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Replication Properties of pIM13, a Naturally Occurring Plasmid Found in *Bacillus subtilis*, and of Its Close Relative pE5, a Plasmid Native to *Staphylococcus aureus*

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A naturally occurring plasmid from *Bacillus subtilis*, pIM13, codes for constitutively expressed macrolide-lincosamide-streptogramin B (MLS) resistance, is stably maintained at a high copy number, and exists as a series of covalent multimers. The complete sequence of pIM13 has been reported (M. Monod, C. Denoya, and D. Dubnau, J. Bacteriol. 167:138–147, 1986) and two long open reading frames have been identified, one of which (*ermC'*) is greater than 90% homologous to the *ermC* MLS resistance determinant of the *Staphylococcus aureus* plasmid pE194. The second reading frame (*repL*) shares homology with the only long open reading frame of the cryptic *S. aureus* plasmid pSN2 and is probably involved in plasmid replication. The map of pIM13 is almost a precise match with that of pE5, a naturally occurring, stable, low-copy-number, inducible MLS resistance plasmid found in *S. aureus*. pIM13 is unstable in *S. aureus* but still multimerizes in that host, while pE5 is unstable in *B. subtilis* and does not form multimers in either host. The complete sequence of pE5 is presented, and comparison between pIM13 and pE5 revealed two stretches of sequence present in pE5 that were missing from pIM13. It is likely that a 107-base-pair segment in the *ermC'* leader region missing from pIM13 accounts for the constitutive nature of the pIM13 MLS resistance and that the lack of an additional 120-base-pair segment in pIM13 that is present on pE5 gives rise to the high copy number, stability, and multimerization in *B. subtilis*. The missing 120 base pairs occur at the carboxy-terminal end of the putative replication protein coding sequence and results in truncation of that protein. It is suggested either that the missing segment contains a site involved in resolution of multimers into monomers or that the smaller replication protein causes defective termination of replication. It is concluded that pIM13 and pE5 are coancestral plasmids and it is probable that pIM13 arose from pE5.

The spread of antibiotic resistance in the environment is of clinical, veterinary, and biological interest. The same or similar plasmids encoding resistances have appeared in more than one species, and clearly such plasmids play a major role in the spread of antibiotic resistance. This study explores the relationship between two naturally occurring plasmids of gram-positive bacteria. pIM13 is a naturally occurring macrolide-lincosamide-streptogramin B (MLS) resistance plasmid originally isolated from *Bacillus subtilis* (9). It has a high copy number in *B. subtilis* (about 200) but a low copy number in *Staphylococcus aureus* (about 10). pIM13 confers constitutive erythromycin resistance on both hosts and forms multimers (12). Because of its high copy number and stability properties, pIM13 has the potential to become a useful *B. subtilis* cloning vehicle. It was noted previously that pE5, even though it is native to *S. aureus*, is almost identical to pIM13 (12). We report here that in contrast to pIM13, pE5 is unstable in *B. subtilis* but stable in *S. aureus*, confers inducible MLS resistance on both hosts (12), and does not multimerize in either host. We also present the complete nucleotide of sequence of pE5, compare it phenotypically with pIM13, and define loci on these plasmids responsible for the observed phenotypic differences. Another plasmid that can be assigned to this family is pNE131. Originally isolated from *Staphylococcus epidermidis*, it has been reported to confer constitutive MLS resistance on that host while being maintained stably and at low copy number (8). The MLS resistance plasmid pE12 was originally described as an in vitro construct derived from the *S. aureus* plasmid pI258 (6). However, we report here that pE12 and pE5 are identical plasmids and that pE12 is not related to pI258 but was probably a contaminant.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The staphylococcal strains used are derivatives of strain NTGC 8325, specifically RN451 (16). The *B. subtilis* strains are derivatives of strain 168. Plasmids are listed in Table 1.

Media and growth conditions. CY broth was used for liquid cultures of *S. aureus*, and VY broth (17) was used for liquid cultures of *B. subtilis*. Cultures were shaken at 37°C (unless otherwise indicated) and monitored turbidometrically with a Klett-Summerson photoelectric colorimeter with a green filter. GL agar (*S. aureus*) (17) or TBAB agar (*B. subtilis*) (Difco Laboratories) was supplemented with antibiotics as indicated. Tetracycline, chloramphenicol, streptomycin, and erythromycin were used at 5 μg/ml unless otherwise indicated.

Preparation of competent cells of *B. subtilis* and transformation with plasmid DNA were carried out as described previously (2). Protoplast transformation was done by the method of Chang and Cohen (1) modified for *S. aureus* (14).

Isolation and analysis of plasmid DNA. DNA was prepared and analyzed as described (18, 20). Plasmid DNA isolated prior to S1 treatment for analysis of single-stranded DNA was obtained by the method of TeRiele et al. (26). Copy

TABLE 1. Plasmids

Plasmid	Relevant phenotype	Source or reference
pIM13	MLS^r	9
pE5	MLS^r	3
pBD347	Cm^r	12; this work
pBD367	MLS^r	This work
pE12	MLS^r	6
pROJ5201	MLS^r	This work

numbers were determined by fluorimetric densitometry of ethidium bromide-stained agarose gels run on sheared whole-cell minilysates of exponentially growing cultures (20).

Restriction mapping and cloning. Restriction enzymes were purchased from New England BioLabs and Boehringer Mannheim Biochemicals. For molecular cloning, specific fragments were extracted from polyacrylamide gels, phenol extracted, ethanol precipitated, and stored lyophilized prior to storage and use (10). For ligation, samples were combined in approximately equimolar ratios and incubated with T4 DNA ligase (Collaborative Research) at a DNA concentration of at least 10 μg/ml and a ligase concentration of 40 U/ml for fragments with complementary ends and at 100 U/ml for fragments with blunt ends. Incubation was at room temperature for 1 to 4 h. Ligated samples were used to directly transform *S. aureus* protoplasts or *B. subtilis* competent cells with selection for the appropriate antibiotic resistance marker. Transformants were screened for plasmid content, and those carrying plasmids of the expected size were used to prepare plasmid DNA for restriction analysis and sequencing.

