Replication of Staphylococcal Multiresistance Plasmids

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Based on structural and functional properties, three groups of large staphylococcal multiresistance plasmids have been recognized, viz., the pSK1 family, pSK41-like conjugative plasmids, and β-lactamase–heavy-metal resistance plasmids. Here we describe an analysis of the replication functions of a representative of each of these plasmid groups. The replication initiation genes from the *Staphylococcus aureus* plasmids pSK1, pSK41, and pI9789::Tn*552* were found to be related to each other and to the *Staphylococcus xylosus* plasmid pSX267 and are also related to *rep* genes of several plasmids from other gram-positive genera. Nucleotide sequence similarity between pSK1 and pI9789::Tn*552* extended beyond their *rep* genes, encompassing upstream divergently transcribed genes, *orf245* and *orf256*, respectively. Our analyses revealed that genes encoding proteins related to the deduced *orf245* product are variously represented, in several types of organization, on plasmids possessing six seemingly evolutionarily distinct types of replication initiation genes and including both theta-mode and rolling-circle replicons. Construction of minireplicons and subsequent functional analysis demonstrated that *orf245* is required for the segregational stability of the pSK1 replicon. In contrast, no gene equivalent to *orf245* is evident on the conjugative plasmid pSK41, and a minireplicon encoding only the pSK41 *rep* gene was found to exhibit a segregational stability approaching that of the parent plasmid. Significantly, the results described establish that many of the large multiresistance plasmids that have been identified in clinical staphylococci, which were formerly presumed to be unrelated, actually utilize an evolutionarily related theta-mode replication system.

Clinical *Staphylococcus aureus* strains often harbor multiple plasmids, ranging from small rolling-circle (RC) replicating plasmids that are cryptic or encode only a single resistance determinant to larger multiresistance and conjugative plasmids (12, 32, 36). Three groups of multiresistance plasmids have been recognized in staphylococci. Isolates from the 1960s and 1970s were commonly found to carry multiresistance plasmids conferring resistance to penicillin and heavy metals or other inorganic ions (48). Such β-lactamase–heavy-metal resistance plasmids characteristically contain the β-lactamase-encoding transposon Tn*552* or a derivative and operons mediating resistance to arsenical, cadmium, and/or mercuric ions (36). Some β-lactamase–heavy-metal resistance plasmids also contain Tn*551*, conferring resistance to macrolide-lincosamide-streptogramin type B antibiotics (33); an IS*256*-bounded composite aminoglycoside resistance transposon, Tn*4001* (30); and/or a *qacA* or *qacB* antiseptic and disinfectant multidrug resistance determinant (30).

pSK41-like conjugative multiresistance plasmids were first detected in strains isolated in the mid 1970s. Such plasmids commonly contain a Tn*4001* hybrid structure (6) and IS*257*-flanked cointegrated copies of small plasmids, such as the aminoglycoside resistance plasmid pUB110 (7). Other determinants encoded by cointegrated episomes carried by pSK41 family plasmids include *smr*, which confers multidrug resistance to antiseptics and disinfectants, and *dfrA*, which mediates

trimethoprim resistance (4). Mupirocin resistance plasmids related to pSK41 have also been identified (31).

Clinical *S. aureus* strains isolated in Australia and the United Kingdom in the 1980s typically contained a multiresistance plasmid related to pSK1 (28, 30, 50, 55). pSK1 family plasmids commonly carry a *qacA* gene (40, 53), Tn*4001* (39), and an IS*257*-containing structure termed Tn*4003*, which encodes trimethoprim resistance (11, 41); some pSK1 family plasmids also possess a Tn*552*-like β-lactamase resistance transposon (17).

Although the replication of staphylococcal RC plasmids has been studied in considerable detail, comparatively little attention has been paid to the replication systems of larger plasmids from this genus, which are presumed to replicate via the theta mode (21, 49). To date, the replication initiation region of only a single staphylococcal multiresistance plasmid has been characterized, that of the β-lactamase–heavy-metal resistance plasmid pSX267 from *Staphylococcus xylosus* (16, 19). To broaden our understanding of the replicative mechanisms of staphylococcal multiresistance plasmids and the evolutionary relationships among them, we have characterized the replication regions from the prototypes of the pSK1 and pSK41 plasmid families and a β-lactamase–heavy-metal resistance plasmid, pI9789::Tn*552*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *Escherichia coli* host strain used was DH5α (F⁻ *endA hsdR17 supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* φ80*dlac*ZΔM15; Bethesda Research Laboratories), while the *S. aureus* host strains used were RN4220 (23) and the rifampin- and novobiocin-resistant strain SK982 (29). The *S. aureus* strain RN1965 (43) was used as a source of a reference set of plasmids for plasmid incompatibility tests (see below). pUC18 (58) and derivatives were used as cloning vectors. The general culture conditions were 37°C on Luria-Bertani (LB) agar or in LB medium (45). Where appropriate, antimicrobial agents were used at the following concentrations: ampicillin, 100 μg/ml; cadmium chloride, 50 μM; erythromycin, 20 μg/ml; gentamicin, 20 μg/ml;

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neomycin, 10 μg/ml; novobiocin, 2 μg/ml; rifampin, 20 μg/ml; tetracycline, 10 μg/ml; trimethoprim, 100 μg/ml.

DNA manipulations. Plasmid DNA was isolated from *E. coli* using a Quantum Prep plasmid miniprep kit (Bio-Rad), while DNA was isolated from *S. aureus* as described by Lyon et al. (28). Protoplast transformation of *S. aureus* was performed as described by Götz et al. (18). Electroporation of *S. aureus* with a Bio-Rad gene pulser was performed as described previously (25). DNA cloning and transformation of *E. coli* was performed using standard techniques (45). Restriction endonucleases and T4 DNA ligase (Promega) were used in accordance with the manufacturer's instructions. PCRs were carried out with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's recommendations. Oligonucleotide primers for PCR and nucleotide sequencing were made with a Beckman Oligo 1000 synthesizer.

The pSK1-derived fragment carried by pSK4829 was amplified with the *rep* upstream primer, 5′-GCGAAGCTTCCCTAGATAATTCTTCTGATAATTTAG-3′, and the downstream primer, 5′-GCGGGATCCTTTTCTGTTGACTTAATTCC-3′, whereas a different upstream primer, 5′-GCGAAGCTTGTTACATTCAATTCATCAGCAAACC-3′, was used to amplify the fragment carried by pSK4833. The *Hin*dIII and *Bam*HI sites (underlined) incorporated into the primers allowed ligation of the restricted PCR products into similarly cleaved pWE180 vector DNA. pWE180 was constructed by ligation of a blunted *Pst*I-*Cla*I fragment of pE194, encoding the *ermC* erythromycin resistance determinant (22), into a blunted *Nde*I site of pUC18. The pSK41-derived fragment carried by pSK5413 was amplified with the *rep* upstream primer, 5′-AATGGATCCATATAGTTTTTGTATACGGTATTC-3′, and the downstream primer, 5′-CTCGGATCCACTAATTTATCATGTCAGTGTTC-3′. The *Bam*HI site (underlined) incorporated into the upstream primer and an internal *Hin*dIII site within the pSK41 sequence (at nucleotide 14485 of GenBank entry AF051916) allowed ligation of the restricted PCR product into similarly cleaved pUC18 vector DNA. *Bam*HI and *Sac*I double digestion of this plasmid, and subsequent ligation with a DNA fragment encoding a *tetA*(K) tetracycline resistance gene (20), amplified from the chromosome of a clinical *S. aureus* isolate (A. E. Simpson, R. A. Skurray, and N. Firth, unpublished data), generated pSK5413.

Nucleotide sequence determination and data analysis. Dideoxy sequencing (46) was performed with SequiTherm cycle-sequencing kits (Epicentre Technologies) or Sequenase version 2 sequencing kits (United States Biochemical) according to the manufacturers' recommendations. All restriction sites were crossed, and all sequence was determined on both DNA strands; derivatives of pBR322 (5) or pUC119 (56) containing pSK1 or pI9789::Tn552 restriction fragments were utilized as double-stranded sequencing substrates. Sequences were stored and assembled with the program SEQUENCHER (Gene Codes Corporation) and analyzed with the Genetics Computer Group (9) and PHYLIP (10) packages maintained by the Australian National Genomic Information Service, University of Sydney. Sequence similarities were assessed with pairwise alignments generated with the program GAP (9) using the Dayhoff 250 PAM matrix (8). Statistical significance (Z) was calculated using the formula $Z = (a - m)/\sigma$, where a is the alignment score, m is the mean of 100 alignment scores where one of the sequences has been randomly shuffled, and σ is the standard deviation of m (37). Z scores greater than 3, 6, or 10 were taken to be indicative of "possible," "probable," or "highly probable" evolutionary relatedness, respectively (26). Phylogenetic trees were drawn by using the program TREEVIEW (34).

