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Sequence and Properties of pIM13, a Macrolide-Lincosamide-Streptogramin B Resistance Plasmid from *Bacillus subtilis*

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We initiated a study of pIM13, a multicopy, macrolide-lincosamide-streptogramin B (MLS) plasmid first isolated from a strain of *Bacillus subtilis* and described by Mahler and Halvorson (J. Gen. Microbiol. 120:259–263, 1980). The copy number of this plasmid was about 200 in *B. subtilis* and 30 in *Staphylococcus aureus*. The MLS resistance determinant of pIM13 was shown to be highly homologous to *ermC*, an inducible element on the *S. aureus* plasmid pE194. The product of the pIM13 determinant was similar in size to that of *ermC* and immunologically cross-reactive with it. The MLS resistance of pIM13 was expressed constitutively. The complete base sequence of pIM13 is presented. The plasmid consisted of 2,246 base pairs and contained two open reading frames that specified products identified in minicell extracts. One was a protein of 16,000 molecular weight, possibly required for replication. The second was the 29,000-molecular-weight MLS resistance methylase. The regulatory region responsible for *ermC* inducibility was missing from pIM13, explaining its constitutivity. The remainder of the pIM13 MLS determinant was nearly identical to *ermC*. The ends of the region of homology between pIM13 and pE194 were associated with hyphenated dyad symmetries. A segment partially homologous to one of these termini on pIM13 and also associated with a dyad was found in pUB110 near the end of a region of homology between that plasmid and pBC16. The entire sequence of pIM13 was highly homologous to that of pE5, an inducible MLS resistance plasmid from *S. aureus* that differs from pIM13 in copy control.

pIM13 is a 2.2-kilobase multicopy, *Bacillus subtilis* plasmid that confers resistance to the macrolide-lincosamide-streptogramin B (MLS) antibiotics. It was isolated and first described by Mahler and Halvorson (24). We explored the properties of pIM13 for three reasons. (i) Molecular cloning in *B. subtilis* is usually attempted with vectors derived from *Staphylococcus aureus*; however, it is possible that vectors based on an indigenous plasmid might minimize the instability problems that often defeat cloning efforts in *B. subtilis*. (ii) Our laboratory maintains an interest in the regulation and evolution of MLS resistance. (iii) Although plasmids native to *S. aureus* have been characterized in some detail (11, 31), little effort has been devoted to studying plasmids from other gram-positive genera, such as the bacilli.

In this communication we present a characterization of pIM13, including its base sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. All *B. subtilis* strains are derivatives of strain 168.

Isolation of plasmid DNA and transformation. Plasmid DNA was isolated from stationary cultures grown at 32°C by the sodium dodecyl sulfate (SDS)-NaCl method of Guerry et al. (14) followed by CsCl-ethidium bromide centrifugation as described previously for *B. subtilis* (9). Preparation of competent cells of *B. subtilis* and transformation with plasmid DNA were carried out as described previously (4). Transformation of *S. aureus* RN451 protoplasts was carried out by the method of Chang and Cohen (3) modified for *S. aureus* (27).

Restriction endonuclease cleavage, restriction mapping, and ligation. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals, New England BioLabs, Inc., and Collaborative Research, Inc., and were used according to the specifications of the suppliers. Mapping of the restriction sites was done by multiple enzyme cutting and analysis of digestion products by electrophoresis on 0.8 or 2% agarose gels with Tris borate buffer (8).

Copy number determination. Plasmid copy numbers were determined by the isolation of [U-^{3}H]thymidine-labeled plasmid and chromosomal DNA from agarose gels as described by Weisblum et al. (48).

Southern blotting and hybridization. DNA probes were labeled by nick translation with [α-^{32}P]dATP and [α-^{32}P]dCTP to specific activities of 1×10^8 to 3×10^8 (35). Transfer of DNA from agarose gels to nitrocellulose membranes was by the procedure of Southern (44). Membranes were prehybridized for 4 h at 65°C in 5× SSC– (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 5× Denhardt reagent (1× Denhardt reagent is 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin) (5) containing 200 μg of denatured salmon sperm DNA per ml and then hybridized at 65°C overnight with the denatured probe in 5× SSC–1.2× Denhardt reagent–100 μg of denatured salmon sperm DNA per ml. After hybridization the filters were washed under conditions of moderate stringency (2× SSC-0.1% SDS followed by 0.1× SSC-0.1% SDS at room temperature). After exposure to X-ray film, the membranes were washed under high-stringency conditions (0.1× SSC-0.1% SDS at 65°C) and reexposed.

Minicell studies. Plasmids were introduced into the minicell strain CU403 by transformation. Minicells were isolated, stored, and used to study incorporation of

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TABLE 1. Strains and plasmids

Strain	Plasmid	Characteristics	Source (reference)
S. aureus			
RN451			R. P. Novick
RN4491	pEM96	*ermA*	28
B. subtilis			
BD630		*hisH2 leumet*	
CU403		*thyA thyB metB divIVB1*	34
BD731	pAM77	*trpC2 thr-5*	F. Barany, 49
BD430	pE194	*trpC2 thr-5*	48
BD431	pE194 *cop-6*	*trpC2 thr-5*	48
BD1002	pE194 *cop-1*	*trpC2 thr-5*	
BD668	pE5	*trpC2 thr-5*	A. I. Stepanov, 6
BD1109	pIM13	*hisH2 leu met*	24
BD366	pUB110	*trpC2 thr-5*	
BD662	pBD90	*trpC2 thr-5*	6

[^{35}S]methionine as described by Shivakumar et al. (42). Minicell lysates were analyzed by discontinuous SDS-polyacrylamide gel electrophoresis as described before (42).

DNA sequence determination. Fragments of pIM13 were cloned into M13mp10 and M13mp11 (26), and the resulting DNA preparations were used for sequencing by the dideoxynucleotide method of Sanger et al. (37) as described by the manufacturer (Amersham Corp.). Thin (0.35 mm) 6% polyacrylamide-urea gels were used (36).