Assay for MLS resistance inducibility. Cells of either *S. aureus* or *B. subtilis* carrying the indicated plasmids were overlaid on a GL agar plate with Tylosin at 10 μg/ml. At the center of the plate a disk saturated with erythromycin was placed. If the MLS resistance is constitutive, the cells will grow on the plate; if it is inducible, than there will be a halo of growth surrounding the erythromycin disc.

DNA sequencing. Determination of nucleotide sequences was done by the dideoxy chain termination method (21) by cloning isolated restriction fragments into either mp10 or mp11 M13 vectors (11).

Southern blotting and hybridization. Single-stranded DNA probes were prepared from M13mp11 clones with the Boehringer Mannheim hybridization primer and [α^{32}P]dATP as specified by the manufacturer. Transfer of DNA from agarose gels to nitrocellulose filters was done by the procedure of Southern (23). Filters were prehybridized for 2 h at 65°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, and salmon sperm DNA (100 μg/ml). Hybridization was performed overnight in 5× SSC–5× Denhardt solution plus radiolabeled probe DNA at 65°C. The filters were washed after hybridization in 2× SSC–0.1% sodium dodecyl sulfate at 65°C with three wash changes, followed by a final rinse at room temperature with 0.1× SSC–0.1% sodium dodecyl sulfate.

RESULTS

Plasmid pedigrees. As stated above, pIM13 was originally isolated in *B. subtilis*, while pE5 is native to *S. aureus*. In addition to these plasmids, a plasmid found in *S. epidermidis*, pNE131 has also been studied by others (8), and based on its sequence, it too is a member of this family. Purified plasmid DNA from both pIM13 and pE5 was used to transform cells of both *S. aureus* and *B. subtilis*. After transformation with pE5, a deletion derivative arose spontaneously in *B. subtilis*. This plasmid, pBD367, was transformed back into *S. aureus*, and its phenotypic properties were analyzed in both hosts. The extent of the spontaneous deletion was determined by restriction analysis followed by nucleotide sequencing, and it was determined that pBD367 was deleted for precisely the same 120 base pairs (bp) that pIM13 was missing from the carboxy-terminal region of the putative pE5 replication protein open reading frame (see below). Figure 1 shows physical and genetic maps of these plasmids and their hypothetical relationships. It was noted while working with pE12, a plasmid originally described as a derivative of the *S. aureus* plasmid pI258 (6), that its restriction map differed from that of pI258 but was very similar to that of pIM13. This plasmid was phenotypically identical to pE5, and subsequent sequence analysis revealed that pE5 and pE12 were identical plasmids.

Sequence features. The complete nucleotide sequences of pE5 and pE12 are presented in Fig. 2. The plasmids were sequenced by the dideoxy chain termination method as described in Materials and Methods. To obtain a completely double-stranded sequence, clones of M13 derivatives mp10 and mp11 were constructed with the following restriction enzymes (the number of fragments for each enzyme is indicated): *Cla*I (one), *Hin*dIII (three), *Taq*I (two), *Hpa*I (two), *Hin*fI (two), *Dde*I (three), *Rsa*I (five), *Mbo*I (three), and 4 of 10 *Alu*I fragments. All restriction sites were crossed during the sequencing.

We noted two open reading frames of greater than 100 amino acid residues. One of these was the erythromycin resistance determinant and was highly homologous to the *ermC* gene of pE194 (12). The other open reading frame encoded a putative replication protein, *repL*. The *repL* reading frame had homology with the only long open reading frame of the cryptic *S. aureus* plasmid pSN2 (7, 8). Based on this homology, the finding that a *Cfo*I-*Mbo*I fragment of pIM13 ligated to a chloramphenicol resistance determinant (the *cat* gene from pC194) was a functional replicon in *B. subtilis* (referred to as pBD347) (12), and the finding that the *Hin*dIII B fragment of pIM13 could be deleted, it is likely that the pSN2 homology defines the minimal replicon. Based on the available sequence data, pE5 is nearly identical with pIM13 and pNE131 as well as with a spontaneously arising plasmid, pBD367. However, sequences present on pE5 were missing from the other three plasmids (Fig. 1 and 2). pNE131 and pIM13 were missing 107 bp found in the *ermC'* leader region. pBD367 and pIM13 were missing 120 bp found at the carboxy-terminal portion of the *repL* reading frame and extending into a large region of dyad symmetry (see below). pE5 had two large regions of dyad symmetry (palindromes) (Fig. 2). One of these, *palA*, had significant homology with pT181, and similar sequences have been identified in most of the small *S. aureus* plasmids analyzed. The pT181 *palA* sequence has been identified as a possible site for lagging-strand initiation for plasmids in *S. aureus* (4). The possibility that it serves the same function in pE5 is discussed below. The other palindrome, called *palB* here, has as yet no defined function and had no homology with plasmids other than pE5 and its close relatives discussed here.

We note here that both pIM13 and pE5 were about 69% A+T and 31% G+C. This more closely resembles the A+T content of the *S. aureus* chromosome (70% A+T) than that of the *B. subtilis* chromosome (57% A+T).