Assay of plasmid segregational stability. An overnight culture of the strain(s) to be assayed, grown in medium selective for the plasmid, was diluted in saline, and a viable count was performed using nonselective LB agar plates. Additionally, 100 μl of the 10^{-2} dilution was used to inoculate 10 ml of LB medium. After overnight growth without selection, this culture was diluted, counted, and subcultured as before; this process was repeated until approximately 100 generations in the nonselective medium were achieved. One hundred colonies from each day's viable-count plates were patched onto media with and without selection for the plasmid so that the proportion of the population retaining the resistance phenotype conferred by the plasmid could be quantitated. DNA was isolated from selected colonies to confirm the absence or presence of the relevant plasmid.

Plasmid incompatibility tests. *S. aureus* strain RN1965 (43) contains RC plasmids representing incompatibility groups (Inc) 3 to 5 and larger plasmids defining Inc1, Inc2, and Inc6. The six plasmids carried by this strain are pRN3025 (Inc1), pRN2003 (Inc2), pT127 (Inc3), pC221 (Inc4), pS177 (Inc5), and pK545 (Inc6), which confer resistance to erythromycin, cadmium, tetracycline, chloramphenicol, streptomycin, and neomycin, respectively. Plasmid incompatibility was determined by using mixed-culture transfer experiments (29) employing RN1965 as the donor and SK982 containing the plasmid under test as the recipient. Transferrants were selected on LB agar containing rifampin, novobiocin, and selection for the incoming plasmid only, and were subsequently patched onto media selective for both incoming and resident plasmids to determine coexistence and hence compatibility. This was confirmed by inoculating the cultures into 10 ml of LB medium selective for only the incoming plasmid, plating them with the same selection for single colonies, and patching them onto LB agar selective for both incoming and resident plasmids; maintenance of the resident plasmid by more than 90% of the cells carrying the incoming plasmid was taken to indicate compatibility. DNA isolations were subsequently performed on representative isolates to confirm the presence of both plasmids.

Nucleotide sequence accession numbers. The nucleotide sequences of the pSK1 and pI9789::Tn552 replication regions are available under the GenBank accession numbers AF203376 and AF203377, respectively.

RESULTS

Replication regions of the multiresistance plasmids pSK1 and pI9789::Tn552. The putative replication regions from the prototype of the pSK1 family of plasmids (12) and the β-lactamase–heavy-metal resistance plasmid pI9789::Tn552 (42, 48) were identified based on similarity to the predicted *rep* region of the conjugative *S. aureus* multiresistance plasmid, pSK41 (4), and the demonstrated *rep* region of the *S. xylosus* β-lactamase–heavy-metal resistance plasmid, pSX267, which is believed to replicate via the theta mode (16). The functionality of the pSK1 and pI9789::Tn552 *rep* regions was subsequently demonstrated in *S. aureus* (see below). The extent of nucleotide sequence similarity among the replication regions of pSK1, pI9789::Tn552, and pSK41, which represent the three groups of large staphylococcal multiresistance plasmids identified to date (12), is illustrated in the multiple sequence alignment shown in Fig. 1; due to the high degree of identity (89%) between the pI9789::Tn552 and pSX267 replication regions, for clarity, only the longer pI9789::Tn552 sequence is shown. The replication initiation genes of these staphylococcal plasmids encode products similar ($17 \leq Z \leq 43$) to those of plasmids from other gram-positive genera, including pLS32 from *Bacillus natto* (52), pLJ1 (51), pLH1 (54), and pSAK1 (GenBank entry Z50862) from *Lactobacillus* species, and the enterococcal plasmids pAD1 (57), pCF10 (44), and pPD1 (14) (Fig. 2A), a number of which have been proposed to utilize the theta mode of replication (see also reference 21). Phylogenetic analysis (Fig. 2B) revealed that the Rep proteins fall into generic clusters, providing no evidence of horizontal transmission of the *rep* genes among these genera.

The phylogenetic analysis also suggested that the pSK1 replicon type is more closely related to that of the β-lactamase–heavy-metal resistance plasmids than it is to that of pSK41-like plasmids. Consistent with this notion, nucleotide sequence similarity between pSK41 and both pSK1 and pI9789::Tn552 is primarily confined to the *rep* gene coding sequences, whereas similarity between pSK1 and pI9789::Tn552 continues beyond this gene in both directions (Fig. 1). Downstream of *rep*, similarity extends across a region, corresponding to sequences beyond nucleotide 2280 of pI9789::Tn552 (Fig. 1), that appears to represent a remnant of a cointegrated RC plasmid which has been described previously on pSX267 (16). This region of similarity, which ends adjacent to an inverted repeat in pSK1, bears greater evidence of genetic drift than the neighboring *rep* coding segment, including two probable deletions within the pSK1 sequence (Fig. 1). Nevertheless, the degree of conservation of this vestige on pSK1 and pI9789::Tn552 argues that the lineages of these plasmids diverged quite recently in evolutionary time.

Upstream of the respective *rep* genes, recognizable nucleotide sequence similarity between pSK1 and pI9789::Tn552 encompasses the intergenic region and a divergently transcribed open reading frame, *orf245* on pSK1 and *orf256* on pI9789::Tn552 (Fig. 1). The deduced products of these genes share statistically significant amino acid sequence similarity with proteins encoded by plasmids from other gram-positive genera, including a large number of lactococcal plasmids related to pUCL22 (13, 47), and the *Tetragenococcus halophilus* plasmid pUCL287 (3). In contrast to the divergent organizations of pSK1 *orf245* and pI9789::Tn552 *orf256* in relation to their respective *rep* genes, the homologous genes on these other plas-
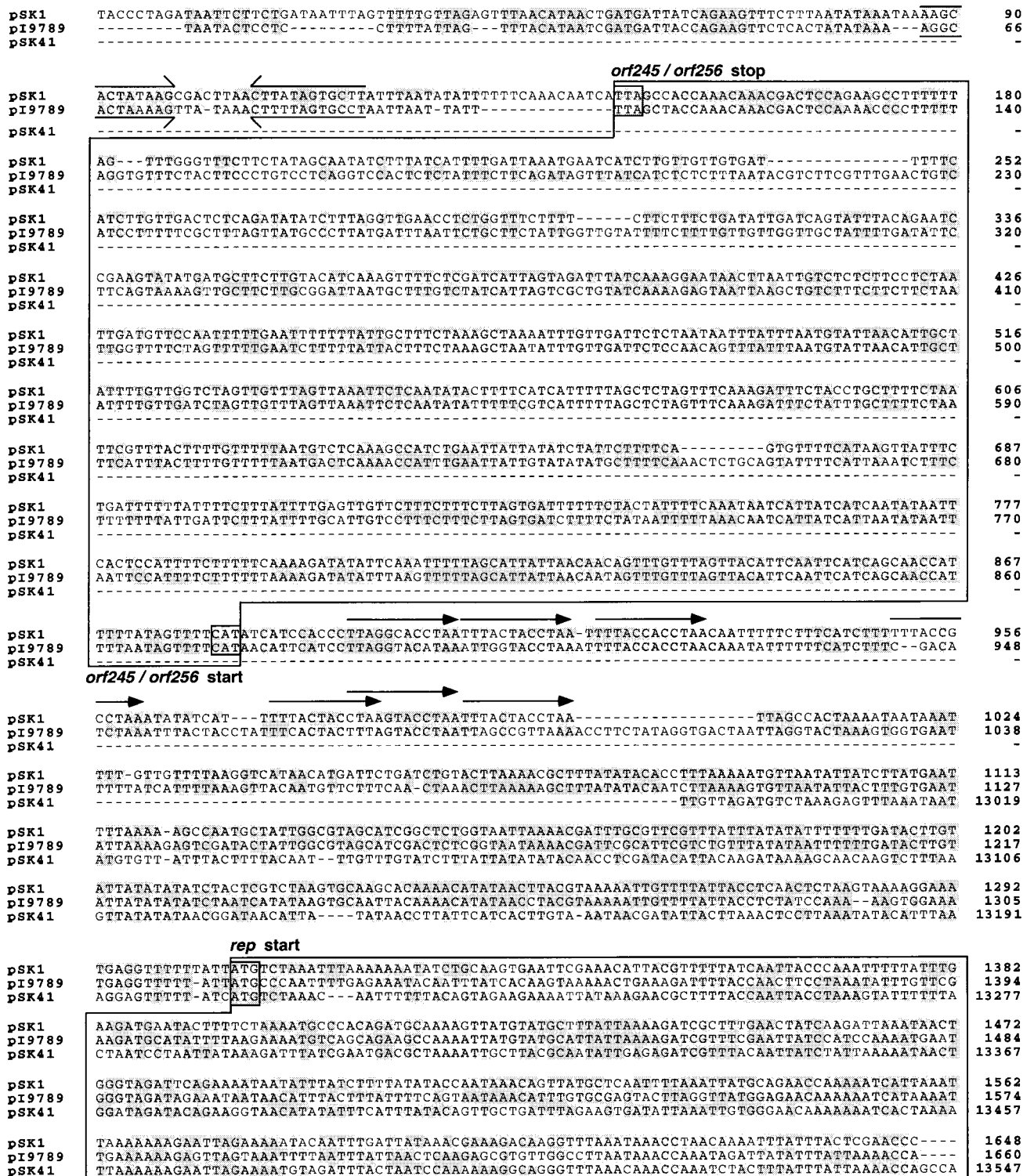
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pSK1    TACCCTAGATAATTCTTCTGATAATTTAGTTTTTGTTAGAGTTTAACATAACTGATGATTATCAGAAGTTTCTTTAATATAAATAAAGC    90
pI9789  ---------TAATACTCCTC----------CTTTTATTAG---TTTACATAATCGATGATTACCAGAAGTTCTCACTATATAAA---AGGC    66
pSK41   ------------------------------------------------------------------------------------------    -
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orf245 / orf256 stop