Western blotting. SDS-polyacrylamide gel electrophoresis was performed on 15% gels prepared essentially as described by Laemmli (22). Proteins were electrophoretically transferred to nitrocellulose (BA 85; Schleicher & Schuell, Inc.) by the method of Towbin et al. (46) by use of an electroblot apparatus (Transphor TE50; Hoefer Scientific Instruments) with the transfer buffer described in the Hoefer catalog and a transfer time of 1 h at 0.9 A. After transfer, the method of Blake et al. (2) was followed for detection of alkaline phosphatase-conjugated anti-rabbit antibody. Primary antibodies were raised in rabbits by use of highly purified *ermC* methylase (40) as the immunogen. The quantitation of the reaction was performed by scanning the reflectance of the blots in a Shimadzu Dual Wavelength Chromatogram Scanner (model CS-910) at 520 nm. The areas under the peaks were determined and were related to the amounts of pure methylase loaded onto parallel lanes as standards. A linear relationship was observed in the range of 1 to 100 ng.

RESULTS

Copy number and agarose gel electrophoresis pattern of pIM13. A preliminary restriction site map of pIM13 was prepared to facilitate DNA sequence determination and other manipulations. The unique *Cfo*I and *Sst*I sites and the two *Hin*dIII sites reported by Mahler and Halvorson (24) were confirmed. The copy number of pIM13 was found to be 150 to 200 in various *B. subtilis* backgrounds. This was markedly different from the reported value of 15 (24). We cannot explain this difference, although the previous workers measured the copy number by a different method (radioactivity incorporated into the covalently closed circular (CCC) DNA fraction in CsCl-ethidium bromide density gradients). pIM13 was introduced into *S. aureus* RN451 by protoplast transformation, and the copy number in that organism was shown to be 30. The agarose gel electrophoresis pattern displayed by pIM13 DNA preparations was unusual (Fig. 1). Only a minor fraction of total plasmid DNA isolated from *B. subtilits* was monomeric CCC DNA; typically the most prominent form was CCC dimer. Higher oligomeric forms were well represented. The molecular copy number of pIM13 in *B. subtilis* was therefore substantially lower than 200. The gel electrophoresis pattern noted was atypical of similarly prepared DNA samples of other plasmids from *B. subtilis* (Fig. 1). The assignment of various bands to the CCC monomer, dimer, and trimer forms of pIM13 was based on their electrophoretic migrations compared with CCC species of known size. Linearization of pIM13 DNA as well as cleavage with a variety of multiple cutting restriction endonucleases followed by size determinations of the resulting fragments also confirmed that our interpretation of the gel (Fig. 1) was correct (data not shown). The size obtained by summing the individual restriction fragments always totaled 2.2 kilobases.

Relationship of pIM13 to other MLS resistance plasmids. We examined the possible homology of pIM13 DNA with that of the plasmids pEM9631, pAM77, pE194, and pBD90. These were used to test for the presence of *ermA*, *ermB*-like, *ermC*, and *ermD* determinants, respectively. (The pAM77 MLS determinant is highly homologous to *ermB* (48a) and is a convenient probe for the *ermB* family of MLS determinants.) Nick-translated pIM13 DNA was used as a probe in Southern blots containing DNA from these plasmids. A weak signal was obtained with pEM9631 (*ermA*), and a strong signal was obtained with pE194 (*ermC*) (data not shown). No hybridization was observed with the *ermD* and *ermB*-like plasmid DNA preparations. The same probe was used to hybridize with Southern blots carrying pE194 DNA cleaved with *Mbo*I and *Hin*fI. Hybridization was observed with *Mbo*I fragments B and D and with *Hin*fI fragment B. These are known to carry the *ermC* gene. No homology was detected with the other *Mbo*I and *Hin*fI fragments, even after prolonged exposure. We conclude that the MLS resistance determinant of pIM13 is highly homologous to *ermC*

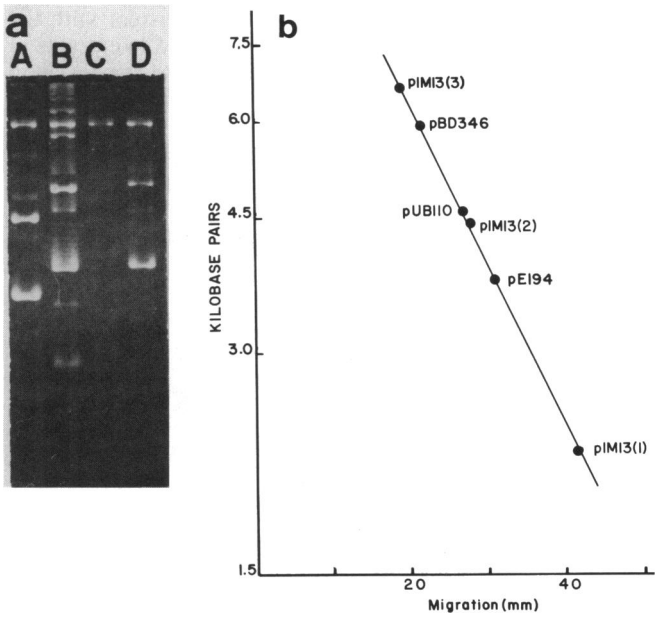


FIG. 1. (a) Agarose gel electrophoresis of pIM13 DNA. Lanes: A, pE194; B, pIM13; C, pE5; D, pUB110. DNAs were electrophoresed in 0.8% agarose gel. These DNAs were isolated from strains BD430, BD1109, BD662, and BD366, respectively. (b) Distance migrated plotted against the log of molecular size.

and that the remainder of pIM13 has no detectable homology with the four tester plasmids.

