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Complete Nucleotide Sequence and Comparative Analysis of pPR9, a 41.7-Kilobase Conjugative Staphylococcal Multiresistance Plasmid Conferring High-Level Mupirocin Resistance▽†

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We have sequenced the conjugative plasmid pPR9, which carries the *ileS2* gene, which had contributed to the dissemination of high-level mupirocin resistance at our institution. The plasmid backbone shows extensive genetic conservation with plasmids belonging to the pSK41/pGO1 family, but comparative analyses have revealed key differences that provide important insights into the evolution of these medically important plasmids and high-level mupirocin resistance in staphylococci and highlight the role of insertion sequence IS*257* in these processes.

Plasmids carrying the *ileS2* gene play an important role in high-level mupirocin resistance (Hi-Mup^r) spread and consequent clinical and epidemiological problems (8, 17, 23, 26). To date, complete nucleotide sequences of two *ileS2*-carrying plasmids have been determined: pUSA03 (37,136 bp; GenBank entry NC_007792) was carried by an epidemic USA300 community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strain (10), and a partially annotated sequence is available for pV030-8 (39,041 bp; NC_010279), which was isolated from a Hi-Mup^r MRSA strain from South Korea. Additionally, the *ileS2*-containing 34-kb plasmid pGO400 has been partially characterized (22). pUSA03, pV030-8, and pGO400 all belong to the pSK41/pGO1 family of multiresistance plasmids capable of promoting the conjugative transfer of resistance genes among bacterial strains (4, 14). In spite of their common genetic backbone, the antibiotic resistance gene contents of these plasmids are extremely diverse (14). The resistance genes are usually flanked by copies of the insertion sequence IS*257*, which has played a key role in the evolution of pSK41/pGO1-like plasmids (4, 13, 14, 32).

Recently we reported intrahospital Hi-Mup^r dissemination among pandemic MRSA strains due to the dispersion of structurally distinct *ileS2*-carrying conjugative plasmids (23). In this study, we have characterized the complete nucleotide sequence of one such plasmid, pPR9, previously named pMUP9 (23). We have undertaken a comprehensive comparative analysis of the DNA sequences of pPR9 and other pSK41 family plasmids, particularly other *ileS2*-carrying plasmids, to ascertain their diversity and to gain insights into the evolution of Hi-Mup^r in staphylococci.

DNA sequence and general overview of pPR9. HUNSC491, the MRSA strain harboring pPR9, was isolated in 2002 at the Hospital Universitario Nuestra Señora de Candelaria (HUNSC), Tenerife, Spain. The strain belongs to ST36-SCC*mec*II, a pandemic genotype that has widely disseminated throughout the HUNSC (24). ST36-SCC*mec*II isolates had acquired different *ileS2*-carrying plasmids (23). DNA of one, pPR9, was obtained by using the Hi-Speed maxi-plasmid purification kit (Qiagen, Valencia, CA), and a shotgun library was constructed at Integrated Genomics Inc. (IG) (11, 12, 28). The pPR9 sequence data were submitted to the IG database and software suite, ERGO, for sequence annotation (2, 9). The predicted proteins were searched using the BLAST algorithm (1), against a nonredundant database at the National Center for Biotechnology Information (NCBI). Protein functional domains were analyzed by searching against the NCBI conserved-domain database (21) and the Pfam database (3).

The complete nucleotide sequence of plasmid pPR9 was determined with 9.4-fold DNA coverage. The overall G+C content was 29.6%, comparable to the observed average G+C value for staphylococci (14). The sequence was found to have a 92% coding ratio with an average open reading frame (ORF) length of 915 bp; the *ileS2* gene, which encodes Hi-Mup^r, is the largest ORF at 3,075 bp. pPR9 possesses a mosaic structure with two clearly delineated regions: first, the backbone-encoding genes putatively involved in the replication, maintenance, and transfer of the plasmid; second, an accessory region containing antibiotic resistance genes and insertion sequences. Two copies of IS*257* are evident (i.e., IS*257*-L and IS*257*-R); *orf4* and *orf42* are gene remnants truncated by IS*257* (Fig. 1). The majority of genes in the pPR9 backbone share more than 90% amino acid identity with homologous regions of pSK41 (4) and other members of the family such as pGO1 (6),

