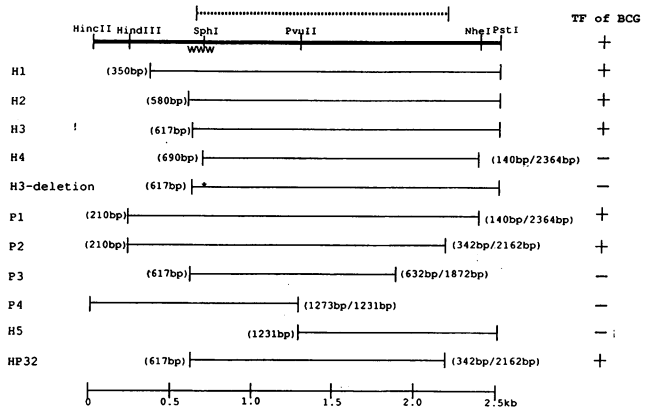

FIG. 3. Determination of the minimum replication region of pMSC262. Span lines indicate the deleted fragments that were inserted into pACYC177. H1 to H5 show the mutants with deletions from the *Hin*cII site, and P1 to P4 show the mutants with deletions from the *Pst*I site. HP32 shows the mutant with 342 bp deleted from the *Pst*I site of H3. H3-deletion is an H3 mutant that has a deletion of 4 bp at the *Sph*I site (*), just at the www repeat sequence. The ability of each mutant to replicate in BCG is shown at the right. The broken line at the top indicates the minimum replication region of pMSC262. TF, transformants.

form BCG S10 to test the ability to replicate in mycobacteria. As shown in Fig. 3, H1, H2, and H3 retained the ability to replicate in BCG but H4 and H5 did not. Similarly, P1 and P2 retained the ability to replicate in BCG but P3 and P4 did not. Therefore, we generated a fragment of HP32 and cloned it into pACYC177. This plasmid was to replicate in BCG, indicating that the minimum replication region in BCG was contained in 1,545 bp (np 617 to 2162). From the transformant, we isolated plasmid DNA that had a structure identical to that of the DNA used for transformation and could transform *E. coli* by using the preparation.

Open reading frame (ORF) analysis. Computer analysis of the minimum replication region of pMSC262 revealed the presence of five possible ORFs which code more than 100 amino acids (Fig. 4). They were designated ORF1, ORF2, ORF3, ORF4, and ORF5.

The initiation codon of ORF3 started at np 624, which is only 7 bp away from the np 617 terminal end of the H3 deletion mutant. Therefore, the promoter region of ORF3 must be deleted in H3. To abolish the effect of any promoter in pACYC177, we cloned the H3 fragment in pACYC177 in the opposite direction. The result showed that the H3 fragment

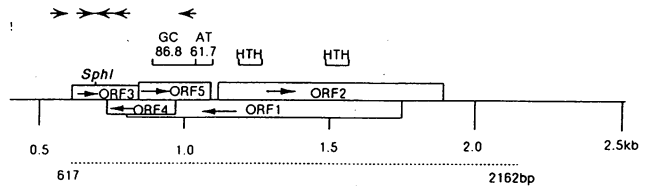

FIG. 4. Structural features of the replication region of pMSC262. Arrows over the sequence show the 18-bp repeat sequences and their orientations. The GC and AT values above the sequence indicate the G+C and A+T contents of the regions. HTH, helix-turn-helix motifs in ORF2. The broken line shows the minimal replication region, and the ORFs in it are indicated by boxes. The translational orientations of the ORFs are shown by the arrows in the boxes.

had the ability to replicate in BCG despite its orientation, suggesting that ORF3 is not essential for replication.