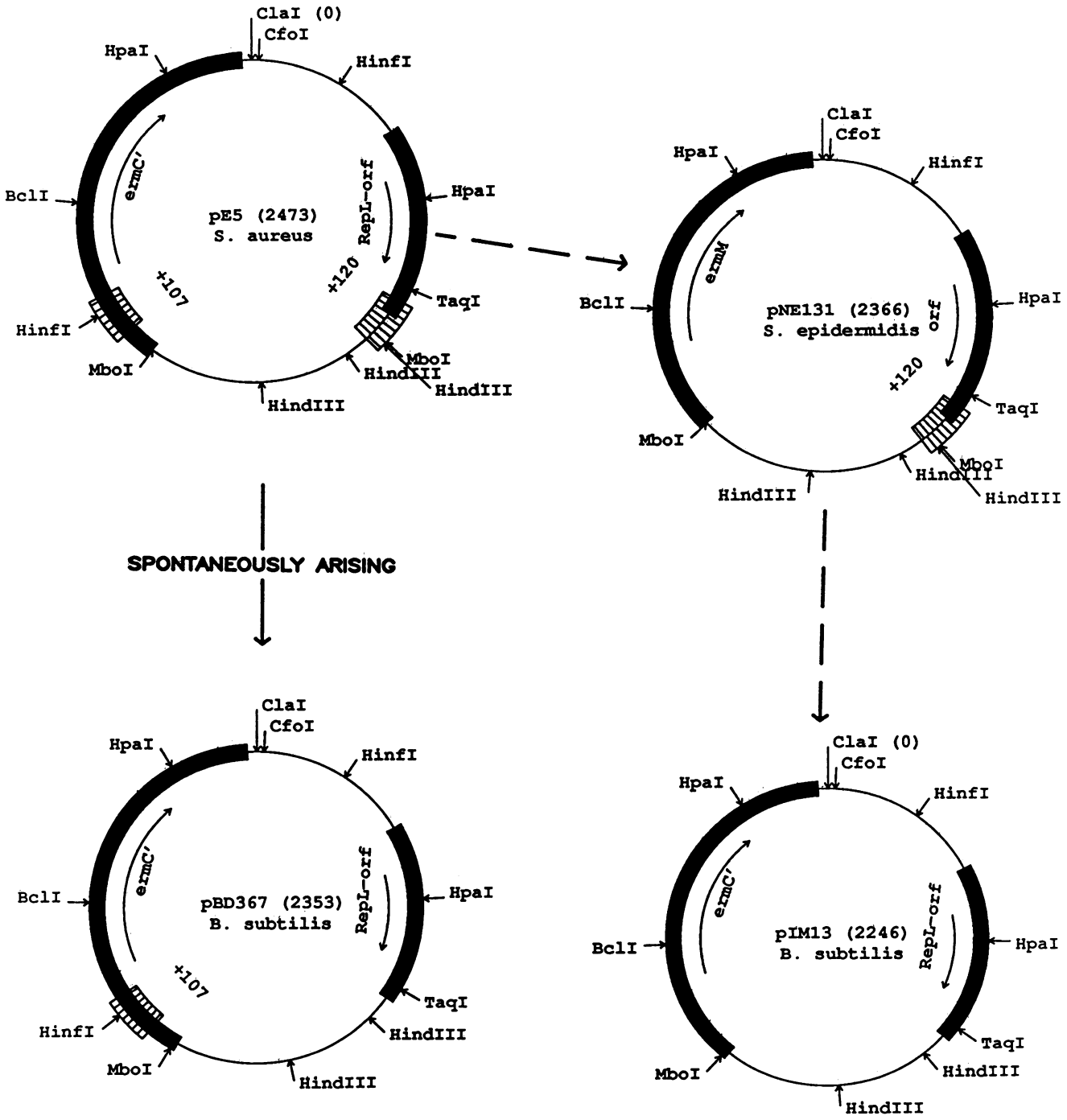

FIG. 1. Physical maps of pE5, pIM13, pNE131, and pBD367. Restriction sites are indicated. The solid boxes indicate the open reading frames (orfs) (*ermC'* and *ermM* represent the MLS resistance determinant, *repL*-orf represents the putative plasmid-encoded replication protein). Hatched areas represent sequences present on pE5 but missing from the other plasmids (in various combinations). The arrows indicate the direction of transcription. The dashed lines indicate a hypothetical "evolutionary" pathway from pE5 in *S. aureus* to pNE131 in *S. epidermidis* to pIM13 in *B. subtilis*. Numbers indicate base pairs.

Phenotypes. Table 2 summarizes the phenotypes of pIM13, pE5, and pBD367 with respect to MLS resistance, copy number, stability, and multimerization. We will discuss each of these aspects individually below.

MLS resistance. pIM13 (and pNE131) expressed MLS resistance constitutively, while pE5 and pBD367 were both inducible for MLS resistance. This was true in both *S. aureus* and *B. subtilis*. The sequence of pIM13 was nearly identical to that of pE5 and pBD367 in the structural gene encoding MLS resistance (*ermC'*), but 107 bp present on pE5 and pBD367 in the leader region were not present in pIM13. From studies of the pE194 *ermC* gene, it is reason-