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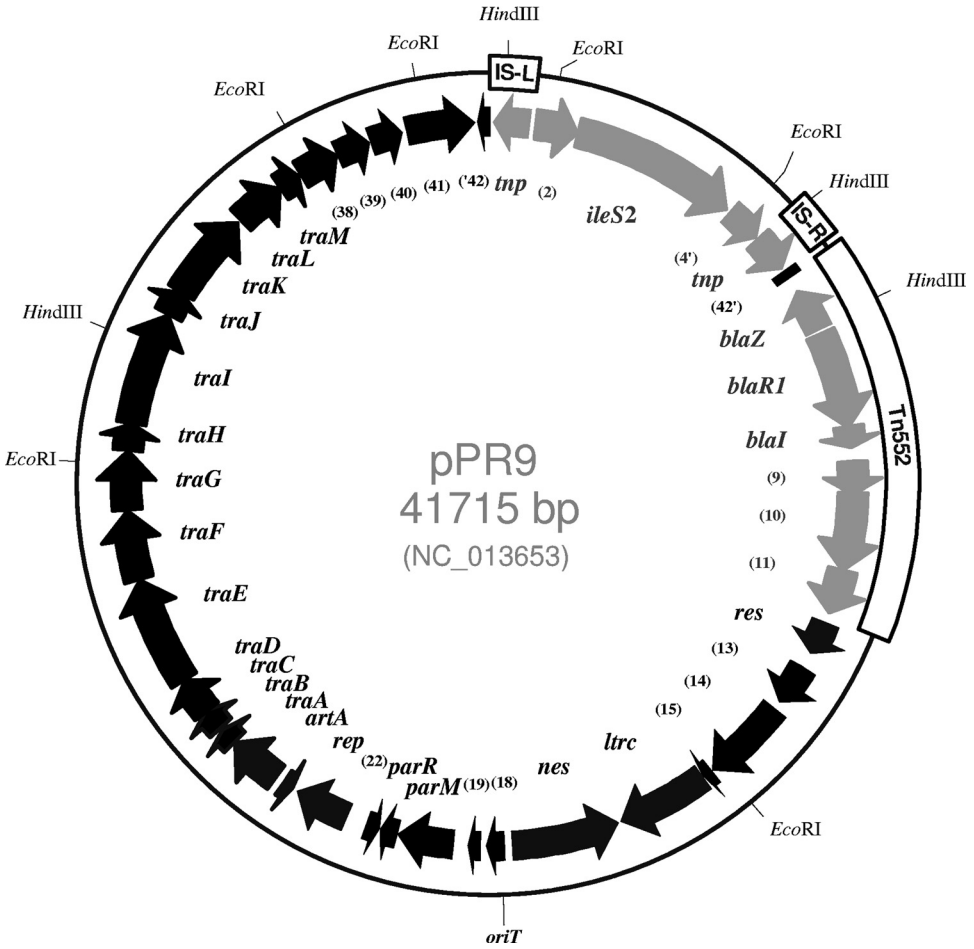

FIG. 1. Physical and genetic map of *Staphylococcus aureus* multiresistance plasmid pPR9 (GenBank entry NC_013653). The approximate positions of the putative origin of transfer (*oriT*), EcoRI and HindIII restriction sites, IS*257* elements (IS*257*-L and IS*257*-R), and Tn*552* are shown on the rim of the outer circle. The first G of the IS*257*-L terminal inverted repeat left (TIR_L) was designated the first nucleotide of the plasmid, and all 42 open reading frames (ORFs; tentatively named *orf1* to *orf42*) likely to represent translated genes are numbered in relation to this site. ORFs are represented by arrows indicating the direction of transcription; *tnp* marks the gene for IS*257* transposase. The accessory region genes are shown in gray, and the plasmid backbone genes are shown in black. All but six ORFs were found to be transcribed in the same direction (clockwise). The nucleotide sequence was verified by comparison of *in silico*-generated restriction maps with experimental data. Also, the numbers and sizes of HindIII and EcoRI restriction fragments that hybridized with specific probes for the *ileS2*, *blaZ*, *traK*, and *tnp* genes corresponded to those predicted from the pPR9 sequence (data not shown). *orf42*, interrupted by insertion of the IS*257*-flanked *ileS2* segment, is indicated by 42′ and ′42. The deletion of the 3′ portion of *orf4* is indicated as 4′.

pLW1043 (32), pUSA03 (10), and pV030-8. pPR9 also contains a complete copy of a Tn*552*-like β-lactamase transposon (25) that shares 94% nucleotide identity with Tn*552* (GenBank entry X52734). As in other pSK41/pGO1-like plasmids (20), the Tn*552*-like element in pPR9 is located within the resolution site of the plasmid's multimer resolution system and is flanked by the 6-bp target duplication sequence 5′-ATAGC G-3′ (Fig. 1 and 2).

