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Nucleotide Sequence and Characterization of Erythromycin Resistance Determinant That Encodes Macrolide 2′-Phosphotransferase I in *Escherichia coli*

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The DNA fragment (3.3 kb) containing the erythromycin resistance determinant was cloned from *Escherichia coli* Tf481A and sequenced. Deletion and complementation analyses indicated that the expression of high-level resistance to erythromycin requires two genes, *mphA* and *mrx*, which encode macrolide 2′-phosphotransferase I and an unidentified hydrophobic protein, respectively.

We previously reported a new resistance phenotype with resistance to high levels of erythromycin (EM) in clinical isolate Tf481A of *Escherichia coli* (12). This resistance was caused by inactivation of macrolide antibiotics by macrolide 2′-phosphotransferase I [MPH(2′)I]. MPH(2′)I is an inducible intracellular enzyme and it inactivates macrolides with a 14-membered ring more strongly than those with a 16-membered ring (13).

In this report, we describe the cloning and nucleotide sequencing of the Em^r determinant. Furthermore, two genes that are required for the expression of high-level resistance to EM are identified.

No transfer of the Em^r determinant of *E. coli* Tf481A to another strain was observed by conjugation (13). By using *E. coli* Tf481A into which the conjugative plasmid RP1 was introduced (4), the Em^r determinant was transferred to other *E. coli* strains by the broth mating method. Restriction analysis of plasmid RP1-EM481 in a transconjugant showed that a 21.9-kb DNA fragment had been inserted into the *Pst*I-D fragment of RP1 (3).

From RP1-EM481, we cloned the Em^r determinant and obtained pTZ3509 and pTZ3519 by inserting the 4.1-kb *Pst*I fragment and the 3.3-kb *Bam*HI-*Pst*I fragment into the cloning sites of pUC119 (19), respectively, (the fragments were in opposite orientations). Both plasmids conferred high-level resistance to EM and the production of MPH(2′)I. By digestion of pTZ3519 and pTZ3509 with exonuclease III (5), deletion derivatives of various sizes were constructed. The nucleotide sequence of the 3.3-kb *Bam*HI-*Pst*I fragment was determined by the dideoxy-chain termination method (15) with *Bca* BEST DNA polymerase (17). The full sequence of 3,267 bp is presented in Fig. 1. Sequence analysis revealed four open reading frames (ORFs) (ORF1 [606 bp], ORF1b [314 bp], ORF1c [462 bp], and ORF2 [1,014 bp]) that extended from the *Bam*HI site to the *Pst*I site and four other ORFs (ORF3 [903 bp], ORF4 [1,164 bp], ORF4b [672 bp], and ORF5 [612 bp]) that extended in the opposite direction (Fig. 2).

To determine the locations of the genes that conferred high-level resistance to EM, we investigated the activity of MPH (2′)I (13) and the MIC of EM (7) for *E. coli* strains carrying the various deletion derivatives (Fig. 2). These results indicated that the *mphA* gene for MPH(2′)I was located in the region from nucleotide 1630 (breakpoint of Δ436) to 2884 (breakpoint of Δ51). ORF2 and ORF3 were located in this region in opposite orientations. The MIC (200 μg/ml) for the strain carrying Δ115 with a deletion from the 5′ end to nucleotide 510 was much lower than the original MIC of >3,200 μg/ml, despite the fact that Δ115 contained *mphA*. The data suggested that some other gene was necessary for the expression of high-level resistance to EM. A complementation analysis was performed to determine the region that encoded this gene, temporarily designated *mrx*. The *Eco*RI-*Hin*dIII fragment of Δ436 containing *mphA* was subcloned into pBR322 (now called pBRΔ436), whereas the *Eco*RI-*Hin*dIII fragment of various deletions from pTZ3509 was subcloned into pSTV28 carrying the replication region of pACYC184 (1, 16) that is compatible with pBR322. *E. coli* carrying both pBRΔ436 and pSTVΔ146 was resistant to high levels of EM, whereas the MIC for the strain carrying both pBRΔ436 and pSTVΔ369 was the same as for the same strain carrying only pBRΔ436 (Table 1). These results indicated that the *mrx* gene was located in the region from nucleotide 266 (breakpoint of Δ37) to 1,593 (breakpoint of Δ146) and that ORF1c was not essential for high-level resistance to EM (Fig. 2). This region contained four ORFs (ORF1, ORF1b, ORF4, and ORF4b) and ORF4 overlapped three other ORFs (Fig. 2).