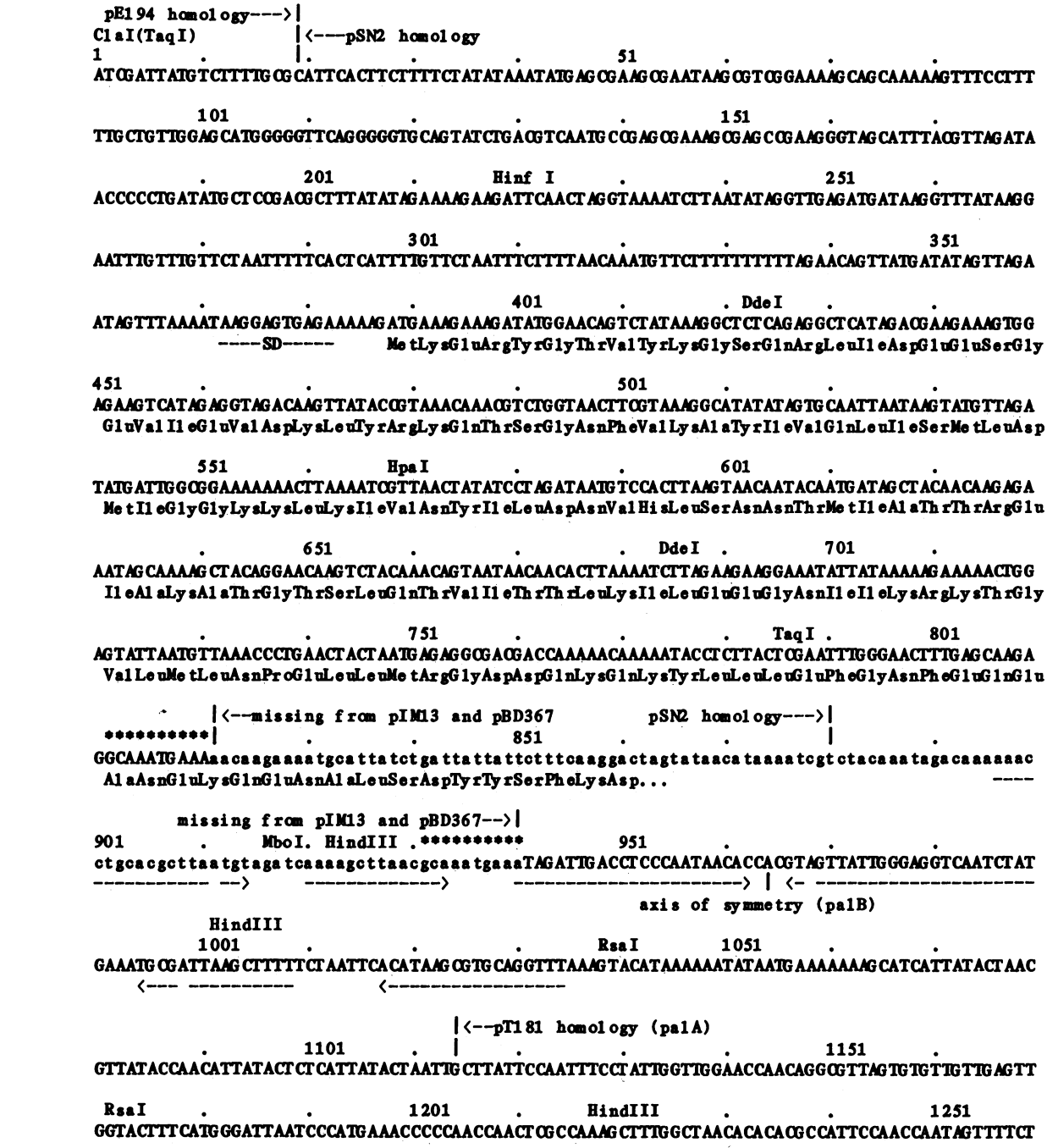

FIG. 2. Complete nucleotide sequence of pE5 numbered from the unique *Cla*I site. Lowercase letters indicate sequences missing from pIM13. Homologies with pT181, pSN2, and pE194 are indicated. Open reading frames with their potential Shine-Dalgarno (SD) sequences are indicated, with the amino acid sequence under the DNA sequence. Axes of dyad symmetry are indicated by converging arrows. *, Ten-base direct repeat.

able to assume that this "deletion" was responsible for the constitutive nature of MLS resistance in pIM13 and pNE131,as pointed out previously (8, 12).

Copy number. Both pIM13 and pBD367 were high-copy-number plasmids in *B. subtilis*, with copy numbers of 200 per cell, and were stable in that host, whereas they both exhibited low copy number (10 per cell) and were unstable in *S. aureus*. pE5 had a low copy number in both hosts but was unstable only in *B. subtilis*. (The copy numbers presented for multimerizing plasmids refer to the total number of monomeric units of those plasmids.) The higher copy numbers of pIM13 and pBD367 were directly traceable to the fact that these plasmids were missing 120 bp that were present in pE5, as shown by the following experiment. The Cm^{r}pIM13 derivative pBD347 was treated with *Mbo*I and *Taq*I, and the smallest fragment was isolated. The following fragments were isolated from pE5: *Mbo*I-B (the *ermC* promoter-bearing fragment), the *Mbo*I-*Taq*I fragment that carries the