Minicell analysis of pIM13. The [^{35}S]methionine-labeled proteins specified by pE194 and by pIM13 in minicells of *B. subtilis* are shown in Fig. 2. The pE194 lysate contained the expected five labeled bands, including the gene product of *ermC*. The latter is an erythromycin-inducible 29,000-molecular-weight protein (29K protein) known to be the rRNA methylase responsible for MLS resistance (40, 42). pIM13 appeared to specify three labeled products. The most prominent corresponded approximately in electrophoretic migration with the 29K *ermC* protein, although it migrated slightly more slowly. Although this small difference was observed repeatedly, we will continue to refer to the pIM13 protein as a 29,000-molecular-weight product. The pIM13 protein was evidently not inducible by a subinhibitory concentration (0.02 μg/ml) of erythromycin or by a concentration as high as 5.0 μg/ml (data not shown). In addition, a faint 23,000-molecular-weight band could be seen, although not in the SDS-polyacrylamide gel reproduced in Fig. 2. This corresponded to a minor pE194 band that has been shown to be an *ermC* product of unknown function (7, 41). The sequence encoding this 23K *ermC* protein overlapped with the coding sequence for the 29K rRNA methylase and was translated in the same reading frame but appeared to initiate at a different position than the 29K protein. Two other products of *ermC* (32K and 18K proteins) that are also encoded by overlapping sequences (7, 41) were not detectable in the pIM13 lysate. An additional visible product of pIM13 was a diffuse band of about 16,000 molecular weight. Our minicell results differed from those of Mahler and Halvorson (24), who observed a minor (noninducible) 29,000-molecular-weight band, a prominent band at 45,000 molecular weight, and an erythromycin-inducible 41K protein, as well as several additional ^{35}S-labeled proteins.

Regulation of the pIM13 rRNA methylase. Western blotting revealed that extracts of strains carrying pIM13 contained a 29K protein that cross-reacted with the antibodies to *ermC* methylase of pE194 (Fig. 3). This was consistent with the appearance of a 29,000-molecular-weight band in extracts of minicells carrying either plasmid (Fig. 2). Also consistent with the minicell results was the apparently constitutive synthesis of the pIM13 protein. Although extracts of pIM13-carrying cultures incubated with and without inducer for only 10 min were compared (Fig. 3), in another experiment we continued the incubations for 4 h. The latter experiment confirmed the constitutivity of the 29K protein synthesis. Finally, C. S. Narayanan and D. Dubnau (unpublished data) have developed an in vitro coupled transcription-translation system that permits induction of the *ermC* rRNA methylase. In this system, the pIM13 protein is synthesized at a high rate that is not affected by subinhibitory concentrations of erythromycin. This is important since the high copy number of pIM13 may result in complete in vivo methylation of 23S RNA without induction, thus preventing erythromycin inducibility (13, 43). The in vitro results with unmethylated ribosomes obviated this possibility. The immunological cross-reactivity of the pIM13 and pE194 29,000-molecular-weight products, together with the base sequence homology evident from the Southern hybridization results, suggests that the products specified by the two plasmids were very closely related. On the other hand, we found that the antibody raised against the *ermC* protein did not cross-react in Western blots with the *ermB*-like or *ermD* enzymes and reacted weakly with the *ermA* product (data not shown). All four proteins exhibit substantial amino acid sequence homology (12, 17, 28). Quantitative Western blotting revealed that the concentration of the rRNA methylase in pIM13 extracts was 3.6 times higher than in extracts of a pE194 *cop-1* derivative. In the cultures used to measure 29K protein content, the copy number of pE194 *cop-1* was 180 and that of pIM13 was 170. The difference in 29K protein content was thus inexplicable on the basis of gene dosage. Furthermore, the measured difference was minimal since the pIM13 product may not have been fully cross-reactive.

Sequence of pIM13. A restriction site map of pIM13, together with the cloning and sequencing strategy used to

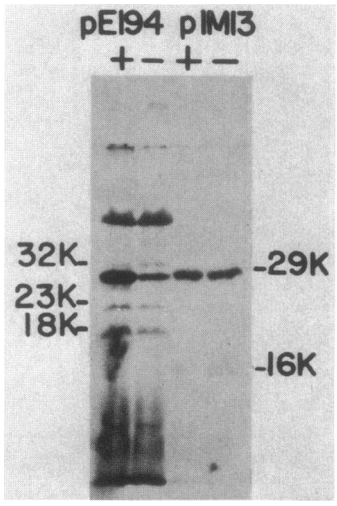


FIG. 2. Minicell analysis of pIM13 and pE194. Minicells from strains carrying either pE194 or pIM13 were incubated with [^{35}S]methionine in the presence (+) or absence (−) of erythromycin (0.02 μg/ml).

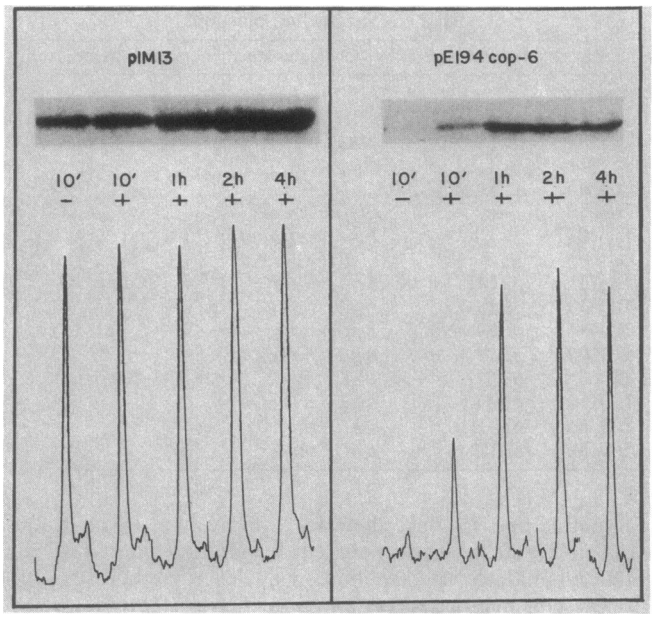


FIG. 3. Western blot analysis of rRNA methylase synthesis. Strains carrying pIM13 or pE194 *cop-6* were grown in the presence (+) or absence (−) of erythromycin (0.02 μg/ml) for the times indicated. Extracts were prepared and analyzed by Western blotting with *ermC*-specific antiserum. Tracings of the blots are shown.