To identify *mphA* and *mrx*, a stop codon was independently introduced into the ORFs of pTZ3519 by site-directed mutagenesis by the method of Kunkel et al. (8). The strain carrying ORF3 TGA18 (TGA at codon position 18) had lost MPH(2′)I activity and was susceptible to EM (Table 1). Therefore, the inactivation of EM by MPH(2′)I was essential for the resistance to EM, and ORF3 corresponded to the *mphA* gene. ORF3 encoded a putative polypeptide of 301 amino acids with a molecular weight of 33,225. This molecular weight is in agreement with that (34,000) previously estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). Additionally, ORF1 TGA99 and ORF4 TGA235 conferred low-level resistance to EM (Table 1). The base substitution in ORF1 TGA99 also changed the Ser (TCA) at codons 116 of ORF4 to a stop codon (TAG). Accordingly, insertion of a stop

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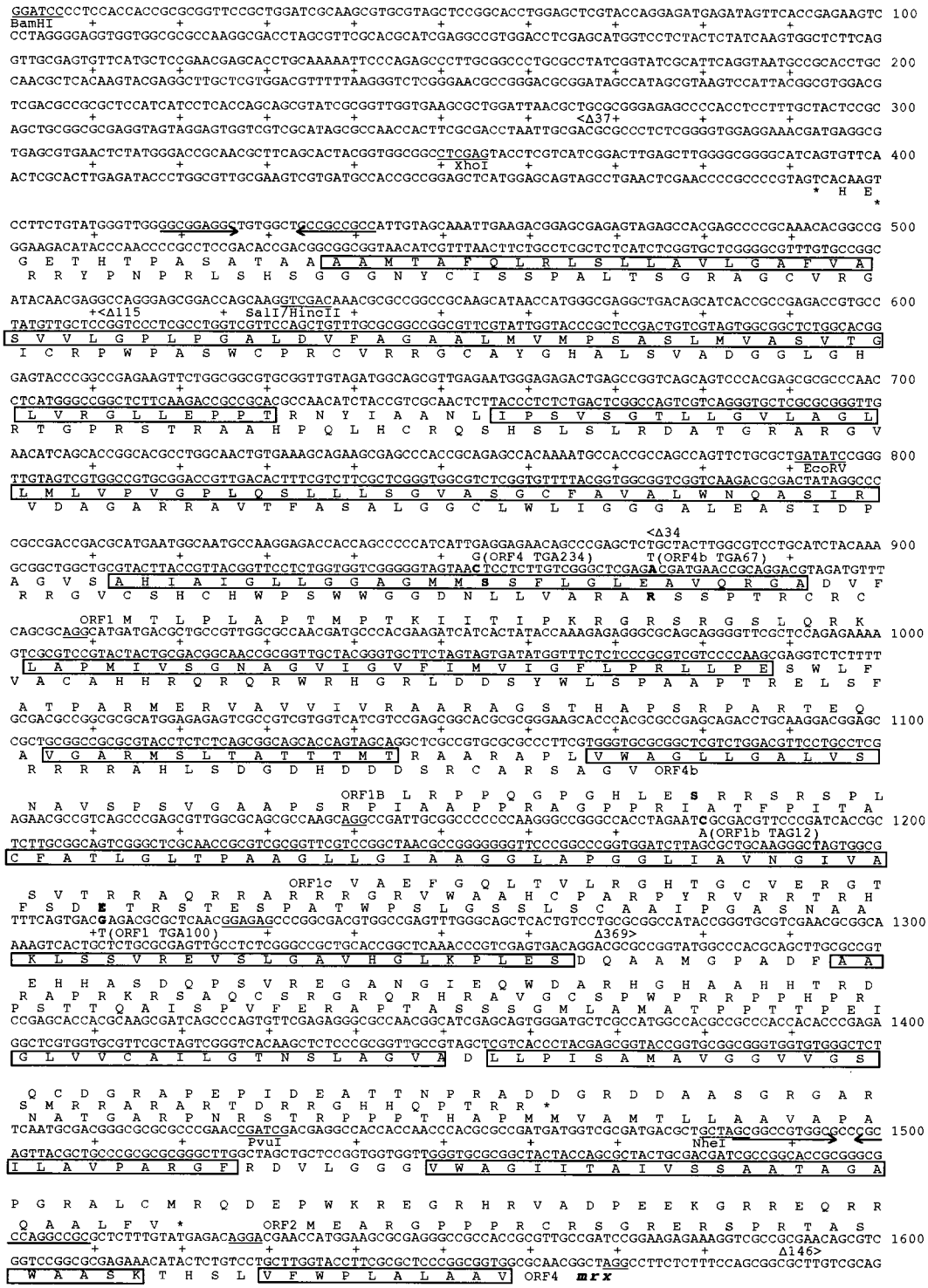