Regarding the other four possible ORFs, we searched for mycobacterial consensus promoter and Shine-Dalgarno sequences (ribosome-binding sites; 8, 23). Only ORF2 had these consensus sequences immediately upstream of the initiation codon. No such sequences were found to be associated with the other three ORFs. This result led us to the conclusion that the other three ORFs are most likely nonfunctional. Also, the P3 mutant, which had a deletion from the 3′ end of ORF2 but not in the other three ORFs, could not replicate in BCG S10. These results supported the importance of ORF2. ORF2 began with GTG (valine) at np 1034 and terminated with a TAG codon at np 1814, showing a G+C content of 68.9%. The mycobacterial consensus −35 (TTGAGA) and −10 (CAT ACG) promoter sequences were at np 947 to 952 and 969 to 974, and the Shine-Dalgarno sequence (GGA) was at np 1236-1238 (Fig. 2). ORF2 encodes a 28.9-kDa protein consisting of 260 amino acids which is basic and hydrophilic (18% arginine, 12% proline, and 12% alanine). Regarding codon usage, we compared that of ORF2 with a mycobacterial codon usage table based on nine genes from the *M. tuberculosis* complex (8). ORF2 had some features of mycobacterial gene structure such as the almost complete absence of AGA-AGG codons for arginine and frequent use of ACG-ACC codons for threonine (82%) and CUC-CUG codons for leucine (81%). Although its A+T content in the third position is a little higher than that of mycobacteria, it is much lower than that of *E. coli*. This protein does not have a signal sequence (data not shown) but has two helix-turn-helix motifs, which are characteristic of DNA-binding regulatory proteins (16). Thus, this ORF2-encoded protein may be involved in the initiation of plasmid replication.

Analysis of the repeat sequences and their relationship with ORF2. Generally, the replication region of plasmids contains two important components: an origin sequence (*ori*) and a gene, *rep*, that encodes a Rep protein. In addition, the typical structural features of *ori* include the presence of clusters of tandemly repeated sequences approximately 20 bp long and segments of DNA exceptionally rich in A and T residues (22). The minimal replicon of pMSC262 also has a cluster of repeat sequences and G+C and A+T-rich regions preceding ORF2 which has the properties of a Rep protein.

As described above, deletion mutant H4, which had been deleted to the *Sph*I site, lost the ability to replicate in BCG (Fig. 3). This suggested strongly that the repeat sequence-rich region (np 617 to 690) is important for replication in BCG.

In the replication regions of many other plasmids, it is known that repeat sequences have a role as *ori* sites. To test the significance of the repeat sequences of our plasmid, we made a mutant with the H3 deletion (Fig. 3), which removes the 4 bp which form the protruding end of the *Sph*I site within one of the repeat sequences. This mutant also lost the ability to replicate in BCG. Therefore, it seems highly likely that this repeat sequence-rich region acts as the replication origin (*ori*) in mycobacteria.

To investigate the relationship between the putative *ori* region and ORF2, we made plasmid pCL100 (Fig. 5). Plasmid pCL100 consisted of an H4 fragment, which is big enough to code for the ORF2 protein, and a *Pvu*II-*Hin*cII fragment, which contains the entire repeat sequence-rich region. We speculated that if the ORF2 protein had a *trans* activity, it would act on the putative *ori* region regardless of its location and replication in mycobacteria would result. However, pCL100 could not replicate in BCG. Two copies of the *Pvu*II-*Sph*I fragment of pMSC262 seemed to interfere with