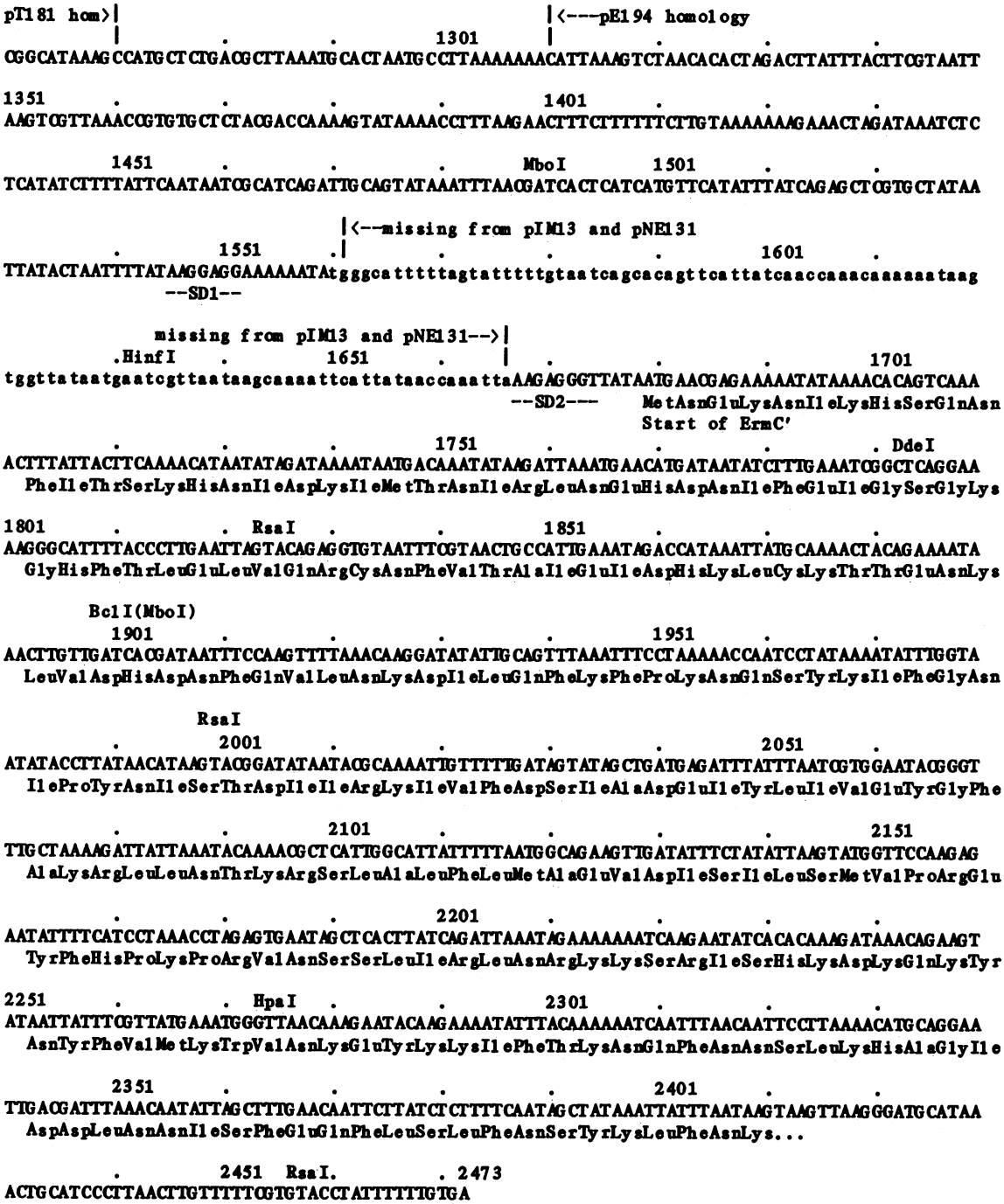

TABLE 2. Plasmid phenotypes

Plasmid	Copy no./cell[a]		Stability[b] (%)		Erythromycin inducibility		Multimerization	
	S. aureus	*B. subtilis*	*S. aureus*	*B. subtilis*	*S. aureus*	*B. subtilis*	*S. aureus*	*B. subtilis*
pIM13	10	200	46	100	−	−	+	+
pE5	10	10	100	48	+	+	−	−
pBD367	10	10	45	100	+	+	+	+

[a] Determined by fluorescence densitometry of ethidium bromide-stained agarose gels run with whole-cell lysates.
[b] Percentage of cells remaining erythromycin resistant after 25 generations of nonselective growth.

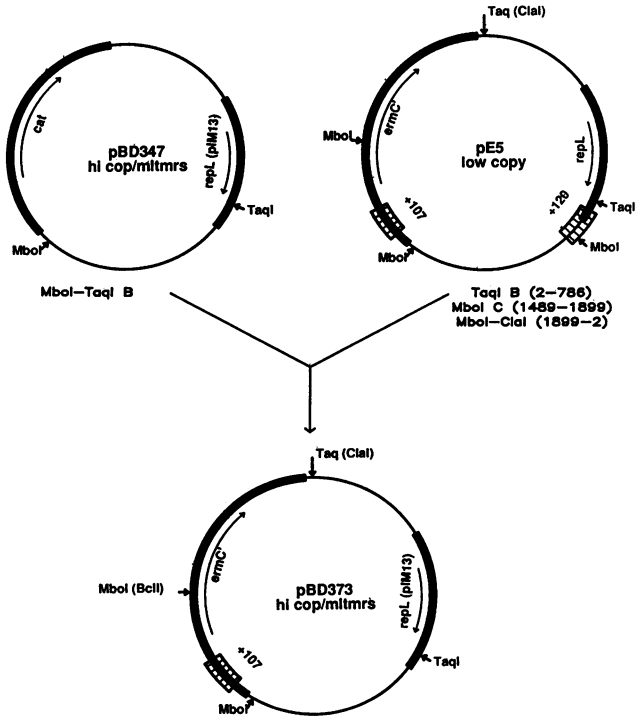

FIG. 3. Construction of pBD373. As described in the text, pBD373 was constructed from the smaller *Mbo*I-*Taq*I fragment from pBD347 as pictured and the *Taq*I-B, *Mbo*I-C, and *Mbo*I-*Cla*I fragments from pE5 (sequence positions are indicated [in base pairs]). hi cop, High copy number; mltmrs, multimers.

remainder of *ermC'*, and a *Taq*I fragment that carries most of the replication fragment. These were ligated and transformed into *B. subtilis*, selecting for Em^r. This cloning is shown schematically in Fig. 3. In effect, this experiment replaced the carboxy-terminal end of the pE5 *repL* coding sequence with the pIM13 sequences which were missing 120 bp from that region. (The pE5 *ermC'* gene remained intact and inducible.) All the clones obtained (37 of 37) were high copy number, multimerized, and were stable in *B. subtilis*. This indicates that the pBD347 fragment used (which was from pIM13 originally) contained the locus responsible (the 120-bp missing segment) for the differences in copy number and multimerization between pIM13 and pE5. It should be noted that the only difference between the *Mbo*I-*Taq*I fragments of pBD347 and pE5 was the absence or presence of this 120-bp segment. By using an in vitro transcription-translation system (15), a 17- to 18-kilodalton (kDa) peptide was observed when pE5 DNA was used as template DNA. This corresponds to the predicted size of the RepL protein. However, when pIM13 DNA was used to direct protein synthesis, a 15- to 16-kDa band was observed (Fig. 4). This is consistent with the idea that RepL for pIM13 is "truncated" because of the missing sequences described above.