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pSK1    ACTATAAGCGACTTAACTTATAGTGCTTATTTAATATATTTTTTCAAACAATCATTAGCCACCAAACAAACGACTCCAGAAGCCTTTTTT    180
pI9789  ACTAAAAGTTA-TAAACTTTTTAGTGCCTAATTAAT-TATT-------------TTAGCTACCAAACAAACGACTCCAAAACCCCTTTTT    140
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    AG---TTTGGGTTTCTTCTATAGCAATATCTTTATCATTTTTGATTAAATGAATCATCTTGTTGTTGTGAT----------------TTTTC    252
pI9789  AGGTGTTTCTACTTCCCTGTCCTCAGGTCCCACTCTCTATTTCTTCAGATAGTTTATCATCTCTCTTTAATACGTCTTCGTTTGAACTGTC    230
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    ATCTTGTTGACTCTCAGATATATCTTTAGGTTGAACCTCTGGTTTCTTTT------CTTCTTTCTGATATTGATCAGTATTTACAGAATC    336
pI9789  ATCCTTTTTCGCTTTAGTTATGCCCTTATGATTTAATTCTGCTTCTATTGGTTGTATTTTCTTTTGTTGTTGGTTGCTATTTTGATATTC    320
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    CGAAGTATATGATGCTTCTTGTACATCAAAGTTTTCTCGATCATTAGTAGATTTATCAAAGGAATAACTTAATTGTCTCTCTTCCTCTAA    426
pI9789  TTCAGTAAAAGTTGCTTCTTGCGGATTAATGCTTTGTCTATCATTAGTCGCTGTATCAAAAGAGTAATTAAGCTGTCTTTCTTCTTCTAA    410
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    TTGATGTTCCAATTTTTTGAATTTTTTTTATTGCTTTCTAAAGCTAAAATTTGTTGATTCTCTAATAATTTATTTAATGTATTAACATTGCT    516
pI9789  TTGGTTTTCTAGTTTTTGAATCTTTTTATTACTTTCTAAAGCTAATATTTGTTGATTCTCCAACAGTTTATTTAATGTATTAACATTGCT    500
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    ATTTTGTTGGTCTAGTTGTTTAGTTAAATTCTCAATATACTTTTCATCATTTTTAGCTCTAGTTTCAAAGATTTCTACCTGCTTTTCTAA    606
pI9789  ATTTTGTTGATCTAGTTGTTTAGTTAAATTCTCAATATATTTTTCGTCATTTTTAGCTCTAGTTTCAAAGATTTCTATTTGCTTTTCTAA    590
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    TTCGTTTACTTTTGTTTTTAATGTCTCAAAGCCATCTGAATTATTATATCTATTCTTTTCA----------GTGTTTTCATAAGTTATTTC    687
pI9789  TTCATTTACTTTTGTTTTTAATGACTCAAAACCATTTGAATTATTGTATATATGCTTTTCAAACTCTGCAGTATTTTCATTAAATCTTTC    680
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    TGATTTTTTATTTTCTTTATTTTGAGTTGTTCTTTCTTTCTTAGTGATTTTTTCTACTATTTTCAAATAATCATTATCATCAATATAATT    777
pI9789  TTTTTTTTATTGATTCTTTATTTTGCATTGTCCTTTCTTTCTTAGTGATCTTTTCTATAATTTTTAAACAATCATTATCATTAATATAATT    770
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    CACTCCATTTTCTTTTTCAAAAGATATATTCAAATTTTTTAGCATTATTAACAACAGTTTGTTTAGTTACATTCAATTCATCAGCAACCAT    867
pI9789  AATTCCATTTTCTTTTTTAAAAGATATATTTAAGTTTTTAGCATTATTAACAATAGTTTGTTTAGTTACATTCAATTCATCAGCAACCAT    860
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    TTTTATAGTTTTCATATCATCCACCCTTAGGCACCTAATTTACTACCTAA-TTTTACCACCTAACAATTTTTCTTTCATCTTTTTTTACCG    956
pI9789  TTTAATAGTTTTCATAACATTCATCCTTAGGTACATAAATTGGTACCTAAATTTTACCACCTAACAAATATTTTTTCATCTTTC--GACA    948
pSK41   ------------------------------------------------------------------------------------------    -
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orf245 / orf256 start

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pSK1    CCTAAATATATCAT---TTTTACTACCTAAGTACCTAATTTACTACCTAA------------------TTAGCCACTAAAATAATAAAT    1024
pI9789  TCTAAATTTACTACCTATTTTCACTACTTTAGTACCTAATTAGCCGTTAAAACCTTCTATAGGTGACTAATTAGGTACTAAAGTGGTGAAT    1038
pSK41   ------------------------------------------------------------------------------------------    -

pSK1    TTT-GTTGTTTTAAGGTCATAACATGATTCTGATCTGTACTTAAAACGCTTTATATACACCTTTAAAAATGTTAATATTATCTTATGAAT    1113
pI9789  TTTTATCATTTTTAAAGTTACAATGTTCTTTCAA-CTAAACTTAAAAAGCTTTATATACAATCTTAAAAGTGTTAATATTACTTTGTGAAT    1127
pSK41   -------------------------------------------------------------------TTGTTAGATGTCTAAAGAGTTTAAATAAT    13019

pSK1    TTTAAAA-AGCCAATGCTATTGGCGTAGCATCGGCTCTGGTAATTAAAACGATTTGCGTTCGTTTATTTATATATTTTTTTGATACTTGT    1202
pI9789  ATTAAAAGAGTCGATACTATTGGCGTAGCATCGACTCTCGGTAATAAAAACGATTCGCATTCGTCTGTTTATATAATTTTTTGATACTTGT    1217
pSK41   ATGTGTT-ATTTACTTTTACAAT--TTGTTTGTATCTTTATTATATATACAACCTCGATACATTACAAGATAAAAGCAACAAGTCTTTAA    13106

pSK1    ATTATATATATCTACTCGTCTAAGTGCAAGCACAAAACATATAACTTACGTAAAAATTGTTTTATTACCTCAACTCTAAGTAAAAGGAAA    1292
pI9789  ATTATATATATCTAATCATAAGTGCAATTACAAAACATATAACCTACGTAAAAATTGTTTTATTACCTCTATCCAAA--AAGTGGAAA    1305
pSK41   GTTATATATAACGGATAACATTA----TATAACCTTATTCATCACTTGTA-AATAACGATATTACTTAAACTCCTTAAATATACATTTAA    13191
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rep start