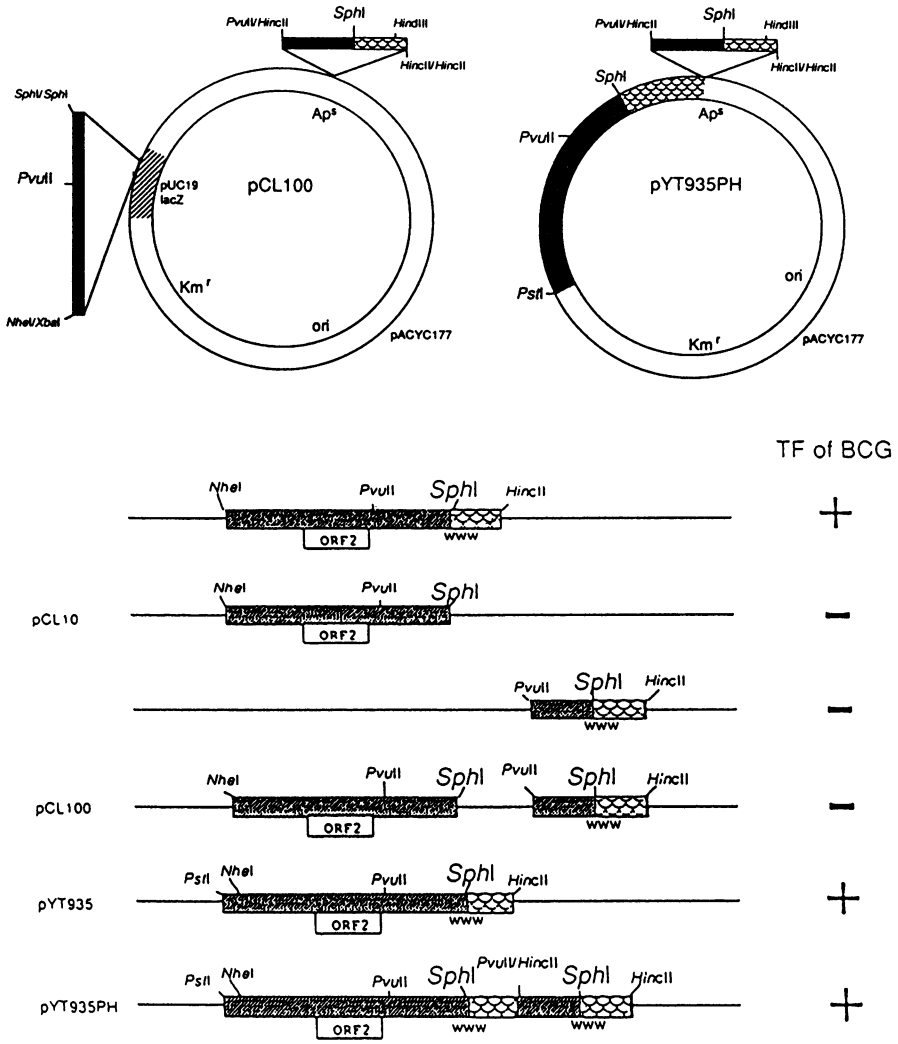

FIG. 5. Functional analysis of the putative *ori* region and ORF2. The structures of pCL100 and pYT935PH and their ability to replicate in BCG were determined. The black boxes indicate the mycobacterial DNA elements, and www shows the repeat sequences around the *Sph*I site. TF, transformants.

replication. However, this possibility was eliminated by the fact that pYT935PH, which also possesses two *Pvu*II-*Sph*I fragments, could replicate in BCG (Fig. 5).

Stability of the pMSC262 replicon in BCG. To test the stability of pMSC262-derived plasmid vector pYT937 in BCG, we subcultured BCG harboring pYT937 on antibiotic-free Ogawa's medium up to 20 times and determined the frequency of kanamycin-sensitive (Km^s) segregants. The results showed that the frequency of segregation was less than 10%.

Compatibility of the pMSC262 replicon with the pAL5000-derived plasmid. The availability of two types of vectors which belong to different incompatibility groups would be very useful as genetic tools. We tested whether a pMSC262-derived replicon is compatible with a pAL5000-derived replicon in BCG. For this purpose, we generated a new pMSC262-derived recombinant plasmid, pMT933, by inserting the *Pst*I-*Hin*cII fragment of pYT935 into pACYC184 (Fig. 1). This plasmid expressed chloramphenicol resistance (Cm^r) when introduced into BCG. By using pMT933 and pYUB75 (a pAL5000-derived plasmid expressing kanamycin resistance [Km^r] [3]), we obtained BCG transformants which expressed both Km^r and Cm^r. By analysis of the plasmid patterns of the transformants, it was revealed that the transformants carried both native plasmids pMT933 and pYUB75.