Multimerization. Plasmid DNA of pIM13 and pE5 was isolated (by dye-buoyant gradient centrifugation) from both *B. subtilis* and *S. aureus* hosts, and equal amounts of DNA were subjected to agarose gel electrophoresis. Figure 5 shows the previously reported phenomenon of pIM13 multimerization. In addition we noted that pIM13 multimerization occurred in both hosts with precisely the same multimerization pattern (Fig. 5, lanes 3 and 4) and that pE5 showed little if any multimerization in either host (Fig. 5,

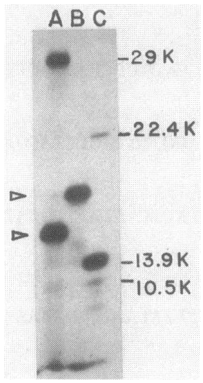

FIG. 4. Translation of pIM13 and pE5 proteins. pIM13 (A), pE5 (B), and ϕ29 (C) DNAs (5 μg each) were used as templates in the *B. subtilis* in vitro coupled transcription-translation system as described previously (15). The products of the ϕ29 template and the constitutively expressed MLS methylase of pIM13 served as molecular weight (10^3) markers. The positions of the plasmid Rep proteins are indicated by arrowheads.

lanes 1 and 2). Multimerization is therefore a property of the plasmid and not the host in which the plasmid resides. The multimers could exist in two possible topological forms, catenanes or covalently closed multimeric circles (concatenates). To distinguish between these two possibilities, the time course of pIM13 digestion with *Cla*I was followed. *Cla*I cleaves pIM13 monomers at a single site. If the multimers were catenanes, then only one new band corresponding to linear monomers (Fig. 6, 4 h) would appear. However, several new bands became visible, probably corresponding to various linear oligomers, indicating that the multimers were covalently linked. Treatment for longer than 4 h and

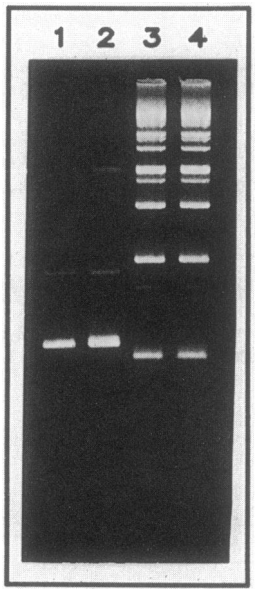

FIG. 5. Approximately equal amounts of purified plasmid DNA were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. Lanes: 1, pE5 isolated from *B. subtilis* BD170; 2, pE5 isolated from *S. aureus* RN451; 3, pIM13 isolated from *B. subtilis* BD170; 4, pIM13 isolated from *S. aureus* RN451.

the use of higher concentrations of *Cla*I resulted in complete conversion to linear monomers (not shown).

Multimer formation, like the copy number effects noted for pBD367 and pIM13 in *B. subtilis*, was associated with the 120 bp missing from the end of the *repL* open reading frame. This was verified by the fragment exchange experiment described above (not shown).

Two possibilities to explain concatamer formation may be entertained: loss of a resolution site (as with ColE1 derivatives missing the *cer* site [24, 25]) or defective termination of rolling-circle replication due to truncation of the replication protein. A third possibility, the involvement of the host generalized recombination system, is unlikely, since the same pattern of multimerization was observed in both *S. aureus* and *B. subtilis* hosts and no difference was noted in *recE* strains of *B. subtilis* (not shown).

Examination of the pE5 and pIM13 sequences (Fig. 2) revealed that the 120-bp region missing from pIM13 contained part of the *palB* palindrome. One attractive hypothesis is that this could be a multimer resolution site and that pIM13 is disrupted in that site. A deletion of the pE5 *Hin*dIII fragment that included bases 923 to 1002 was constructed in vitro. The resulting plasmid (pROJ5201) did not multimerize. In fact, no phenotypic difference between pROJ5201 and pE5 was noted. Therefore, disruption of the *palB* dyad was not sufficient to cause multimerization.

Replication protein. We noted that the *repL* open reading frame was truncated in pIM13 and pBD367. Actually, the last 14 amino acid residues (Lys-Gln-etc.) of pE5 were replaced by two (Ile-Asp) in pIM13. In addition, a large region of dyad symmetry (*palB*) was partially disrupted, as noted above. The pIM13 chloramphenicol-resistant derivative pBD347 (described above) was cleaved at its unique *Hpa*I site (located in the *repL* open reading frame), subjected to Bal31 treatment, religated, and transformed back into *B. subtilis*. Autonomous plasmids were not recovered after transformation into a plasmid-negative host. However, when transformed into a strain carrying pIM13, Cm^r colonies arose. Analysis of the plasmid DNA present in these strains revealed a complex pattern suggesting the presence of both pIM13 and another plasmid (presumably a pBD347 derivative), both of which multimerized. While it was possible to obtain Em^r Cm^s segregants, Em^s Cm^r segregants did not arise. Retransformation with the plasmid DNA isolated from one of these strains yielded 73 Em^r colonies, of which all were Cm^s, when selection was for erythromycin resistance. Selection for chloramphenicol resistance gave 12 colonies, all Cm^r Em^r. These data suggest that interruption of the open reading frame inactivates the pBD347 replicon, but that a *trans*-acting factor supplied by pIM13 can restore the missing function. It is therefore likely that RepL is required for plasmid replication and provides a *trans*-active function.