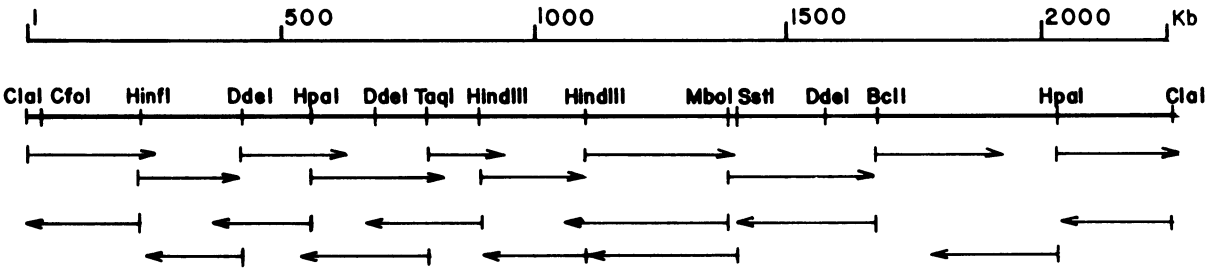


FIG. 4. Sequencing strategy. The restriction fragments indicated by the arrows were cloned into M13 derivatives. Sequencing by the dideoxynucleotide procedure was performed in the directions shown by the arrows.

determine the DNA base sequence of this plasmid, is shown in Fig. 4. The sequence of pIM13 (Fig. 5) consisted of 2,246 base pairs. The sequence of all but about 200 base pairs was determined on both strands. All restriction sites were crossed. The sequence contained 31.2% G+C, typical of the A+T-rich plasmids found in *S. aureus*, *Streptococcus* spp., and *Bacillus* spp. The pIM13 sequence contained three open reading frames (ORF) of greater than 200 base pairs. ORF1 (position 861 to 577) has no obvious Shine-Dalgarno (SD) sequence (38) near its beginning and will not be discussed further.

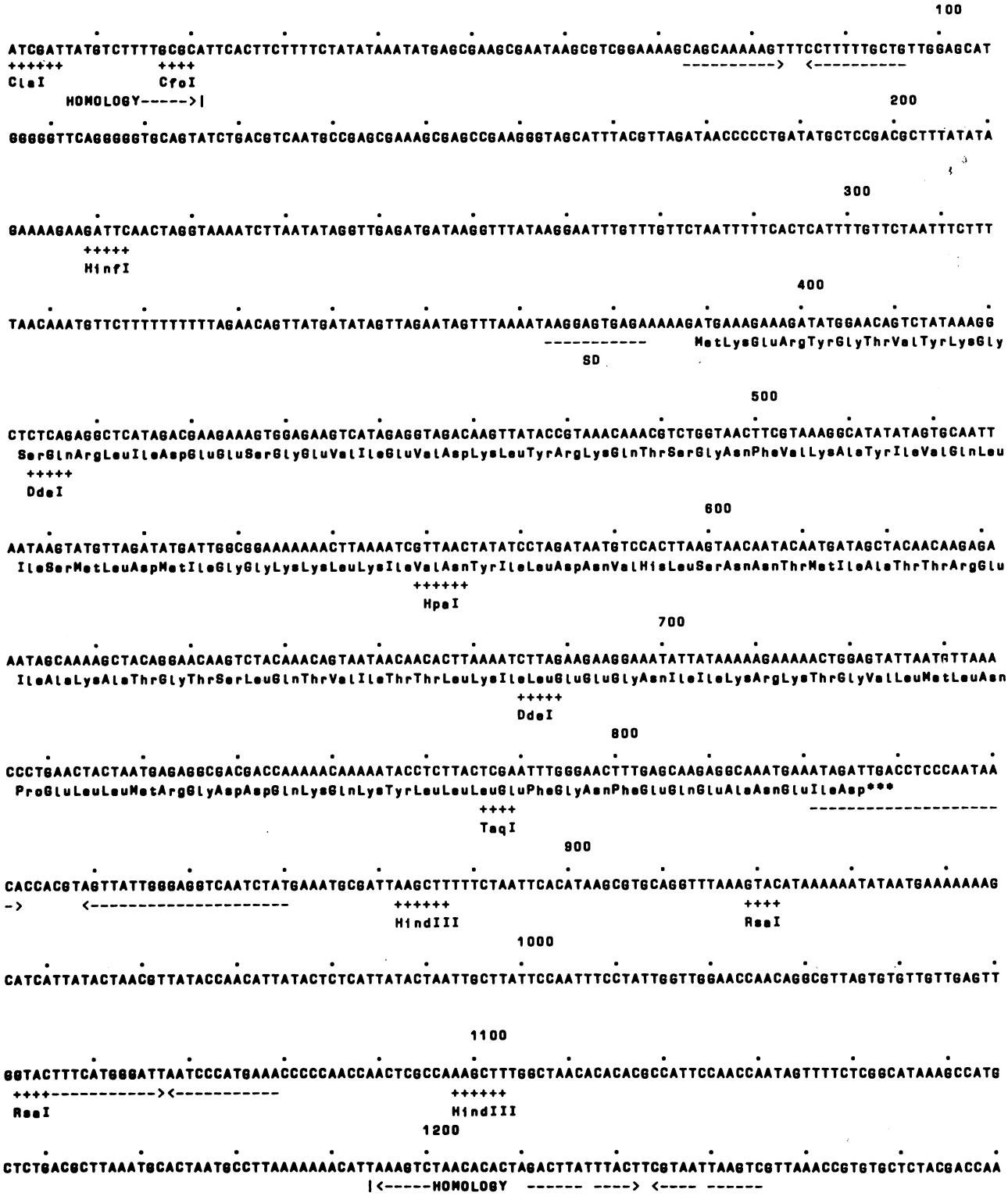
ORF 2 (positions 1420 to 2184) bore a striking resemblance to the sequence encoding *ermC* (10, 18). Based on comparison with the latter gene, it appeared that the pIM13 MLS resistance determinant began with an AUG codon at position 1453 and was preceded by an SD sequence from position 1440 to 1448. The latter was identical to SD2 that preceded *ermC* and was required for its synthesis. *ermC* expression is regulated by a translational attenuation mechanism that requires the presence of a transcribed leader segment in which SD2 is sequestered by base pairing and is thus inactive (10, 18). Translation of an upstream 19-amino-acid peptide in the presence of inducing erythromycin results in ribosomal stalling, opening of the base-paired (attenuated) structure, and freeing of SD2, allowing initiation of methylase synthesis. In the constitutively expressed pIM13 determinant, the leader sequence involved in secondary structure formation (25, 29) as well as the peptide coding sequence was absent. The missing sequence corresponded to the sequence 2856 to 2752 in the numbering system of Horinuchi and Weisblum (18). However, SD1 and SD2 remained intact. The sequence of pIM13 was consistent with deletion of the missing segment from somewhere between the positions 1438 and 1440. (We do not mean to conclude that the pIM13 MLS gene was derived from *ermC* by a deletion event.) In any event, the constitutive high-level expression of the 29K protein by pIM13 was adequately explained by the absence of the regulatory sequences. Expression may also have been enhanced by the presence of tandem SD sequences (SD1 and SD2).