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pSK1    TGAGGTTTTTTATTATGTCTAAATTTAAAAAAAATATCTGCAAGTGAATTCGAAACATTACGTTTTTATCAATTACCCAAATTTTTATTTG    1382
pI9789  TGAGGTTTTT-ATTATGCCCAATTTTGAGAAATACAATTTATCACAAGTAAAAACTGAAAGATTTTACCAACTTCCTAAATATTTGTTCG    1394
pSK41   AGGAGTTTTT-ATCATGTCTAAAC---AATTTTTTACAGTAGAAGAAAATTATAAAGAACGCTTTTACCAATTACCTAAAGTATTTTTTA    13277

pSK1    AAGATGAATACTTTTCTAAAATGCCCACAGATGCAAAAGTTATGTATGCTTTATTAAAAGATCGCTTTGAACTATCAAGATTAAATAACT    1472
pI9789  AAGATGCATATTTTAAGAAAATGTCAGCAGAAGCCAAAATTATGTATGCATTATTAAAAGATCGTTTCGAATTATCCATCCAAAATGAAT    1484
pSK41   CTAATCCTAATTATAAAGATTTATCGAATGACGCTAAAATTGCTTACGCAATATTGAGAGATCGTTTACAATTATCTATTAAAAATAACT    13367

pSK1    GGGTAGATTCAGAAAATAATATTTATCTTTTATATACCAATAAACAGTTATGCTCAATTTTAAATTATGCAGAACCAAAAATCATTAAAT    1562
pI9789  GGGTAGATAGAAATAATAACATTTACTTTATTTTCAGTAATAAACATTTGTGCGAGTACTTAGGTTATGGAGAACAAAAAATCATAAAT    1574
pSK41   GGATAGATACAGAAGGTAACATATATTTCATTTATACAGTTGCTGATTTAGAAGTGATATTAAATTGTGGGAACAAAAAAATCACTAAAA    13457

pSK1    TAAAAAAAGAATTAGAAAAATACAATTTGATTATAAACGAAAGACAAGGTTTAAATAAACCTAACAAAATTTATTTACTCGAACCC----    1648
pI9789  TGAAAAAAGAGTTAGTAAATTTTAATTTATTAACTCAAGAGCGTGTTGGCCTTAATAAACCAAATAGATTATATTTATTAAAACCA----    1660
pSK41   TTAAAAAAGAATTAGAAAATGTAGATTTACTAATCCAAAAAAGGCAGGGTTTAAACAAACCAAATCTACTTTATTTATTAAAACCAGCCA    13547
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FIG. 1. Nucleotide sequence alignment of the replication regions of pSK1, pI9789::Tn552, and pSK41. The pSK41 sequence shown corresponds to nt 12991 to 14216 of GenBank entry AF051917. Nucleotides present in two or more sequences at any one position are shaded, and insertions or deletions are denoted by dashes. The nucleotide sequences are numbered on the right. Translation start and stop codons of the indicated genes (outlined) are boxed. Directly repeated sequences are indicated by arrows, whereas inverted repeats are shown by half arrows.

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pSK1    -----ACATATGA------------------CAAGGAACTAATAAATTCTAAGTTCCAGAACAAAGAATTTATTAGT-------TCCAGA    1708
pI9789  -----AATTATGATATTGAAGCAAGTCTTACCAAGGAACTTCCAAATTCACAGTTCCAGAACAATGAATTTGGAAGT-------TCTAGA    1738

pSK41   TAACTAAAAATGATATTTATGAAATAGATAAGGCTGAAAATGAAGTTGAGGCCTTACAAGACAAGGAAGTGTCAAAAGGACACGTCCAGA    13637


pSK1    ACTAATAAATCATCAGTTCAAGAACTAATAAATTCTAA-GTCAAGTGATACTGATTTTAATAATACTGAATATATAGAGACTAAGAATAA    1797
pI9789  ACTGTGAATTTAAGTGGTCAAGAACTTCCAAATTCACA-GTCTAATGATACTGAGTTAAATAATACTAATTATATAGAAAATGAGGATAA    1827

pSK41   AATGTCAAAAGGACACGTCTAGAAATGTCAAAAGGACACGTCTAGAAATGTCAAA--AGGACACAAATGATACTGATTTTATTGATAC    13725


pSK1    TGATACGAATTATACGAATGAT---ACATCTAACA---TGATTTCTAAGAATTCTCATTCGAATCATACAAATCATCAACAAACCGAATT    1881
pI9789  TGATACGTATGATATGAATGATATTTATATCTAATACTTTAATAAATAATAATCATCATTCGAATCATACAAATCATCAAAAAACTGAATT    1917
pSK41   TGATTTTATTGATACTGAAAGTAATGATATGAATAAATATGAATGATCAAATCAACATTCAAATCATTCAAATCACTTTTCAAATATTCA    13815


pSK1    TAATAATGATGCCTTAAAAATTCCAGGCGCTTGAAGAATTACCTTCGCAAATCAAATCTTATGTAAGTAATTTTGAAATTAAAGACATCCG    1971
pI9789  TAATAATGACGCCCTTAAGTTCCAAGTACTCGAAGAATTGCCACAACAATTTCAAGACTATTTAAGTAAATTTGAAATTAGAGAAATTCG    2007
pSK41   TGATAAAGAATCTCTTAAATATATAGAACTTCAAGAACTCCCCGAATTAATTAAAAGTTATATTAATAATTTCAGCTATGAAGAAGTTAA    13905


pSK1    TATTATTAAAAGTATCTTTACTCAAGGGGAAAAAGTCATTTAATAATACACATGATACATATTACCGTTTAGAAGACGTCGAATTTGAACT    2061
pI9789  GATTATTAAAAGCGTATTACTCAAAGGGAAAAAGTCATTTAATACTTCACATGATACTTATTATCGTTTAGAAGATGTTGAGTTTGAAAT    2097
pSK41   AAGTATTAAATCTGTTATTTTAAAAAGCTAAAAAATCATTTAATAACAAATACGATACTTTCTATATGTTAGAAGATATAGATGAAGAATT    13995


pSK1    TGTAAGTGTTTTAAAAACGTTTTAAAG-CCATGTTGCTACAAAAAAATGAAACCGTTGAAACTATGCAAGGCTATTTAATGCAATCAATTA    2150
pI9789  TGTAAGTGTATTGAAACGTTTTAAAG-CGATGTTGCTACAAAAGAATGAAACCGTTGAAGCTATGCAAGGTTATTTAATGCAATCCATTA    2186
pSK41   ACTATTGGTTTTTAAAACGATTTAAAGGTTATCTTGTTAAAAAACAA-GAAAAAGTAGCTAATATGGAAGGTTATTTAATGAGAAGTATCA    14084


pSK1    AAGCTGAACTTGAAGAAATACATGCATTAAATATGCGTCGTCAAAACATA--CCTCAATACAATATCTTTAATCAA-TAA CTCAAATAAT   2237
pI9789  AAGCTGAACTTGAAGAAACACATGCCCTTTATATGCGCCGTAAAAACATG--AAACAATATAATATCTTTAATCAA-TAA TCAAATGAT   2273
pSK41   TTGCTGAACTTGAAGAAATGCACTCAACTATTATGAGAAGAAAAAATATGGAAAACAATCCATTATCTCTATTTAATTAG TTT--TGAA   14172
                                                                                           rep stop


pSK1    CTTACAACAATCAAAACAACATCAAAATTTGGAATTAAGTCA--------------------------------------------------   2279
pI9789  TTTATAACAATCAAAGAGACATCAAAATATTCCGTTAAATCATCTGACTTTTTAACTGATGACGACGAAAAAAATCAAGAAATTTTAAGT   2363
pSK41   GTTA-AATTTTCAAAAAAAAG--AGATTATACACCTAAAAAGGTGTA----------------------------------------------   14216