Mechanism of replication. It has been reported by TeRiele et al. (26) that many small plasmids of gram-positive bacteria accumulate strand-specific, circular, single-stranded DNA. This effect is noted most prominently in *B. subtilis* strains harboring the plasmids of *S. aureus*. Gruss et al. (4) inferred that a structure on the plasmid pT181 which they refer to as *palA* may be a lagging-strand initiation site in *S. aureus* but not in *B. subtilis*, explaining why the degree of single-stranded DNA accumulation is greater in *B. subtilis* than in *S. aureus* for these plasmids. Sequences similar to the *palA* palindrome have been found on 10 of the 11 completely sequenced *S. aureus* plasmids (including pE5). If the hypothesis of Gruss et al. is correct, then it is likely that plasmids with this *palA* structure replicate by a similar mechanism

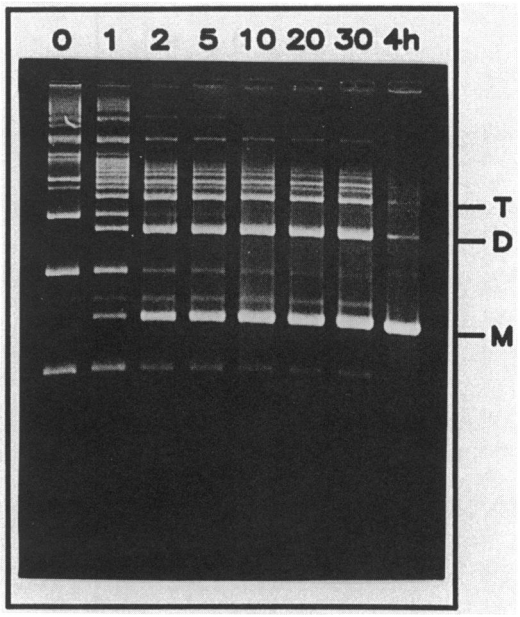

FIG. 6. Agarose gel electrophoresis of pIM13 DNA digested with *Cla*I at 10 U/ml as a function of time (in minutes, except for the rightmost lane, a 4-h time point). The 1% agarose gel was stained with ethidium bromide, and DNA was visualized with UV light. Note the appearance of linear monomer (M), dimer (D), and trimer (T) bands. After 4 h most of the DNA was in the linear monomer form.

(assymetric rolling-circle replication with a *palA* lagging-strand initiation site), although a D-loop mechanism has not been precluded. Because pIM13 and pE5 have this structure, we would predict that pIM13 and pE5 would accumulate strand-specific, single-stranded DNA in *B. subtilis*. Figure 7 shows that this was the case. DNA was isolated from *B. subtilis* cells harboring pIM13 or pE5 and either treated with nuclease S1 or left untreated. As can be seen for both pIM13 and pE5, there were S1-sensitive bands on the Southern blot. In addition, there appeared to be a single S1-sensitive band for pE5 but several for pIM13, consistent with the existence of single-stranded multimers. The probe used in this experiment was single-stranded pIM13 DNA obtained by cloning *Cla*I-digested pIM13 DNA into the mp11 *Acc*I site. When the opposite orientation of the insert was used as a probe, the S1-sensitive bands were not visible, indicating that this DNA represents a unique single strand. The S1-resistant bands were identical with this probe (results not shown).

DISCUSSION

This study presents data which strongly indicate that a plasmid isolated from *B. subtilis*, pIM13, has probably arisen from the *S. aureus* plasmid pE5. At the very least, these plasmids are coancestral. The data to support our contention that pE5 is an antecedent of pIM13 follow. pIM13 was identical, base for base, to pE5 with the exception of 227 bp present on the pE5 genome but missing from pIM13 (in stretches of 107 and 120 bp). The plasmid pBD367 arose spontaneously when pE5 DNA was transformed into *B. subtilis*. pBD367 was deleted for the same 120 bp that were present on pE5 but missing from pIM13. We have shown that the differences in stability, copy number, and multimerization between pE5 and pIM13 were associated with the

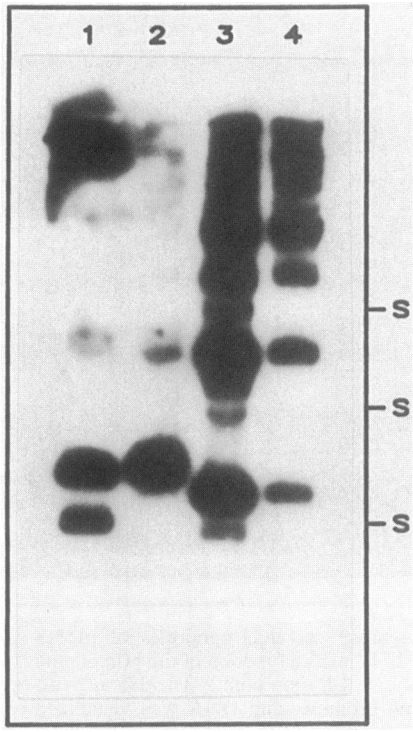

FIG. 7. Autoradiograph of a Southern blot. DNA was isolated by the method of Riele et al. (21) and either left untreated or treated with S1 nuclease. The DNA was separated by agarose gel electrophoresis and transferred to nitrocellulose filters. Single-stranded probes were prepared as described in the text. Lanes: 1, pE5 from *B. subtilis*, untreated; 2, pE5 from *B. subtilis*, treated with S1 nuclease; 3, pIM13 from *B. subtilis*, untreated; 4, pIM13 from *B. subtilis*, treated with S1 nuclease. The bands marked S were S1 sensitive and were therefore single-stranded.