To test the compatibility of both plasmids, the transformants expressing both Km^r and Cm^r were cultured by using 7H9 broth for 10 and 30 generations in the absence of antibiotics and then checked for the presence of antibiotic resistance. The number of cells which expressed both Km^r and Cm^r was almost equal to that of the cells resistant to either kanamycin or chloramphenicol. The results indicate that these two plasmids coexisted in BCG for at least 30 generations.

Replication in *E. coli*. It was reported that pAL5000 could replicate in a *E. coli* K-12 *pol* mutant strain. To test whether the replication region of pMSC262 has the ability to replicate in *E. coli*, we did the following two experiments. First, we generated two small plasmids from pYT937 by using *Nhe*I: one containing the mycobacterial replication region and a Km^r gene and the other containing the *E. coli* replication region, pACYC177, and an ampicillin resistance gene (Fig. 1). After transformation of *E. coli* K-12 strain C600 with these plasmids, only ampicillin-resistant (Ap^r) transformants were recovered.

Next, we tested the replication ability of pYT937 and pYT72, which consisted of an 11.3-kb fragment of pMSC262 and pACYC177 (5). This was done with *E. coli* K-12 strain JG112 lacking the *polA* function, because pACYC177 itself does not replicate in the *pol* mutant *E. coli* K-12 strain. The result showed that pYT937 and pYT72 could not replicate in *E. coli* K-12 strain JG112. These two results indicated that the mycobacterial replication region of pMSC262 did not work in *E. coli*.

Transformation of other mycobacterial strains. In addition to *M. bovis* BCG, pYT937 transformed *M. phlei* and *M. fortuitum*. However, pYT937 did not replicate in *M. smegmatis* J15cs and mc^{2}155. In contrast, pYT72 and pYT923 (Table 1), which contained the region of pYT935 upstream of the *Hin*cII site, replicated in *M. smegmatis* J15cs but not in mc^{2}155 (data not shown).

DISCUSSION

We previously reported (5) the construction of a series of *Mycobacterium-E. coli* hybrid plasmids and obtained a small shuttle vector, pYT937. Extrachromosomal replication of pYT937 in BCG had been demonstrated by hybridization analysis and transformation of *E. coli* (data not shown).

In the present study, we determined the nucleotide sequence of the mycobacterial replication region. As described in Results, we met some difficulties in sequencing the region around the *Sph*I site. It is known that large segments of DNA (>1.0 kb) inserted into an M13 phage vector tend to generate deletions and rearrangements. In addition, the region that we sequenced has a high G+C content and a lot of repeat sequences. To get a correct sequence, we generated several small fragments (<0.5 kb) with *Nar*I, *Hin*dIII, *Sph*I, *Pvu*I, and *Pvu*II within the *Pvu*II-*Hin*cII region and analyzed them. These data were combined with the sequence data obtained from the mutants obtained by using exonuclease III-mung bean nuclease digestion kit and used to confirm the sequence.

We have determined the DNA sequence of the minimum replication region of mycobacterial plasmid pMSC262. As far as we know, at 1.6 kb, it is the smallest mycobacterial replicon ever reported. The G+C content of this replicon is 71.8%, which is within the usual range for mycobacterial DNA (1, 19).

At least two important components appear to be contained in this region: a putative *ori* region with a cluster of repeat sequences and a gene that codes for a putative Rep protein. This structural feature is similar to other plasmids of gram-positive and gram-negative bacteria (9, 15). It is not known whether the minimum region of pMSC262 possesses other functions usually found in the replication regions of other bacteria (for instance, incompatibility, stability, etc.).