pSK1    ------------------ACAGAAAACGTACGTTAACTTATG-TGTAGGACTTAAGCAAAATATAAACATTTTAAACT-AGAAGAGGGCA   2349
pI9789  GATCTAGAAAAGGTTTGTATCGAAAACGTATGTTAAGTTATGGTGGATTACTTAAACAAAAGCATAAAATTTTAAACTTAGACGATGCCG   2453
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    AAGATG---ACCTTATTTTTACAAGAAAGGCGTTGAATAATTGCTAACCAAT--------------------------------------   2398
pI9789  AAGATGGTAATTTGATTAATACAAGTGATGAAGATAAAACGACAGACGAAGAAGAAAAAGCACATTCGATTACAGCAATTTGGAATTTCG   2543
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -----------------------------------------------------------------------------------------   -
pI9789  AAAAGCAAAATTATTATTTAAAAAAATTTAAGGAGTTAAAAAATATGAACACTAAAATTTTAACTGGAATGACTGGAAGTTCTTTAGAACGA   2633
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -----------------------------------------------------------------------------------------   -
pI9789  AAAATCAATAATTTTATTAATGATAACCAAATCGAAGTCATTGATATTAAGTTTTCAAGTTCTGTATTTTATTTGGGGTAATGATTATT   2723
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -----------------------------------------------------------------------------------------   -
pI9789  TATAAATAATTAAAGAGTTTAAGACATTAGTGATGACTGATGTCTTTTTTTACTGTTTTTTTATAAAAAAGTACTGGCTTATTTTTGTGA   2813
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -----------------------------------------------------------------------------------------   -
pI9789  CAAATGCAATATATTGTGTCACAAAAGTGTGACACTTAAGCTTTTTATGACCCCACATAAAAATATTGTTGAAGCTTTGAATTGTCTGGC   2903
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -----------------------------------------------------------------------------------------   -
pI9789  TTTGCCAGATACCCCATGTATACATGGGGTCAAATTTCCCTTATGCTCTTACGGAGTTACGGAGTTCTTAGAGAAAAAATAAAAATTCTC   2993
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    -------------------------------------------------------------------TTCTATGCCAATTTTTAAATTTGCATGTAAC   2429
pI9789  GATTTTGATAAAAAATGTCTTGCAGGAATTTAGAAAAACATGGGGGAAGTTTTAAAGGATTTTATGCTAACTTTTAA-TTTGCATGTAAA   3082
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    TGGGCAATGTCTTAAAAAAATCGACACTGGATTTGCTCAAATTTTATGTTGTGAAATTAAAATATATTTATTTGGCTCTCATTTGCGTTTT   2519
pI9789  TGGGCAGTGTTTAAAAAAAATAGTCATTGGTTTTATTTAAAATTTTGTTTTTGAAAATAGCATATATGTATTTGGTTCATATTTGCGTTTT   3172
pSK41   -----------------------------------------------------------------------------------------   --


pSK1    AAGAGTTTATGTAATTGTTCAGGTATAAAAACTATATAAAATACTCTTAATTCTTTAAAATGCCCCTTTAAAATTAAAAATAAAGGTATTC   2609
pI9789  AAGAGATTATGTAGGT-TTTAGATAGAAAACTATATGATTTACCCCTAAATCTTTAAAATACCCCCTTAAAATTCAAAATAAAGGTATTT   3261
pSK41   -----------------------------------------------------------------------------------------   -