The conjugal proficiency of pPR9 (5×10^{-6} to 1×10^{-7} transconjugants per donor cell) was demonstrated using filter mating experiments (4) and is in the range of those reported for other plasmids of the same family (22, 29). Transconjugants carrying pPR9 showed mupirocin MICs of $\geq$1,024 μg ml^{-1}, confirming the Hi-Mup^r phenotype conferred by pPR9. In curing experiments (30), loss of pPR9 was observed at low frequencies, indicating that pPR9 is normally inherited stably.

pPR9 encodes a novel chimeric *rep* gene. To date, all plasmids belonging to the pSK41/pGO1 family have utilized highly conserved RepA_N-type replication initiation genes, which are associated with plasmids or phage found in low-G+C content Gram-positive bacteria (31). pPR9 contains a putative *rep* gene (*orf23*) which encodes a protein of 341 amino acids. Surprisingly, sequence alignment between the deduced pPR9 and pSK41 Rep proteins revealed that amino acid similarity was restricted to their C-terminal ~155 amino acids (see Fig. S1A in the supplemental material), a region most conserved between RepA_N proteins from the same genus and therefore thought to possess a host-specific function (31). The lack of similarity evident at the N-terminal end of the proteins is noteworthy because it corresponds to the RepA_N domain of pSK41 Rep, which is believed to mediate binding to four repeated sequences in the origin of replication termed Rep boxes