The coding sequences of *ermC* and the pIM13 element were highly homologous, with alteration of only 7 of 732 base pairs. These resulted in a total of five amino acid differences between the two determinants (Table 2). The calculated molecular weight of the pIM13 protein was 28,912 and that of the *ermC* methylase was 28,947. The amino acid compositional differences were presumably responsible for the slight difference noted in electrophoretic migration between the *ermC* and pIM13 proteins. The sequence known to constitute the *ermC* promoter was present unaltered in pIM13, as was the dyad thought to constitute the *ermC* transcriptional terminator (10; Fig. 5). In fact, the sequence in pIM13 from position 1194 to 21 was largely homologous to the pE194 sequence between positions 3103 and 1924. In addition to the differences noted above, several others were present (Table 2). Within the entire homologous region, there was a total of 11 base substitutions. Except for the absence of the *ermC* leader sequence in pIM13, there were no other deletions.

The termini of the homologous region occurred near regions of dyad symmetry in both the pE194 and pIM13 sequences (Fig. 5 and 6). A dyad was common to both plasmids near each end of the homologous segment. One was located between positions 2186 and 2220. It probably represented the *erm* transcriptional terminator. The other dyad was located between the pIM13 positions 1211 and 1235. An additional dyad symmetry was noteworthy. It occurred between positions 1282 and 1307 in the pIM13 sequence, 81 base pairs from the terminus of the region of homology with pE194 (Fig. 5). Remarkably, this hyphenated dyad was inverted in pIM13 with respect to its orientation in pE194 (Fig. 6).

ORF 3 was located between positions 365 and 826. Near the beginning of this ORF was an SD sequence (373 to 383) followed by an appropriately spaced AUG codon. Translation of this frame would have resulted in a protein of molecular weight 16,620, in close agreement with the diffuse band of about 16,000 molecular weight detected in the minicell experiments (Fig. 2). In vitro transcription-translation experiments with CCC pIM13 DNA and restriction fragments derived from this plasmid confirmed that a protein of this apparent molecular weight was encoded by the DNA segment containing this ORF (C. S. Narayanan and D. Dubnau, unpublished data).

Relationship to pE5. pE5 is a 2.4-kilobase MLS resistance plasmid isolated by A. I. Stepanov from a clinical strain of *S. aureus* in Moscow, USSR (Stepanov, personal communication). The resistance element in pE5 is very similar to *ermC* by the criteria of base sequence homology in Southern blots (6) and restriction site mapping (A. G. Shivakumar and D. Dubnau, unpublished data). MLS resistance is conferred inducibly by pE5 and with the same induction specificity displayed by *ermC*. Only the MLS resistance elements of pE194 and pE5 are homologous; the replication regions of these plasmids exhibit no detectable homology on Southern blots (6). The copy number of pE5 in *B. subtilis* was 4 to 5 (data not shown). We found that the restriction site maps of pE5 and pIM13 were nearly identical (Fig. 7), except that pE5 appeared to contain two small inserts not present in pIM13. Uncleaved pE5 was slightly larger than pIM13 (Fig. 1). The first insert corresponded in position and size to the *ermC* regulatory sequence missing from pIM13, and its presence in pE5 probably accounted for the inducible nature of the MLS resistance expressed by that plasmid. The



second insert was within the C-terminal portion of the pIM13 ORF 3.

Essential replication region. The approximate limits of the region of pIM13 essential for replication and maintenance were determined. As expected, the *erm* determinant was not essential, since the minor segment from the *Cfo*I site at position 17 to the *Mbo*I site at 1369 could be removed and replaced with a chloramphenicol resistance gene with no apparent effect on the stability or copy number of the resulting plasmid. The *Hin*dIII B fragment (positions 882 to 1098) was removed by self-ligation of a *Hin*dIII-cleaved preparation of pIM13 DNA and transformation into a com-