pSK1    TAATTTTTAAAATATACTTAGATAAAAATAAAAAAGCTAACTCAAAATGAGCGAGCTTTTTATTTCAATATAATTGGGTGCGTCATATTGA   2699
pI9789  ATAATTTGAGTAAGTCTTGTGATAAAGTTTTTTTAAAAAGGAG------------TTTTTATTTTGAATAAAGACACAGTGTTAGATAAA   3339
pSK41   -----------------------------------------------------------------------------------------   -
```

FIG. 1—*Continued.*

A

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pSK1   --------------------------MSKFKKISASEFETLRFYQLPKFLFEDEYFSKMPTDAKVMYALLKDRFELSRL  53
pI9789 --------------------------MPNFEKYNLSQVKTERFYQLPKYLFEDAYFKKMSAEAKIMYALLKDRFELSIQ  53
pSX267 --------------------------MPNFEKYNLSQVKTERFYQLPKYLFEDIYFKKMSAEAKTMYALLKDRFELSIQ  53
pSK41  --------------------------MSK-QFFTVEENYKERFYQLPKVFFTNPNYKDLSNDAKIAYAILRDRLQLSIK  52
pCF10  --------------------------MENFQFYKSKEIYREKYYQMPKVFFTNEMYKSLSNDAKIAYMLLKDRFDNSVK  53
pPD1   --------------------------MENFQFYKSKEIYREKYYQMPKVFFTNEVYKSLSNDAKIAYMLLKDRFDNSVK  53
pAD1   --------------------------MNNFKFFNVNRIYNELFYQFPKVLIASDKYKKMSDSTKIAYMLLKARLEFAVQ  53
pLH1   MLLTLTNIILTIDFKALQKFAYTSFLEVFFMQRYSSTQINSELFWKFPKFLSENKKYADLSNDDRVAYMLIKDRYRYSLS  80
pLJ1   ----------------------MEVFFMRRYSATQINAELFWKFPKFLSINKKYVGLTNDDRMAYMLIKDRYRYSLS  55
pSAK1  ----------------------MNPINSSFKFYQATKVYGTLFFQFPKVLLYSPTYKNLSAEAKLAYVILKDRLEYSLH  57
pLS32  --------------------MSKYFTANNLDSLVFYQVPKVLLIGDKYKTMNPNAMKLYIILIDRIKLSMM  51
cons                             PK            Y        R

pSK1   NNWVDSENNIYLLYTNKQLCSILNYAEPKIIKLKKELEKYNLIINERQGLNK--------PNKIYLLEPTY---D-----  117
pI9789 NEWVDRNNNIYFIFSNKHLCEYLGYAEQKIIKLKKELVSFNLLTQERVGLNK--------PNRLYLLKPNY---DIEASL  122
pSX267 NEWVDKNNNIYFIFSNKHLCEYLGYAEQKIIKLKKELVSFNLLTQERVGLNK--------PNRLYLLKPNY---DVEGSR  122
pSK41  NNWIDTEGNIYFIYTVADLEVILNCGNKKITKIKKELENVDLLIQKRQGLNK--------PNLLYLLKPAITKNDIYEID  124
pCF10  NNWVDSEDNIYFIYTVAELMTLLNCREGKVSKIKKELEAVNLLKQKKGRVKKMNGKIETTPNLLYLGKPNVTSEDVFKIN  133
pPD1   NNWVDSEDNVYFIYTVAELMTLLNCREGKVSKIKKELEAVNLLKQKKGRVKKINGKIETTPNLLYLGKPNVTSEDVFKIN  133
pAD1   RNQVDENGNVYFTFTISELGEVLDCGKQRVLAIKSQLEEYGLLVQKQMGFNKTLGK--NNPNRLYLAELEVTENDIYQLQ  131
pLH1   NNWIDEKGDVYVYFTIEELRELLHVGKNKVTRVKQHLMDYDLLEIEKQGFDPKKKK--NFPDRIYLLQPDYDPTDLISIS  158
pLJ1   NNWIDKDKNVYVYFTIDDLKELLHVGKNKVTRIKQHLIDYGLLEIVKQGFDPQNKK--NYPDRIYLLQPEYDPTDL--IS  131
pSAK1  NDWVDENGNIYFIFSNTELQQILNCSEPKVIKTKKELEQANLLFQKKMGFDPKMKR--NNPNRLYLGDLNVSATDVYKRE  135
pLS32  NNWKDELGRYYVRLSIEKGSELLGFSDSTFKRAKKLAKYELLEEKREGMNK--------SNILYPLMLEYTEEDIYRLN  123
cons         D    Y       L    L      KL        L            Y       D

pSK1   -----------KELINSKFQNKEFISSRTNKSSVQELINSKSSDTDFNNTEYIETKNNDTNYTNDTSN--MISKNSHSNH  184
pI9789 -----------TKELPNSQFQNNEFGSSRTVNLSGQELPNSQSNDTELNNTNYIENEDNDTYDMNDIISNTLINNNHHSNH  192
pSX267 -----------TKELPNSQFQNNEFGSSRTMNLSGQELPNSQSNDTDLSNTDYIETENNDTYDMNDTYNNLISHDN--SDH  190
pSK41  KAENEVEALQDKEVSKGHVQKCQKDTSRNVKRTRLEMSKGHTNDTDFIDTDFIDTESNDMNNMNDT--------NQHSNH  196
pCF10  E---------EQNNVNPVIAKIANTEKVNDI-NENPVIAKIANTEKVNDI---NENPVIAKIADNLFYTSYLDT----LD  196
pPD1   E---------EQNNVNPVIAKIANTEKVNDI-NENPVIAKIANTEKVNDI---NENPVIAKIADNLFYTSYLDT----LD  196
pAD1   T---------FDDTMQENVDKSERMKIIPTP-QRKTNAESLGNQEGMKIIPCQNVDKSEGMKISTVFNNLNTDT----ID  197
pLH1   QASQVSAL--EQSGFPKMGSRGSNQGSLDIK-GKSDSEICNKGTSALEQSDFPKMGTNKDKNNSDTIKDTDTDTQKWNFS  235
pLJ1   QSSHASAL--EQSGIPKMGTRYQNEGNLDNK-GKSDSENCNKDTSALEQSGIPKMGANKDNNSSDTIKDTIKDTDQWNFS  208
pSAK1  NEASQSTISSATSGTKNSLARHEMPQSLATN-GTKNSLARDKVPQSLATSG-TKNSLVQYKDLETQARDNKETKKLDFS  213
pLS32  -----------NEVDDLLIKAEETAKNIDKNWMGQNELSSNERIGQNELSGKVQIEPSREVKLNHPDGSKRTTNNNNSIK  192
cons

pSK1   TNHQQTEF--NNDALKFQALEE-----------LPSQIKSYVSNF--EIKDIRIIKSILLKGKKSFNNTH-----DTYYR  244
pI9789 TNHQKTEF--NNDALKFQVLEE----------LPQQFQDYLSKF--EIREIRIIKSVLLKGKKSFNTSH-----DTYYR  252
pSX267 TNHQQTEF--NNDALKFQLLEE----------LPKQLQDYLSKF--EIREIRIIKSVLLKGKKSFNNAH-----DTFYR  250
pSK41  SNHFSNIH--DKESLKYIELQE----------LPELIKSYINNF--SYEEVKSIKSVILKAKKSFNNKY-----DTFYM  256
pCF10  TLDTKKEAL--QQQLLLDQFSEV-----QENTFLSKDSLKFIAAFSDTIKEAHEMVGTIIRAKTKVEKEY-----NVVLI  264
pPD1   TLDTKKEVL--QQQLLLDQFSEV-----QENTFLSKDSLKFIAAFSDTIQEAHEQVGTIIRAKTKVEKEY-----NIVLI  264
pAD1   TIDTEKERL--QQQLLLDQFSEV-----QENTFLNKDSLKFIAAFSNTIQEAHEQVGTIIRAKTKVEKEY-----NLVLI  265
pLH1   PDSYPKEIVVKQNKDLLKHLSSTLTDDKDLPMFLNNESIDLITLWFKTPEAAHDCISVILNAAANDSRKEAMEQIGRHELY  315
pLJ1   TNNYTPEQVTAQNQDLLSHLGETLTGDKEAPMFLNKDSINLIAKWFRTPEGASECISTILNAANDSRKNAESQIGHHELY  288
pSAK1  TNQYSPEIIRKQNQDLVRRAKDYLPESANGGLFLNKEGVELLGLWCRSPKQMRRFLGIILNA----KKAVEREHEGTAIV  289
pLS32  NNFNNNE---NNLVNKELTREETIHNLISEYM------------------------------KKGLPKEV------CIMV  233
cons

pSK1   LEDVEFELVSVLKR-----FKAMLLQKNETVETMQGYLMQSIKAELEEIHALNMRRQNIPQ---------YN-IFNQ--  306
pI9789 LEDVEFEIVSVLKR-----FKAMLLQKNETVEAMQGYLMQSIKAELEEIHALYMRRKNMKQ---------YN-IFNQ--  314
pSX267 LEDVEFELVSVLKR-----FKAMLLQKNETVEAMQGYLMQSIKAELEEIHALNMRRQNMKQ---------HN-IFNQ--  312
pSK41  LEDIDEELLLVLKR-----FKGYLVKKQEKVANMEGYLMRSIIAELEEMHSTIMRRKNMENN--------PLSLFN---  319
pCF10  GEDYQEE----IDKCLRRVMHKIKTD--STVKSPKGLFYKSFYNLFVECALEKENVKN-RDNSKGSEITLHNWLEK----  333
pPD1   GEDYQEE----IDKCLRRVMHKIKTD--STVKSPKGLFYKSFYNLFVACALEKENINN-GNKSKGSEITLHNWIEQ---  333
pAD1   GEDYQEE----IDKCLRRVMHKIKTD--STVKSPKGLFYKSFYNLFVECALEKKSQLNKKSNSNTPGVITHNWVENQ---  336
pLH1   FEDCNNELKREITRKLRRYFNKMRTAKPGEIKNPRSYLYTTMRNTFDKWQNDILVAEKEDSDNNKK-------------  381
pLJ1   FEDYNNELKRMITNRLRRYFNKMRTAKDGKIKNPKNYLYVSMRNMFDKWQNDVLMAEKDKANNKD--------------  353
pSAK1  LDDPRCQ--EMINKTMRRFPNVLRSDNK-KINNVENYLFGAMKETLVAYWNKSLITTNGGSLNEF--------------  351
pLS32  LDEVE--------------------KTPDVKNFGAYLRTCLDNALHKRNLKYGNIDFKGKKINGQVPF-YNWLDN---  287
cons
```
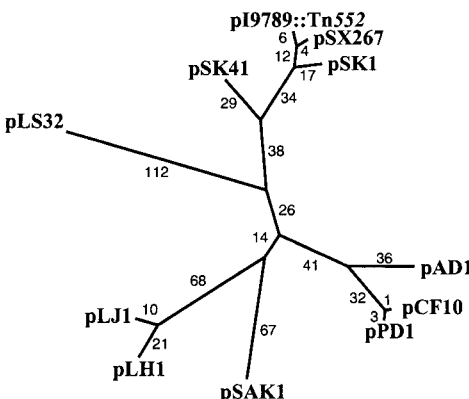
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FIG. 2. (A) Multiple sequence alignment of the deduced pSK1 and pI9789::Tn552 *rep* products and related proteins. The amino acid sequences of the replication initiation proteins from the indicated plasmids were obtained from the following GenBank entries: pSX267, X92404; pSK41, AF051916; pCF10, L14285; pPD1, D78016; pAD1, L01794; pLH1, AJ222725; pLJ1, J04240; pSAK1, Z50862; and pLS32, D49467. Residue numbering is shown on the right. Amino acids common to five or more sequences at any position are shaded, globally conserved residues are indicated in the consensus (cons) line, and insertions or deletions are denoted by dashes. (B) Phylogenetic analysis of the Rep proteins shown in panel A. The unrooted tree was constructed by using the programs PROTDIST and NEIGHBOR (10) from the multiple alignment generated by PILEUP (9) shown in panel A. Branch lengths in arbitrary units are indicated. All nodes had bootstrap values of greater than 80%, and an equivalent tree topology was obtained with the program PROTPARS (10).

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mids are located immediately downstream of, and probably cotranscribed with, their cognate plasmid replication initiation genes, which do not appear to be evolutionarily related to those of the staphylococcal plasmids.

Gering et al. (16) have demonstrated that the origin of pSX267 replication resides within its *rep* coding sequence and suggested that repeats present within *rep* may be involved. pSK1, pI9789::Tn*552*, and pSK41 possess equivalent arrays of direct and inverted repeats within their respective *rep* genes (Fig. 1). The pSK1 replication region also contains a series of direct repeats located upstream of *orf245*. Seven copies matching the 12-nucleotide (nt) consensus sequence (C/T)-T-(A/T)-(A/G)-(C/G)-(C/T)-(A/G)-C-C-T-A-A are evident (Fig. 1). The significance of these repeats is unknown at this time, although it is tempting to speculate that they might be involved in the regulation of *orf245* and/or replication. However, the 12-bp repeats appear to be less well conserved on pI9789::Tn*552* and pSX267 (Fig. 1) and are outside the minimal replicating fragment defined for the latter plasmid (16).