Separation of the putative *ori* sequence from ORF2 resulted in loss of replication ability. This was not due to the presence of two copies of the *Pvu*II-*Sph*I fragment, which contains the putative *ori* region, because the pYT935PH plasmid also possesses two copies of the *Pvu*II-*Sph*I fragment but replicated in BCG. This result indicates that the *cis* relationship between ORF2 and the putative *ori* region is important in mycobacterial replication. Furthermore, the repeat sequences of the putative *ori* region may not have the ability to exert incompatibility. Further analysis is required to elucidate the precise mechanism of mycobacterial plasmid replication.

Another mycobacterial plasmid, pAL5000 (4.8 kb), has been used to construct *Mycobacterium-E. coli* shuttle vectors. Two groups have reported the entire nucleotide sequence of pAL5000, but with significant differences (10, 19). However, both groups showed the presence of a repeat-rich sequence (putative *ori*) and an ORF which may be involved in plasmid replication. These results suggest that structural features of the replication region of pAL5000 are similar to those of pMSC262, even though no sequence homologies between these plasmids are present. However, by Southern hybridization, we found sequences homologous to pMSC262 in chromosomal DNAs of *M. scrofulaceum* and *M. kansasii*, but not in those of *M. avium*, *M. bovis* BCG, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. phlei*, *M. smegmatis*, and *M. tuberculosis* H37 Rv and Ra (unpublished data). It will be interesting to determine the significance of these homologous sequences.

Copy number is one of the important characteristics of a vector plasmid, because it may influence the stability of the plasmid and the quantity of the cloned gene product. Although we could not determine the precise copy number of pYT937 in BCG, plasmid recovery from BCG transformants suggests that the copy number of pYT937 is lower than that of pAL5000.

Although the copy number of pYT937 in BCG appears small, the plasmid was stable in host cells. After repeated subcultures on Ogawa's egg medium, we detected only a small number of plasmid-free segregants. Since pMSC262 originated from slowly growing mycobacteria, it seems plausible that the slow growth rate of BCG may offer conditions adequate for plasmid replication.

We found that the pMSC262 replicon and the pAL5000-derived replicon can coexist in BCG. This suggests that the two plasmids belong to different compatibility groups. In addition, we confirmed that pYT937 could also replicate in *M. phlei* and *M. fortuitum*. Therefore, our plasmids would be useful for introduction of genes from a variety of organisms into the mycobacteria.

Recently, Villar and Benitez (27) reported that the regions of pAL5000 essential for replication in *M. fortuitum* and *M. smegmatis* are different. A similar phenomenon was also found regarding pYT937. In *M. smegmatis*, pYT937 could not replicate but pYT923, which contains ca. 1.8-kb regions upstream of the *Hin*cII site, could replicate. Analysis of the region upstream of the *Hin*cII site is in progress. We believe that our plasmids will also be useful in elucidating the mechanism of plasmid replication in mycobacteria.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid provided by the Ministry of Education, Science and Culture of the Japanese Government (03670228), the Sasagawa Memorial Health Foundation, and the Japan Health Sciences Foundation.

We thank W. R. Jacobs, Jr., for providing plasmid pYUB75 and T. Miki for the gift of *E. coli* strains. We also thank M. Ogawa and K. Mizue for technical assistance.

REFERENCES

1. **Baess, I.** 1982. Deoxyribonucleic acid relatedness among species of rapidly growing mycobacteria. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **90**:371–375.
2. **Barletta, R. G., D. D. Kim, S. B. Snapper, B. R. Bloom, and W. R. Jacobs, Jr.** 1992. Identification of expression signals of mycobacteriophages Bxb1, L1 and TM4 using the *Escherichia-Mycobacterium* shuttle plasmids pYUB75 and pYUB76 designed to create translational fusions to the *lacZ* gene. J. Gen. Microbiol. **138**:23–30.
3. **Baulard, A., C. Jourdan, A. Mercenier, and C. Locht.** 1992. Rapid mycobacterial plasmid analysis by electroduction between *Mycobacterium* spp. and *Escherichia coli*. Nucleic Acids Res. **20**:4105.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
5. **Goto, Y., H. Taniguchi, T. Udou, Y. Mizuguchi, and T. Tokunaga.**