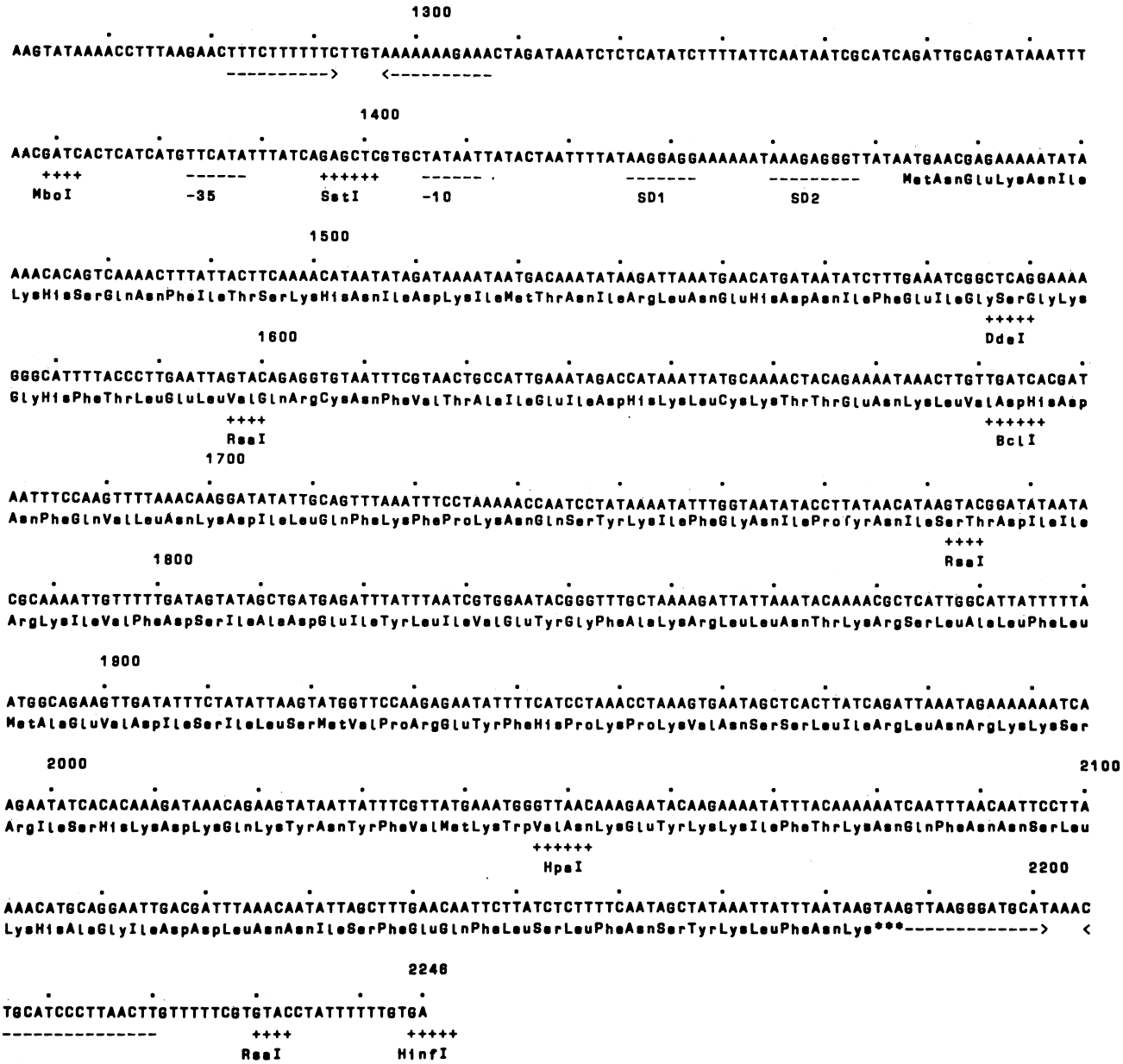


FIG. 5. Sequence of pIM13. The limits of homology with pE194 are indicated at positions 21 and 1196. SD sequences and the *erm* promoter are underlined. Regions of twofold symmetry are indicatd by converging arrows.

petent culture of *B. subtilis* BD630 with selection for erythromycin resistance. The circularized *Hin*dIII A fragment that resulted was fully capable of stable replication with a high copy number. A similar experiment in which a *Hin*fI digest of pIM13 was treated with DNA polymerase Klenow fragment to fill in the overlapping termini and then self-ligated failed to yield a miniplasmid derivative. (In addition to the *Hin*fI site indicated in Fig. 7, a second *Hin*fI site overlapped the unique *Cla*I site.) A *Bal* 31 digestion experiment (not shown) from the *Cfo*I site at position 17 suggested that removal of about 100 base pairs in the clockwise direction would yield a viable plasmid. The *Cfo*I site itself was dispensable. Thus, the essential region for replication and maintenance of this plasmid was contained within the segment defined by the *Cfo*I site (nucleotide 17) and the *Hin*dIII site (position 882).

DISCUSSION

We documented the presence of an *ermC*-like element in a *B. subtilis* plasmid. The strain carrying pIM13 was identified as *B. subtilis* by standard taxonomic criteria (24). *ermC* was originally identified in *S. aureus*, and this determinant therefore appears to be both geographically and phylogenetically widespread. pE5 was isolated in Moscow, pE194 was isolated in Bucharest (20), and pIM13 was isolated in the United States. An additional plasmid, pE12, is extremely similar to pE5 and was isolated in West Germany (S. Projan, personal communication).

Although the MLS resistance determinants on pE194, pIM13, and pE5 were quite similar, the remainder of pE194 bore no resemblance to the other plasmids in base sequence. This relationship is partly analogous to that between pUB110

TABLE 2. Comparison of homologous regions in pIM13 and pE194

Position		Base (amino acid) or rearrangement[a]	
pIm13	pE194	pIM13	pE194
1263	3034	G	C
1308	2989	C	T
1348	2949	A	C
1578	2613	G (Gly)	C (Gly)
1600	2591	C (Gln)	A (Lys)
1748	2443	T (Phe)	A (Tyr)
1813	2378	G (Asp)	A (Asn)
1885	2306	T (Phe)	C (Leu)
1982	2209	A (Asn)	G (Ser)
2019	2172	G (Gln)	A (Gln)
2232	1959	A	G
1282–1307	3015–2090	Inversion	Inversion
1438–1440	2856–2752	Deletion	