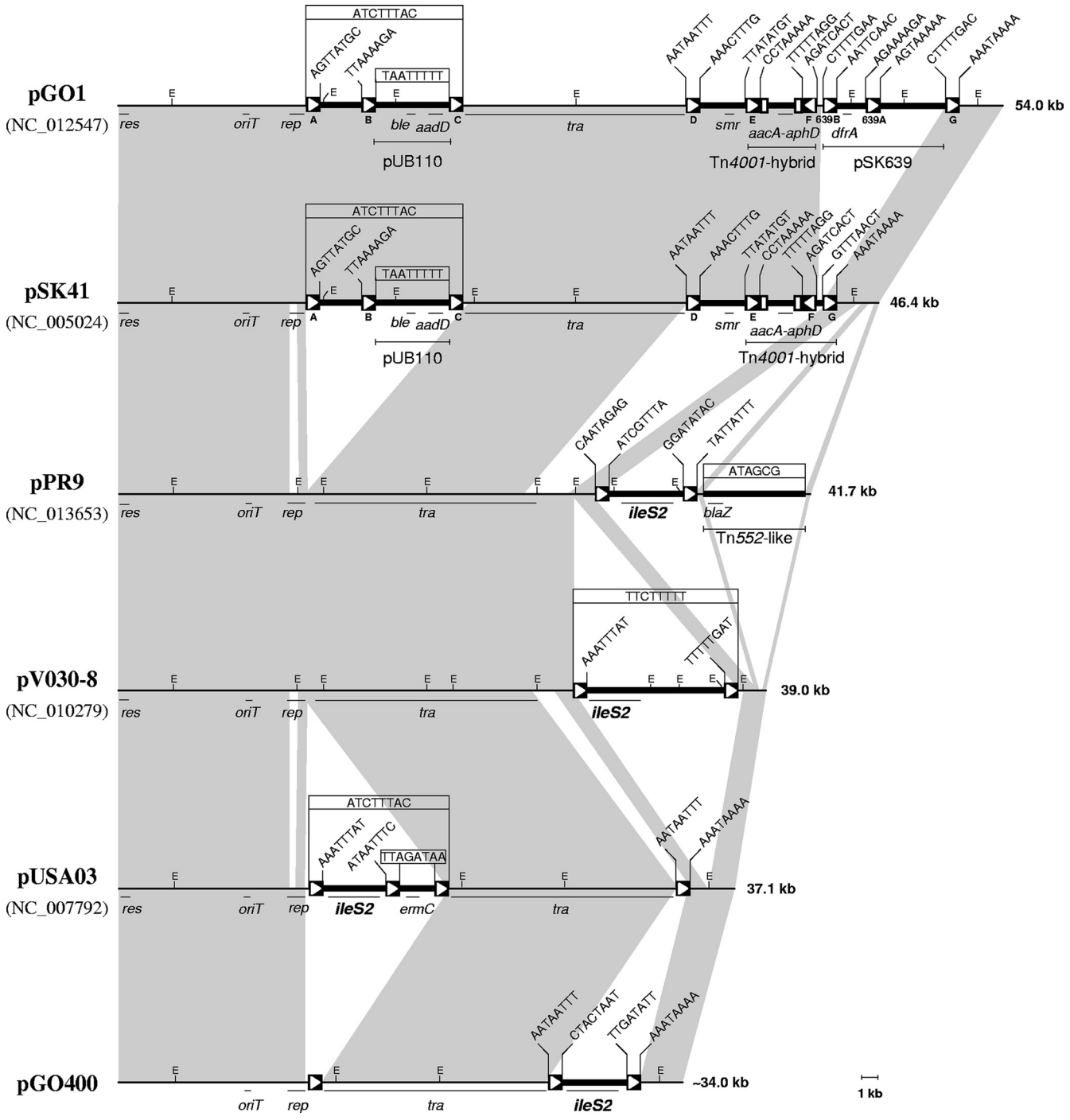

FIG. 2. Relationships between representative pSK41/pGO1 family plasmids. Linear physical and genetic maps of staphylococcal conjugative plasmids pGO1 (6), pSK41 (4), pPR9, pV030-8, pUSA03 (10), and pGO400 (22) are presented, with accession numbers given in parentheses; plasmid sizes are shown on the right. Shaded connections between plasmid maps illustrate that they all share a conserved basic backbone. The plasmid accessory regions are shown as a thick line in each map. The genetic loci shown are *aacA-aphD* (gentamicin-tobramycin-kanamycin resistance), *aadD* (kanamycin-neomycin-paromomycin-tobramycin resistance), *ble* (bleomycin resistance), *dfrA* (trimethoprim resistance), *smr* (antiseptic and disinfectant resistance), *ileS2* (high-level mupirocin resistance), *blaZ* (penicillin resistance), *ermC* (macrolide, lincosamide, and streptogramin B resistance), *tra* (conjugative transfer functions), *rep* (replication initiation), *res* (resolvase), and *oriT* (origin of conjugative DNA transfer). The positions and extents of the cointegrated copy of the plasmids pUB110 and pSK639, the Tn*4001*-IS*257* hybrid structure, and the Tn*552*-like transposon are indicated. Inverted and truncated copies of IS*256* associated with the Tn*4001* hybrid structures are represented by open boxes, whereas IS*257* elements are represented as solid boxes containing an arrowhead indicating the direction of the transposase transcription and hence the element's orientation. Where known, the 8-bp sequences adjacent to each IS*257* element are indicated. IS*257* element designations for pGO1 and pSK41 are taken from the work of Caryl and O'Neill (6) and Berg et al. (4), respectively. The location of *oriT* is based on homology to pGO1, where it has been mapped precisely (7). Recognition sites for the restriction endonuclease EcoRI (E) are shown. A distance scale in kb is given below the figure.