1991. Development of a new host vector system in mycobacteria. FEMS Microbiol. Lett. **83**:277–282.

6. **Hermans, J., C. Martin, G. N. M. Huijberts, T. Goosen, and J. A. M. De Bont.** 1991. Transformation of *Mycobacterium avium* and *Mycobacterium smegmatis* with the broad host-range Gram-negative cosmid vector pJRD215. Mol. Microbiol. **5**:1561–1566.
7. **Hinshelwood, S., and N. G. Stoker.** 1992. An *Escherichia coli-Mycobacterium* shuttle cosmid vector, pMSC1. Gene **110**:115–118.
8. **Jeremy, W. D., and P. Abhay.** 1990. Mycobacterial gene expression and regulation, p. 173–198. *In* J. McFadden (ed.), Molecular biology of the mycobacteria. Academic Press, Ltd., London.
9. **Kües, U., and U. Stahl.** 1989. Replication of plasmids in gram-negative bacteria. Microbiol. Rev. **53**:491–516.
10. **Labidi, A., E. Mardis, B. A. Roe, and R. J. Wallace, Jr.** 1992. Cloning and DNA sequence of the *Mycobacterium fortuitum* var. *fortuitum* plasmid pAL5000. Plasmid **27**:130–140.
11. **Lazraq, R., S. Clavel-Sérès, H. L. David, and D. Roulland-Dussoix.** 1990. Conjugative transfer of a shuttle plasmid from *Escherichia coli* to *Mycobacterium smegmatis*. FEMS Microbiol. Lett. **69**:135–138.
12. **Lee, M. H., L. Pascopella, W. R. Jacobs, Jr., and G. F. Hatfull.** 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and bacille de Calmette-Guérin. Proc. Natl. Acad. Sci. USA **88**:3111–3115.
13. **Miller, J., J. Manis, B. Kline, and A. Bishop.** 1978. Nonintegrated plasmid-folded chromosome complexes. Plasmid **1**:273–283.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Novick, R. P.** 1989. Staphylococcal plasmids and their replication. Annu. Rev. Microbiol. **43**:537–565.
16. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53**:293–321.
17. **Radford, A. J., and A. L. M. Hodgson.** 1991. Construction and characterization of a *Mycobacterium-Escherichia coli* shuttle vector. Plasmid **25**:149–153.
18. **Ranes, M. G., J. Rauzier, M. Lagranderie, M. Gheorghiu, and B. Gicquel.** 1990. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: construction of a "mini" *Mycobacterium-Escherichia coli* shuttle vector. J. Bacteriol. **172**:2793–2797.
19. **Rauzier, J., J. Moniz-Pereira, and B. Gicquel-Sanzey.** 1988. Complete nucleotide sequence of pAL5000, a plasmid from *Mycobacterium fortuitum*. Gene **71**:315–321.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
22. **Scott, J. R.** 1984. Regulation of plasmid replication. Microbiol. Rev. **48**:1–23.
23. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *E. coli* 16s ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
24. **Snapper, S. B., L. Lugosi, A. Jekkel, R. E. Melton, T. Kieser, B. R. Bloom, and W. R. Jacobs, Jr.** 1988. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. USA **85**:6987–6991.
25. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4**:1911–1919.
26. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F, Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351**:456–460.
27. **Villar, C. A., and J. Benitez.** 1992. Functional analysis of pAL5000 plasmid in *Mycobacterium fortuitum*. Plasmid **28**:166–169.
28. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
29. **Zainuddin, Z. F., Z. M. Kunze, and J. W. Dale.** 1989. Transformation of *Mycobacterium smegmatis* with *Escherichia coli* plasmids carrying a selectable resistance marker. Mol. Microbiol. **3**:29–34.