[a] Encoded amino acids are given only for the methylase sequence. The locations of the inverted palindrome are given, as are the positions in the pIM13 sequence from within which the regulatory region appeared to be missing.

and pBC16. The former is a kanamycin resistance plasmid isolated from *S. aureus* (21). The latter is a tetracycline resistance plasmid that appears to be widespread among the bacilli (1, 33). These plasmids are extremely homologous, except that they carry distinct drug resistance determinants. It appears likely that not only does exchange occur between bacilli and staphylococci but a form of horizontal modular evolution can occur in which resistance elements and replication modules can exchange or be substituted by recombination. It is possible that in the case of pIM13 and pE194 this exchange occurred by a transposonlike mechanism and that subsequent deletion events have removed most of the transposon sequences. Alternatively, non-transposon-mediated illegitimate recombination mechanisms may have been responsible. There is abundant evidence that the machinery for such events exists in both *B. subtilis* and *S. aureus* (15, 16, 23, 32, 45, 47). We have observed that a particular *rec*-independent deletion event in *B. subtilis* occurs between direct repeats and in close association with regions of hyphenated dyad symmetry (15). We have further observed that the *rec*-independent recombination responsible for the chromosomal integration of pE194 (16) often occurs in close association with regions of hyphenated dyad symmetry (L. Dempsey and D. Dubnau, unpublished data). In this connection, the association of both termini of the pE194-pIM13 homologous segment with dyad symmetry elements is noteworthy (Fig. 6B and C). The sequence of pUB110 bore a region (positions 1504 to 1560) in which 46 of 57 base pairs were identical to a portion of pIM13 (positions 1298 to 1354) (Fig. 6D). The latter region of pIM13 included one of the dyads that was also in pE194 and was associated with one of

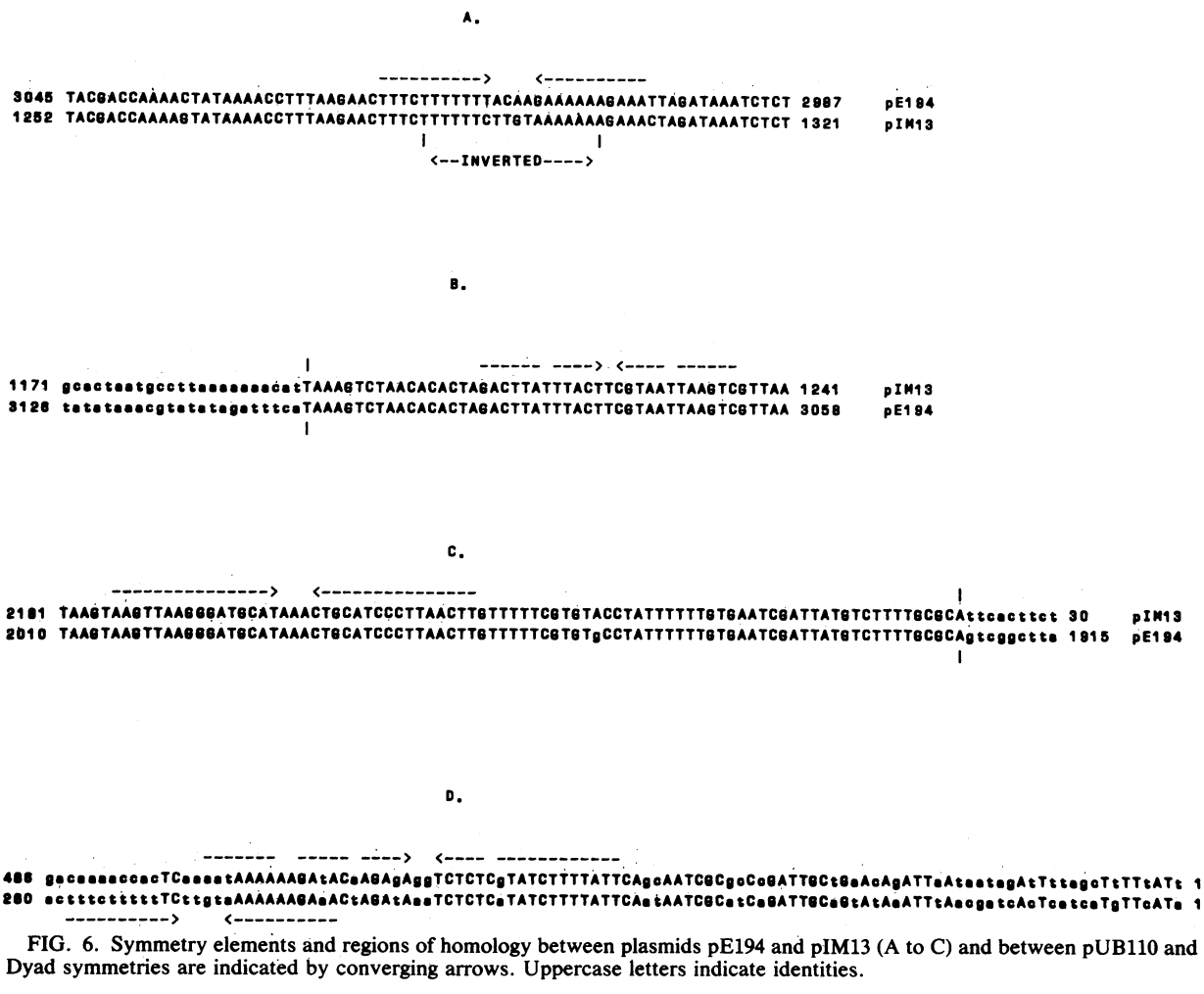


FIG. 6. Symmetry elements and regions of homology between plasmids pE194 and pIM13 (A to C) and between pUB110 and pIM13 (D). Dyad symmetries are indicated by converging arrows. Uppercase letters indicate identities.