120-bp deletion/insertion. In addition, pNE131, a plasmid isolated from *S. epidermidis*, was the same as pE5 except that it was missing 107 bp in the *ermC'* leader region (but intact elsewhere). pIM13 was also missing those 107 bp, and this deletion rendered pIM13 and pNE131 MLS resistance constitutive. Both pIM13 and pE5 had an A+T composition of 69%, which is similar to that of the *S. aureus* rather than the *B. subtilis* chromosome. The two plasmids both had *palA*, a potential lagging-strand initiation site in *S. aureus* but not *B. subtilis*. Both pIM13 and pE5 accumulated single-stranded DNA in *B. subtilis*. It is therefore likely that pIM13 arose from pE5, probably through a pNE131-like intermediate.

It has long been observed that molecular cloning in *B. subtilis* is problematic because of structural instability (i.e., spontaneous deletions). Ehrlich has proposed that this is caused by the fact that the vector plasmids used in such clonings accumulate large amounts of single-stranded DNA in *B. subtilis* and that such single-stranded DNA is recombinogenic owing to its invasiveness (S. D. Ehrlich, personal communication). Ehrlich has predicted and observed that short regions of homology can serve as recombinational hot spots. (Although Hahn and Dubnau have shown that homologies do not, a priori, produce deletions [5].) The 120-bp deletion in pBD367 occurred at a stretch of 10 bp directly repeated in the pE5 sequence (see the starred bases in Fig. 2), and we have demonstrated that pIM13 and pE5 accumulate single-stranded DNA. We also noted that there was some homology between the pE5 deletion site, a site in pUB110-pC194 cointegrates that is a recombinational hot spot (5), and a sequence on pTB19 (a *Bacillus stearothermophilus* plasmid) which is at a junction between regions of homology and nonhomology with pUB110 (13). These potential intraplasmid recombination sites are shown in Fig. 8. An alternative hypothesis to explain the observed deletions is that short stretches of homology associated with palindromic regions can result in deletions. In the example described here, the 10-bp direct repeat was associated with the *palB* palindrome. Both the pUB110 and pTB19 sites were within 120 bp of a large dyad. It is also possible that this region is in the pIM13/pE5 origin of replication and represents a possible nick site for 3' extension (a similar role has been suggested for the Hahn and Dubnau and pTB19 sites; M. F. Gros, H. T. Riele, and S. D. Ehrlich, submitted for publication).

It has been noted previously that plasmids with high copy numbers in *S. aureus* (both mutant and wild type) have either comparable or lower copy numbers in *B. subtilis*. Wild-type pT181 has similar copy numbers in both hosts, but some copy mutants, most notably pT181-*cop608*, have much higher copy numbers in *S. aureus* than in *B. subtilis* (19). Wild-type pE194 has a copy number of 55 per cell in *S. aureus* but about 6 per cell in *B. subtilis* (6, 22). Both wild-type and copy mutant pC221 replicons have lower copy numbers in *B. subtilis* than in *S. aureus* (S. Projan, unpublished results). Therefore, the copy number effects of pIM13 and pBD367 are somewhat unusual in that these plasmids have their high copy number in *B. subtilis*. Given the copy number data, however, the stabilities of these plasmids are understandable. Since pE5 occurs naturally in *S. aureus*, it is not surprising that it is stable in that host. When transferred to *B. subtilis*, pE5 maintained its copy number but became unstable, an observation consistent with the data obtained for all other *S. aureus* plasmids in *B. subtilis*. On the other hand, the copy number of pIM13 was much higher in *B. subtilis* than in *S. aureus*. Thus, even if this plasmid were partitioned randomly, it would be expected to be more stable than pE5. The instability of pIM13 in *S. aureus* also is logical, since its copy number remained low and it multimerized, therefore lowering the number of segregating units. (Instability caused by multimerization has been reported for ColE1 replicons [24, 25].)

We have identified a *trans*-acting activity required for plasmid maintenance. The open reading frame associated with this activity has been designated *repL*. It was previously pointed out that RepL has little or no homology with the known Rep proteins of the plasmids of gram-positive bacteria. In addition, the RepL open reading frame specified a peptide of 18 kDa, while the other Rep proteins are in the 35-kDa range.

Perhaps the most unusual phenomenon associated with pIM13 was the formation of covalent multimers. As stated above, multimerization also occurs in ColE1 replicons, but

```
tAtCAAGAt---AA-GAAAgAa
tAtCAAGAa---AA-GAAAgAa
gAgCAAGAggcaAAtGAAAaAc
```

FIG. 8. Comparison of sequences involved in intraplasmid recombinations. The sequences are expressed from 5' to 3', homologous bases are aligned, and nonhomologous bases are in lowercase letters. Top line, pUB110-pC194 site (5); middle line, pTB19 junction (13); bottom line, pE5 (this work).

there are significant differences between pIM13 and ColE1 multimerization. (i) ColE1 multimerization was increased in a *recA* background, while the multimerization properties of pE5 and pIM13 were unaffected in a *B. subtilis recE* strain (24, 25). (ii) When individually isolated multimers of pIM13 were used to transform either *S. aureus* or *B. subtilis*, the original pattern of multimers was restored (not shown), whereas *cer* mutant ColE1 multimers tended towards larger multimers in a similar experiment (24). (iii) pIM13 was stable in *B. subtilis*, while multimerizing ColE1 derivatives were less stable than their parent plasmids.

Multimerization occurred regardless of host with both pIM13 and pBD367 because of the 120-bp deletion in the carboxy-terminal region of the *repL* coding sequence. The major possibilities are that a resolution site is deleted or that the truncated RepL protein of pIM13 is defective in termination of replication. At present neither possibility has been eliminated.

In conclusion, we have shown that pIM13, a *B. subtilis* plasmid, has probably descended from pE5, a plasmid native to *S. aureus*. In studying the relationships between these plasmids, we have identified a putative replication protein, RepL, and have determined the regions on pIM13 responsible for high copy number, multimerization, and constitutive MLS resistance.

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