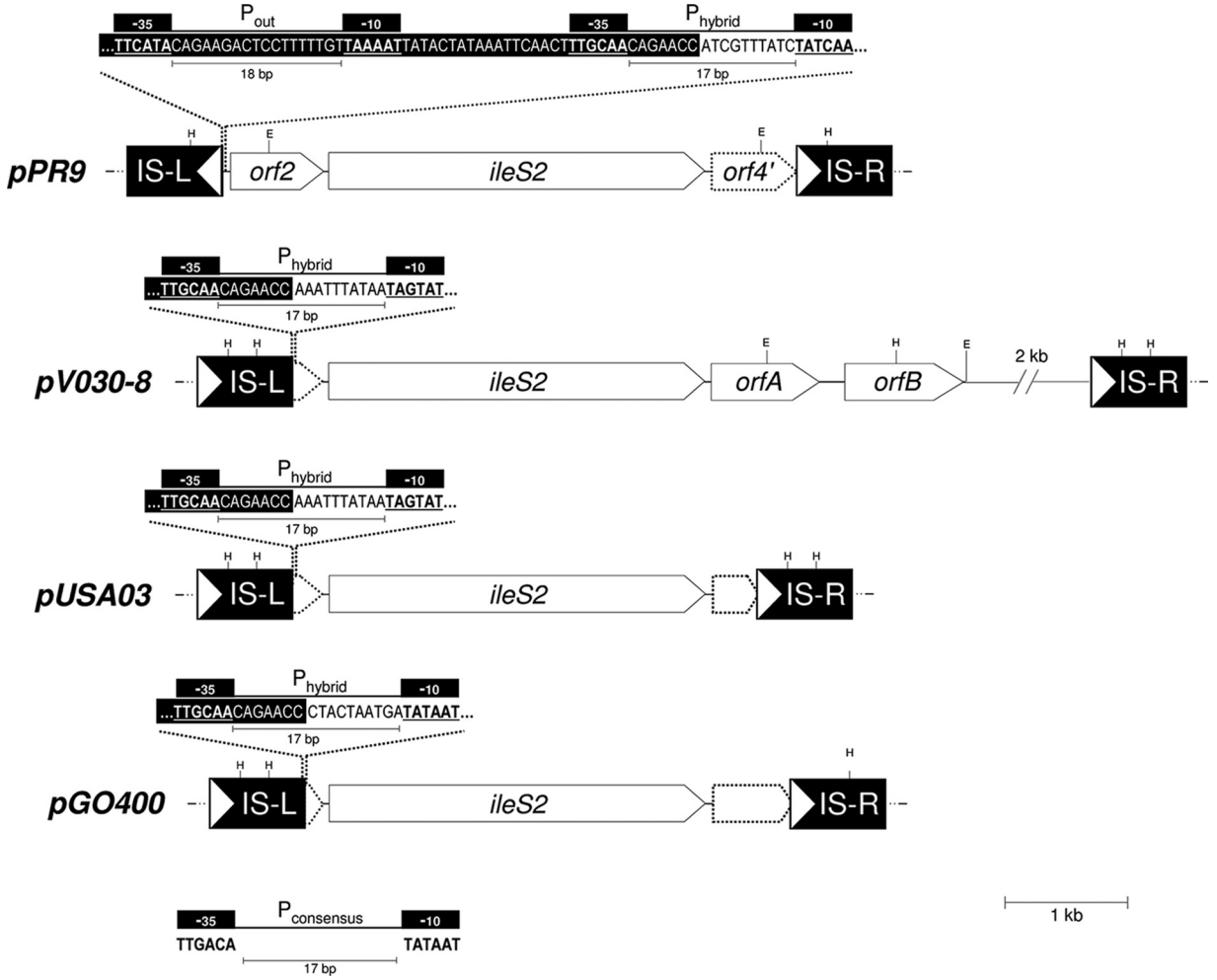

FIG. 3. Structural organization of the regions encompassing the *ileS2* genes of plasmids pPR9, pV030-8, pUSA03, and pGO400. The respective plasmid names are shown on the left. Restriction endonuclease cleavage sites are abbreviated as follows: E, EcoRI; H, HindIII. IS*257* elements (IS-L and IS-R) flanking the *ileS2* gene are represented by solid boxes; the white arrows indicate the direction of IS*257* transposase transcription. The *ileS2* gene and the predicted ORFs upstream and downstream are represented as arrows with the arrowhead indicating their orientation. A segment of approximately 2 kb on pV030-8 has been omitted for clarity. Truncated ORFs are shown using a dotted outline. The positions of the −35 and −10 sequences for the putative promoters P_{hybrid} and P_{out} are shown above each plasmid. The spacing between the −35 and −10 promoter sequences is indicated below each putative promoter. A distance scale in kb is given below the figure.

(18, 31). Intriguingly, the N-terminal 174 amino acids of pPR9 Rep share significant similarity to the N-terminal ends of numerous hypothetical phage proteins that are annotated as replication proteins (Fig. S1A).

With the exception of the unrelated DNA segments that encode the N-terminal ends of the respective Rep proteins, nucleotide sequence comparison of the entire pPR9 and pSK41 replication regions revealed a high degree of identity (greater than 97% over 727 nucleotides [nt]), including in the upstream intergenic region that contains the pSK41 *rep* promoter and encodes the antisense regulator RNAI (see Fig. S1B in the supplemental material) (19). The DNA segment that has been "replaced" in pPR9 encompasses the *rep* start codon through to just after the four pSK41 Rep boxes. Notably, the unique segment in pPR9 contains an array of five 15-bp direct repeats, the last four in tandem organization, at a position equivalent to the pSK41 Rep boxes. It would therefore seem possible that in the pPR9 lineage of the pSK41 plasmid family, which also includes pV030-8, there has been a recombination event that has swapped the segment encoding the probable DNA binding domain of its Rep protein for that of a phage-like protein, together with its likely DNA target sequences.