FIG. 1. Nucleotide sequence of the 3.3-kb *Bam*HI-*Pst*I fragment and the deduced amino acid sequence. The putative ribosome-binding sites (2) and various restriction sites are underlined. Inverted repeats (pair of arrows), deletion breakpoints (< or >), and stop codons (asterisks) are indicated. Base changes and amino acid replacements are indicated by bold lettering. The region homologous to the IS*176* element and the terminal 14-bp inverted repeat are shown by italic lettering and a dotted arrow, respectively. The hydrophobic regions in Mrx are boxed.

codon in ORF4 caused a decrease in the resistance to EM. ORF4 downstream from the breakpoint of Δ146 has several potential initiation codons, the first at nucleotide 1,557. ORF4 was theoretically capable of encoding a polypeptide of 388 amino acids with a molecular weight of 38,238. To identify the products that were expressed from pTZ3519, we carried out minicell analysis. The MPH(2′)I from ORF3 was identified, but no specific product from any other ORF was detected

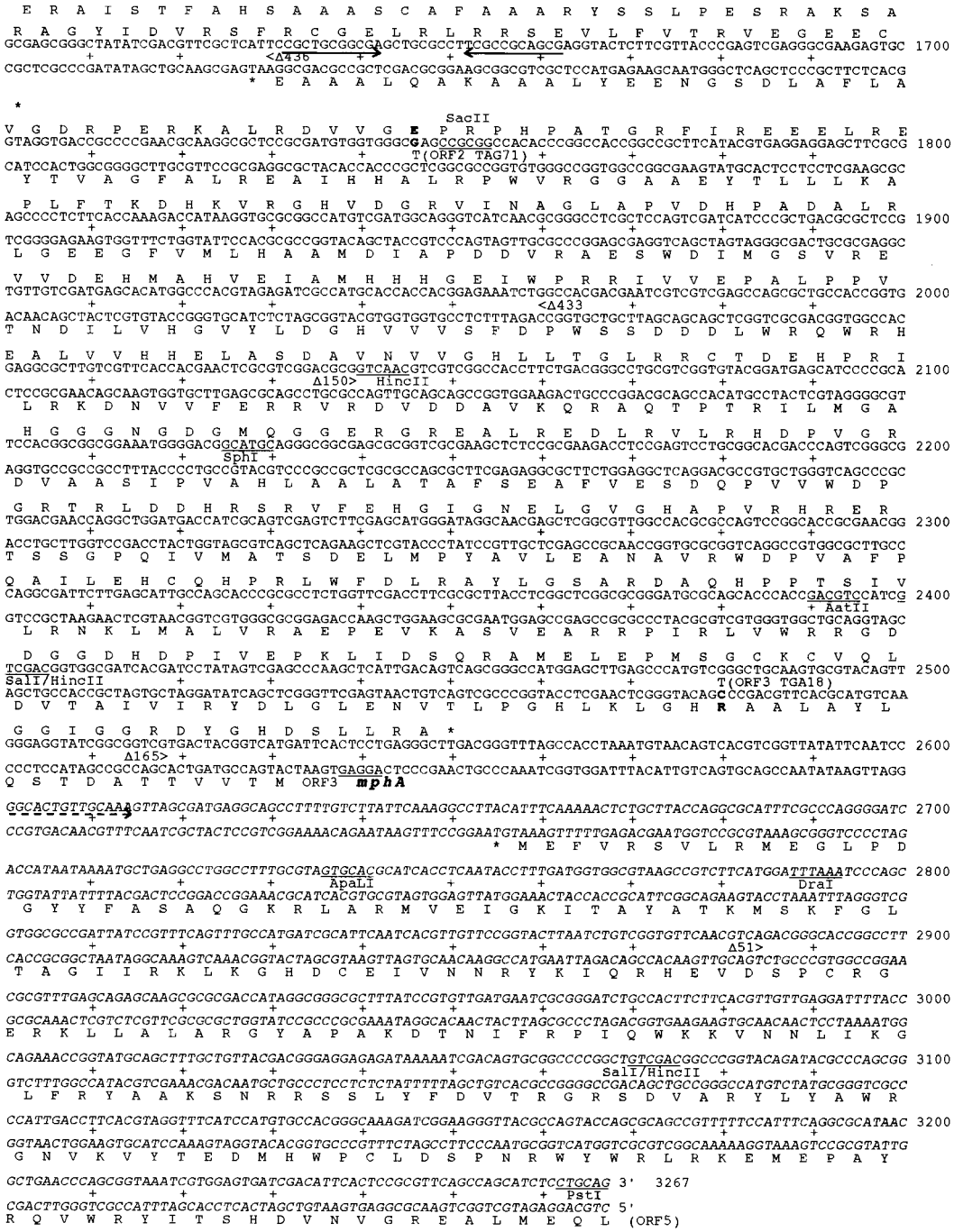

FIG. 1—*Continued.*

(data not shown). Also, no amino acid sequence predicted from any other ORF on the 3.3-kb DNA fragment exhibited significant homology to those of published genes. However, the results of the complementation and mutation analyses indicate that ORF4 corresponded to the *mrx* gene that is required for the expression of the high-level resistance to EM, mediated by MPH(2′)I. Although the precise biological function of Mrx produced from *mrx* remains unknown, the hydrophobicity analysis by the method of Kyte and Doolittle (9) showed that Mrx might contain approximately 10 hydrophobic domains.

The 3′-flanking region from nucleotide 2601 to the 3′ end was identical to IS*176* (IS*26*), an insertion usually associated with those of aminoglycoside resistance genes (Fig. 1) (6, 10, 14, 18). A part (ORF5) of an ORF in IS*176* was also found in the 3′-flanking region. Furthermore, the G+C content of the 3.3-kb fragment was 62%, and this value was higher than that (50%) of *E. coli* (11). These results suggest that the Em^r determinant including *mphA* is carried by a transposon and originated from organisms other than *E. coli*. The production of MPH(2′)I in *E. coli* Tf481A was induced by EM (data not shown). Identification of the gene