Functional analysis of the pSK1 replication region. To confirm the functionality of the pSK1 replication region identified above and to investigate the role, if any, of *orf245* in the process, two minireplicons, pSK4829 and pSK4833, were constructed in *E. coli* using the plasmid pWE180. This plasmid is a derivative of pUC18 (59) containing an erythromycin resistance determinant, *ermC*. Although *ermC* confers resistance in both *S. aureus* and *E. coli*, the ColE1-derived replicon of pWE180 is functional only in the latter. The two pSK1 DNA segments amplified and separately cloned into appropriately restricted pWE180 corresponded to the *rep* gene and upstream intergenic region in the case of pSK4833 (nt 842 to 2287 [Fig. 1]) and a larger fragment also encompassing *orf245* in pSK4829 (nt 3 to 2287 [Fig. 1]). pSK4829 and pSK4833 plasmid DNA isolated from *E. coli* was able to transform *S. aureus* RN4220 cells to erythromycin resistance via protoplast transformation. Plasmid isolation and agarose gel electrophoresis demonstrated the presence of pSK4829 and pSK4833 DNA in the RN4220 transformants, confirming that both of these plasmids contain a replication origin that functions in *S. aureus*. It should be noted that a recombinant plasmid equivalent to pSK4833, but containing the pI9789::Tn*552 rep* region rather than that from pSK1, was similarly shown to replicate in *S. aureus* RN4220.

The replication proficiency of pSK4833 in *S. aureus* established that *orf245* is not essential for replication initiating at the pSK1 origin and suggests that *rep* is the only coding sequence absolutely required, as has been shown to be the case in the related replication region of pSX267 (16). However, the *orf245* homolog encoded by pUCL287, *repB287*, although similarly dispensable for replication, has been shown to influence the segregational stability and copy number of this plasmid (3). To investigate the possibility that *orf245* plays an equivalent role in the replication of pSK1, the segregational stabilities of pSK4829 and pSK4833 in the absence of selection were determined and compared to that of pSK1 in its entirety.

As shown in Fig. 3A, after 100 generations of growth without plasmid selection, pSK1 was maintained by approximately 80% of an *S. aureus* RN4220 population. pSK4833, encoding only the pSK1 *rep* gene and upstream intergenic sequence, exhibited significantly lower segregational stability, being entirely lost from the bacterial population by approximately the 80th generation. In comparison, pSK4829, which additionally encodes *orf245*, was found to be almost as stable as the parent plasmid, pSK1. These data strongly suggest that *orf245* contributes to the stable maintenance of pSK1 in *S. aureus*.

Functional analysis of the replication region from the conjugative multiresistance plasmid pSK41. The results discussed
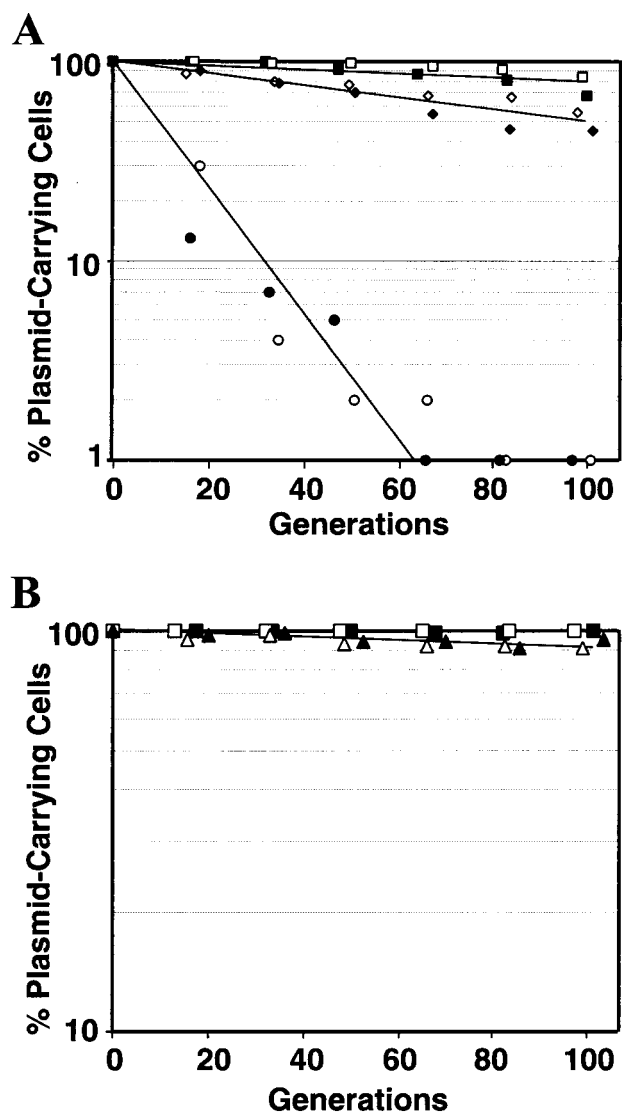

FIG. 3. Segregational stabilities of pSK1, pSK41, and derivatives in *S. aureus* strain RN4220. The values indicate the proportion of cells in a population, cultured in the absence of plasmid selection for the indicated number of generations, subsequently able to grow when plated on medium containing selection for the plasmid. Open and solid shapes distinguish data for each of two independent experiments. (A) Values for pSK1, pSK4829, and pSK4833, tested in *S. aureus* strain RN4220, are denoted by squares, diamonds and circles, respectively. The presence of pSK1 was indicated by growth in the presence of 100 μg of trimethoprim/ml, whereas retention of pSK4829 or pSK4833 was indicated by growth in the presence of 20 μg of erythromycin/ml. (B) Values for pSK41 and pSK5413, tested in *S. aureus* strains SK982 and RN4220, respectively, are denoted by squares and triangles, respectively. The presence of pSK41 was indicated by growth in the presence of 20 μg of gentamicin/ml, whereas retention of pSK5413 was indicated by growth in the presence of 10 μg of tetracycline/ml.

above implicating *orf245* in the stable maintenance of pSK1 prompted us to investigate the replication system of pSK41, since this plasmid possesses a related *rep* gene but lacks an *orf245* homolog. However, in an equivalent position and orientation, pSK41 possesses a gene, *orf86*, whose deduced product contains a putative helix-turn-helix DNA-binding domain (4), a feature shared by Orf245 and homologs (3, 47). We therefore intended to construct two pSK41 minireplicons, one containing pSK41 *rep* and the upstream intervening sequence and the other containing *orf86* through *rep*, to determine if *orf86* plays a role equivalent to that of *orf245* on pSK1; how-
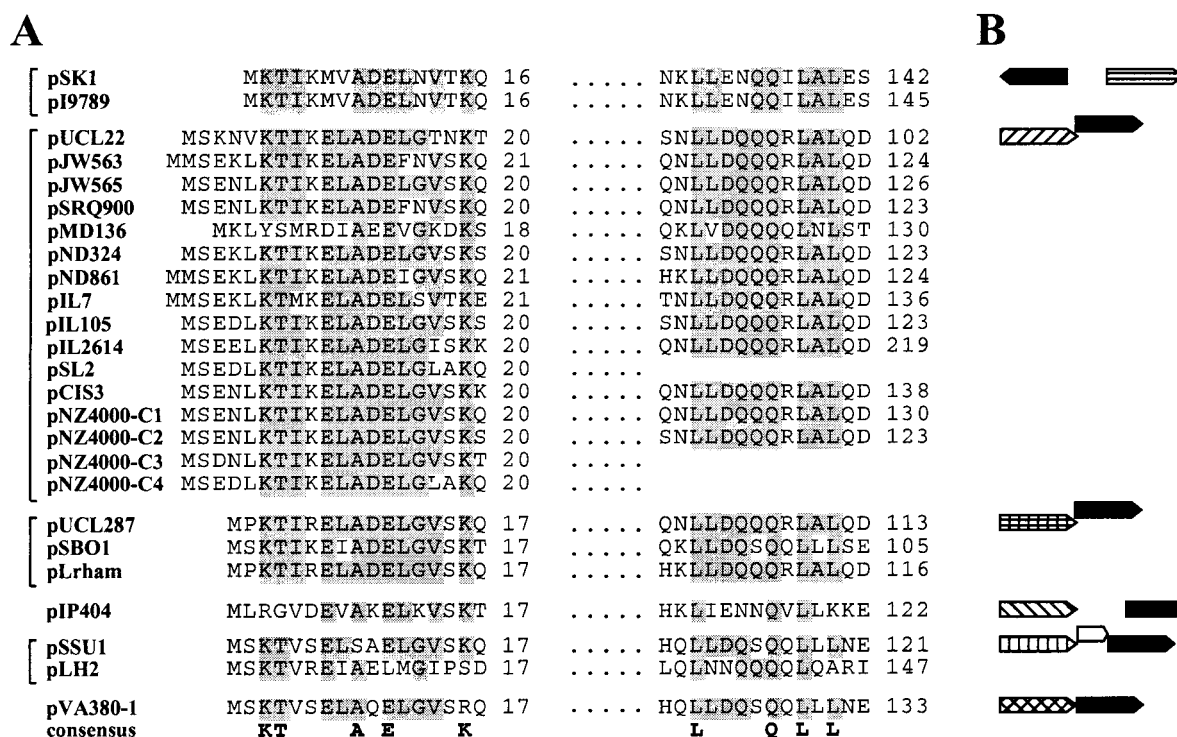
A