To confirm the functionality of the unusual pPR9 replication region (nt 23236 to 24606; GenBank entry NC_013653) (see Fig. S1B in the supplemental material), it was cloned into the *Escherichia coli* vector pSK5299 (15). The resulting plasmid, pSK6846, could indeed replicate in *S. aureus* RN4220, and copy number analysis (15) indicated that it was maintained at approximately 5 copies per cell (data not shown), which is lower than that previously determined for the pSK41 minireplicon pSK5413 (approximately 7 copies per cell) (5). Notably, pSK6846 and pSK5413 were found to be compatible (data not shown).

Comparative analysis of *ileS2*-carrying plasmids. Strikingly, although *ileS2* is ubiquitously flanked by IS*257* in plasmids conferring Hi-Mup^r, the segment occupies a unique position

within each plasmid backbone (Fig. 2). In pPR9, the *ileS2* segment is inserted into *orf42* (Fig. 1), which corresponds to *orf138* of pSK41. In pUSA03 and pGO400 the *ileS2* segments are associated with IS*257*s present at either end of their respective *tra* regions (10, 22), whereas in pV030-8, the segment is inserted into the gene corresponding to pPR9 *orf41* (equivalent to pSK41 *orf423*, which is located immediately upstream of *orf138*). An important implication of these findings is that the DNA segment responsible for Hi-Mup^r in staphylococci has been incorporated into pSK41/pGO1-like plasmids on at least four independent occasions, once again underlining the capacity of IS*257* to mediate the capture of resistance determinants. The pathways by which the *ileS2* segments have been incorporated into these plasmids cannot be inferred with any certainty; nonetheless, flanking 8-bp target duplications present in pV030-8 and pUSA03 (Fig. 2) imply that transposition has contributed to the process, and this is supported by the identity or near-identity of the flanking IS*257*s (data not shown).

Compared to pSK41 and pGO1, *ileS2*-carrying plasmids have a simpler structure with fewer copies of IS*257* (Fig. 2). Notably, unlike all previously described family plasmids, pPR9 and pV030-8 lack IS*257*s flanking their *tra* genes. In the other plasmids, the distal gene in the *tra* region, named *orf55* in pSK41, is truncated by the adjacent IS*257*. This gene is intact in pPR9 (*orf38*) and encodes a putative 243-amino-acid protein. The organization of pPR9 and pV030-8 suggests that these plasmids belong to a distinct lineage that diverged prior to the acquisition of IS*257* elements at the *tra* termini in an evolutionary branch that has given rise to other known family members. This notion is consistent with the previous suggestion that region 1 and the *tra* region were probably contiguous in the pSK41 progenitor (4).

Heterogeneity of the regions surrounding the *ileS2* gene. pPR9 is unique in that the IS*257*s flanking *ileS2* are in inverted orientation with respect to each other (Fig. 3). pPR9 has the most intact form of the IS*257*-*ileS2* upstream region. The upstream (L) copies of IS*257* in pV030-8, pUSA03, and pGO400 truncate *orf2*. pV030-8 has the largest *ileS2* downstream region, and *orfA* is truncated to various degrees in pPR9 (*orf4′*), pUSA03, and pGO400 by IS*257*-R. Even with the heterogeneity in extents, the flanking sequences that are present, up to the IS*257* boundaries, are 100% identical between the plasmids, implying acquisition from a common source but various truncations. The organization suggests that *ileS2* was part of a multigene operon.

Interestingly, the various arrangements of IS*257*-L raise the possibility that different IS*257*-derived hybrid promoters (P_{hybrid}s) could drive transcription of *ileS2* in the various plasmids, which might have consequences in terms of resistance expression (Fig. 3). It has been proposed that a potential P_{hybrid}, which is a better match to the canonical consensus than the promoter predicted initially (16), drives *ileS2* transcription in pGO400 (27). Similarly, pUSA03 has a good candidate for a different P_{hybrid}, which is likewise present in pV030-8. pPR9 also has a potential P_{hybrid}, although it is a suboptimal match to the consensus. However, the inverted orientation of IS*257*-L in pPR9 raises the possibility that a previously identified complete promoter within IS*257*, P_{out} (27), might be responsible for the *ileS2* transcription in this plasmid (Fig. 3). We are investigating these possibilities.

Nucleotide sequence accession number. The annotated sequence of pPR9 has been deposited in the GenBank database under accession number NC_013653.

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