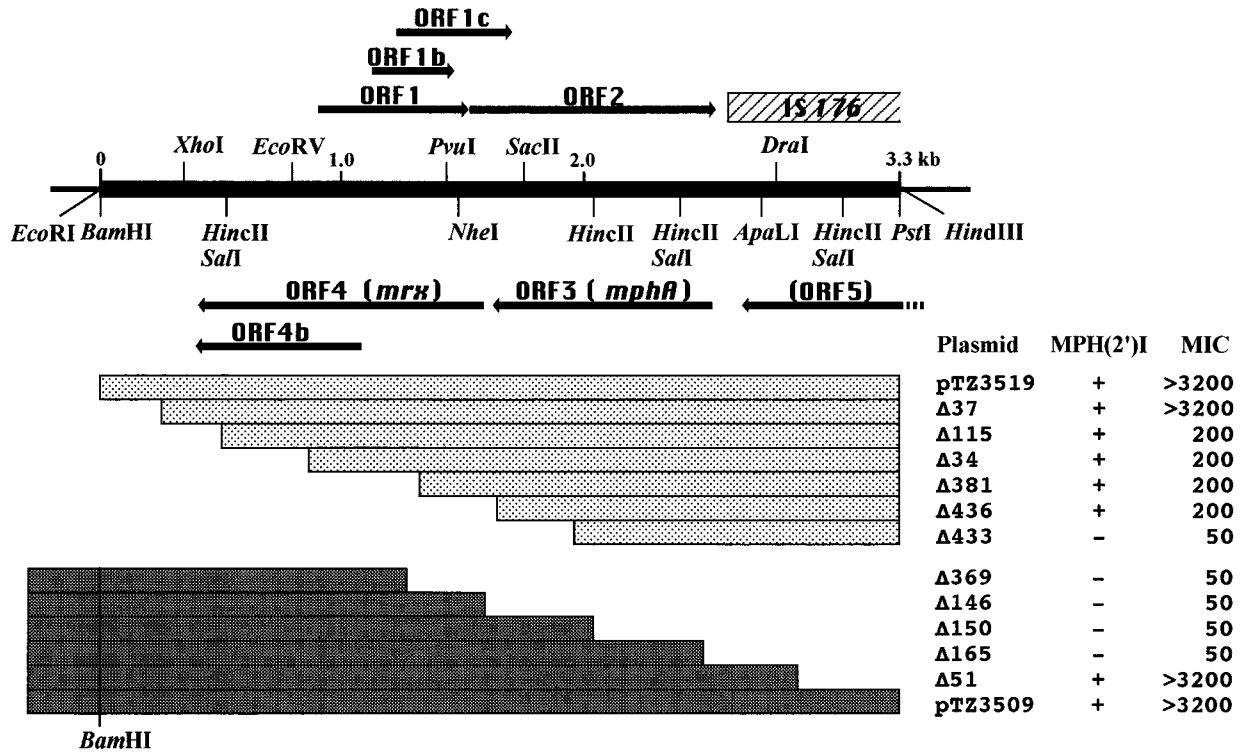

FIG. 2. Restriction map of the 3.3-kb DNA fragment and the properties of deletion derivatives constructed in vitro. The thin line on the restriction map represents the sequence of the pUC119 vector. The arrows indicate the locations and the orientations of the ORFs. The hatched box represents the region homologous to part of the IS*176* element. The dark and light bars indicate the DNA regions of deletion derivatives of pTZ3519 and pTZ3509, respectively. MPH(2′)I activity was detected (+) or not detected (−). MIC of EM is shown in micrograms per milliliter.

that regulates the expression of *mphA* and its product are in progress.

Nucleotide sequence accession number. The nucleotide sequence of the 3.3-kb *Bam*HI-*Pst*I fragment and the deduced amino acid sequence have been deposited in DDBJ, EMBL, and GenBank under accession number D16251.

TABLE 1. EM MICs and MPH(2′)I activities of *E. coli* MV1184 carrying different plasmids

Plasmid	MIC[a]	MPH(2′)I[b]
pTZ3519 with the following mutations:	>3,200	295
ORF1 TGA100	200	218
ORF1b TAG12	>3,200	278
ORF2 TAG71	>3,200	317
ORF3 TGA18	50	0[c]
ORF4 TGA235	200	326
ORF4b TGA67	>3,200	317
pBRΔ436	200	201
pSTVΔ146	50	0[c]
pBRΔ436 + pSTVΔ146	800	234
pSTVΔ369	50	0[c]
pBRΔ436 + pSTVΔ369	200	273
None	50	0[c]

[a] The MIC of EM is shown in micrograms per milliliter.

[b] A solution of crude enzyme (13) was prepared from an *E. coli* strain that was grown in the presence of EM (25 μg/ml). The specific activity of MPH(2′)I (in units per milligram of protein) is shown. One unit results in inactivation of 1 nanomole of oleandomycin per hour.

[c] A value of 0 is actually <0.5 unit.

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