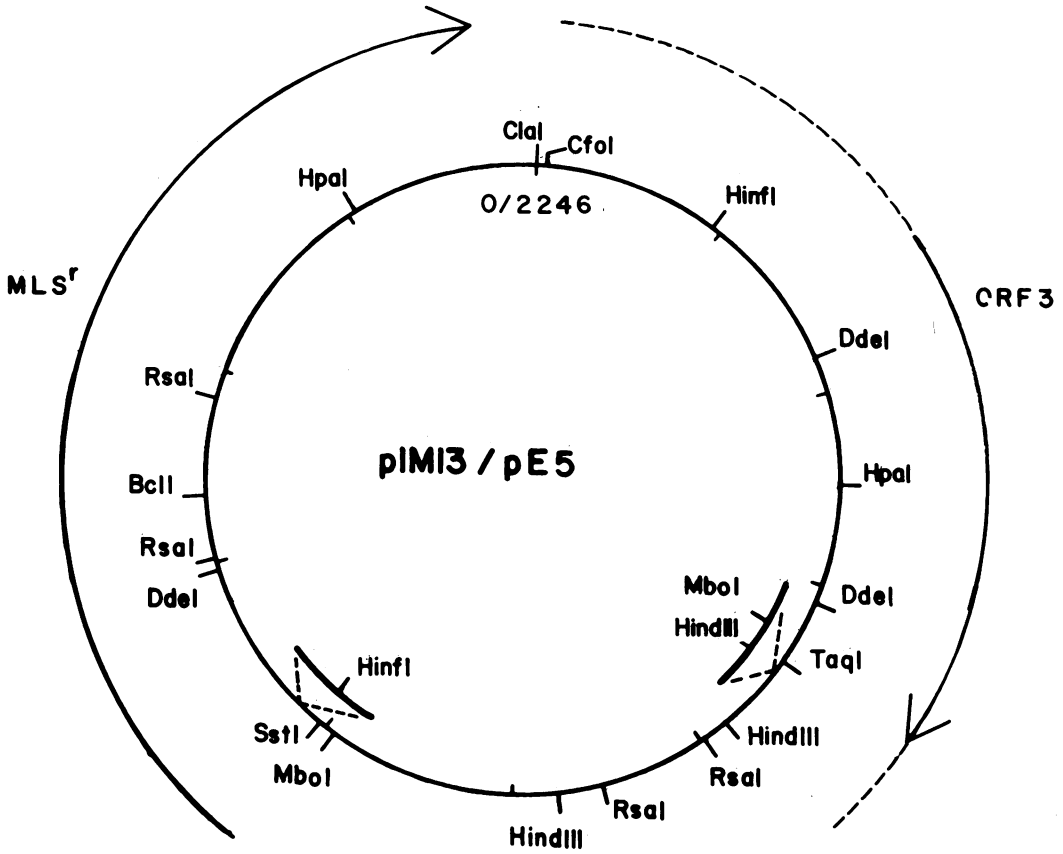


FIG. 7. Physical maps of pIM13 and pE5. The solid circle illustrates the map of pIM13. The two inner segments represent the inserts that are found in pE5. The solid outer arcs represent the two major ORFs in pIM13 and their directions of transcription. The dotted arcs delimit the essential replication segment of pIM13. 2246, Molecular size of pIM13.

the termini of the pIM13-pE194 homologous segment (Fig. 6A). Furthermore, the corresponding sequence in pUB110 (25a) also contains a hyphenated dyad symmetry that overlaps the one in pIM13 (Fig. 6D). It is interesting that the pUB110-pIM13 homology occurs near the point at which exchange of the kanamycin and tetracyline genes occurs between pUB110 and pBC16. Based on published restriction maps, it is possible to estimate that the maximum distance between that point and the homologous region is 120 base pairs (39). It appears that regions of homology between otherwise unrelated pairs of plasmids are associated with hyphenated dyad symmetries and are located near the sites of recombinational events in which plasmid gene substitutions appear to have occurred.

In pIM13, 1003 of 2246 base pairs were devoted to the expression of MLS resistance. We showed that the functions required for plasmid replication and maintenance were contained within a 865-base-pair segment defined by the unique *Cfo*I site and the *Hin*dIII site at position 882. This was roughly the size of the essential replication region of other small multicopy plasmids and included ORF 3. It is likely that ORF 3 encodes a replication protein. Such a product is required for the replication of the *S. aureus* plasmid pT181 (30) and almost certainly is required for pE194 replication as well (11; R. Villafane and D. Dubnau, unpublished data). The predicted ORF 3 product bore no obvious amino acid sequence homology to the replication proteins of these two plasmids or to any of the replication proteins potentially encoded by the *S. aureus* plasmids pUB110 and pC194 (19, 25a). We found that the otherwise nearly identical plasmids pE5 and pIM13 differed strikingly in copy control and that pE5 contained an approximately 100-base-pair insert within ORF 3. It will be interesting whether this insert maintains the reading frame and is responsible for the difference in copy control.

The remarkable distribution of pIM13 DNA in agarose gels deserves discussion. When DNA fragments ranging in size from 1 to 14 kilobases were inserted into pIM13, the agarose gel pattern of the resulting plasmid DNA shifted to a typical one, in which the CCC monomer was the predominant form (not shown). It is possible that the unusual pIM13 pattern was due to a relatively slow resolution of replication products. Insertion of DNA may have increased the replication time relative to the resolution time, thus retarding the accumulation of unresolved replication intermediates.

One motive for our investigation of pIM13 was the possibility that this plasmid will be useful as the basis of new cloning vectors for use in *B. subtilis*. We observed that pIM13 was stably inherited in this organism and seemed to tolerate the acquisition of at least several kilobases of foreign DNA without a marked reduction in copy number. We are presently exploring the value of this plasmid as a cloning vector.

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ADDENDUM IN PROOF

After this manuscript was submitted, we noted that Lampson and Parisi (J. Bacteriol. **166**:479–483) observed a deletion in the *S. epidermidis* MLS-resistance plasmid pNE131 that is identical to that in the *erm* gene of pIM13.

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