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  ┌ pSK1        MKTIKMVADELNVTKQ 16  ..... NKLLENQQILALES 142
  └ pI9789      MKTIKMVADELNVTKQ 16  ..... NKLLENQQILALES 145

  ┌ pUCL22      MSKNVKTIKELADELGTNKT 20  ..... SNLLDQQQRLALQD 102
  │ pJW563     MMSEKLKTIKELADEFNVSKQ 21  ..... QNLLDQQQRLALQD 124
  │ pJW565      MSENLKTIKELADELGVSKQ 20  ..... QNLLDQQQRLALQD 126
  │ pSRQ900     MSENLKTIKELADEFNVSKQ 20  ..... QNLLDQQQRLALQD 123
  │ pMD136       MKLYSMRDIAEEVGKDKS 18  ..... QKLVDQQQQLNLST 130
  │ pND324       MSEKLKTIKELADELGVSKS 20  ..... SNLLDQQQRLALQD 123
  │ pND861     MMSEKLKTIKELADEIGVSKQ 21  ..... HKLLDQQQRLALQD 124
  │ pIL7       MMSEKLKTMKELADELSVTKE 21  ..... TNLLDQQQRLALQD 136
  │ pIL105      MSEDLKTIKELADELGVSKS 20  ..... SNLLDQQQRLALQD 123
  │ pIL2614     MSEELKTIKELADELGISKK 20  ..... QNLLDQQQRLALQD 219
  │ pSL2        MSEDLKTIKELADELGLAKQ 20  .....
  │ pCIS3       MSENLKTIKELADELGVSKK 20  ..... QNLLDQQQRLALQD 138
  │ pNZ4000-C1  MSENLKTIKELADELGVSKQ 20  ..... QNLLDQQQRLALQD 130
  │ pNZ4000-C2  MSENLKTIKELADELGVSKS 20  ..... SNLLDQQQRLALQD 123
  │ pNZ4000-C3  MSDNLKTIKELADELGVSKT 20  .....
  └ pNZ4000-C4  MSEDLKTIKELADELGLAKQ 20  .....

  ┌ pUCL287     MPKTIRELADELGVSKQ 17  ..... QNLLDQQQRLALQD 113
  │ pSBO1       MSKTIKEIADELGVSKT 17  ..... QKLLDQSQQLLLSE 105
  └ pLrham      MPKTIRELADELGVSKQ 17  ..... HKLLDQQQRLALQD 116

    pIP404       MLRGVDEVAKELKVSKT 17  ..... HKLIENNQVLLKKE 122

  ┌ pSSU1       MSKTVSELSAELGVSKQ 17  ..... HQLLDQSQQLLLNE 121
  └ pLH2        MSKTVREIAELMGIPSD 17  ..... LQLNNQQQQLQARI 147

    pVA380-1     MSKTVSELAQELGVSRQ 17  ..... HQLLDQSQQLLLNE 133
    consensus    KT   A E    K             L   Q L L
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B

FIG. 4. (A) Multiple amino acid sequence alignment of segments conserved in Orf245 and related proteins. Residues present in at least 75% of the sequences at any one position are shaded, and amino acids present in at least 90% are indicated in a consensus line at the bottom. The dots denote sequences between the conserved regions that have been omitted for clarity. The amino acid sequences were obtained from the following GenBank entries: pUCL22, X60454; pJW563, X85168; pJW565, Y12736; pSRQ900, AF001314; pMD136, AF069302; pND324, U44843; pND861, AF034786; pIL7, Z25475; pIL105, AF116286; pIL2614, U90222; pSL2, X56550; pCIS3, AF153414; pNZ4000 (C1 to C4), AF036485; pUCL287, X75607; pSBO1, AB021464; pLrham (unnamed plasmid from *Lactobacillus rhamnosus*), AF037091; pIP404, M32882; pSSU1, AB019522; pLH2, X81981; and pVA380-1, L23803. For each segment, the amino acid number of the rightmost amino acid of a sequence is shown. Proteins from plasmids possessing related replication initiation genes are bracketed. The Orf245-like protein from pSL2, and two of the four homologs carried by pNZ4000, appear to be truncated and therefore do not possess the second conserved region. (B) For the top plasmid of each replicon class shown in panel A, the genetic organization of the gene encoding the Orf245-like protein, with respect to its associated *rep* gene, is represented diagrammatically. Distinct *rep* genes are represented by different patterned boxes, whereas *orf245* homologs are denoted by solid boxes, and the genes' directions of transcription are indicated by arrowheads on the boxes; note that genes are not drawn to scale.

ever, despite numerous attempts we have not been able to obtain a recombinant plasmid containing *orf86*, either in the presence of *rep* or in isolation. pSK5413 was constructed by ligating an amplified fragment corresponding to nt 12795 to 14727 of pSK41 (GenBank entry AF051917), encoding the *rep* gene and the upstream intergenic region, into pUC18 and the subsequent insertion of the staphylococcal tetracycline resistance determinant, *tetA*(K) (20), as a marker. pSK41 was maintained in a population with almost absolute fidelity after 100 generations (Fig. 3B). After electroporation into *S. aureus* RN4220, segregational stability assays indicated that pSK5413 was maintained almost as efficiently as pSK41 (Fig. 3B), suggesting that *orf86* does not contribute to the segregational stability of pSK41 and that the replication machinery of pSK41 requires no accessory gene equivalent to pSK1 *orf245* for maximal efficiency.

Previous studies suggested that pSK1 belongs to staphylococcal plasmid incompatibility group 1 (Inc1) (27), which includes α and γ family β-lactamase–heavy-metal resistance plasmids such as pI9789 (48) and pSX267 (19). To investigate the incompatibility classification of the pSK41 replicon, a mixed-culture transfer experiment using the multiplasmid strain RN1965 (43) as the donor and SK982 harboring pSK5413 as the recipient was performed. These studies demonstrated that plasmids from the non-RC plasmid incompatibility groups, Inc1, Inc2, and Inc6, could each coexist with pSK5413, indicating that pSK41 does not belong to any of these groups.

pSK41 may therefore belong to either of the two non-RC Inc groups that we were unable to test, Inc7 or Inc15, or define a new incompatibility group.

DISCUSSION

The nucleotide sequence identity evident among pSK1, pI9789::Tn*552*, pSX267, and pSK41 is consistent with incompatibility classifications determined here and previously (19, 27, 48). In particular, the sequence repeats present within the *rep* genes, which are likely to represent the replication origin of each plasmid, are largely conserved on the Inc1 plasmids, pSK1, pI9789::Tn*552*, and pSX267, whereas the repeats present within the *rep* gene of pSK41, which does not belong to Inc1, differ considerably (Fig. 1). As is the case for pSX267, database entries for three other staphylococcal plasmids contain incomplete sequences of probable *orf245*-like genes. It is therefore likely that the multiresistance plasmids, pSK156 (35), pIP630 (1), and pIP1156 (2), all belong to the same plasmid replication complex as pSK1 and pI9789::Tn*552*.

The staphylococcal multiresistance plasmids described here represent the third type of theta-mode replicon to possess an *orf245*-like gene, with pUCL22- and pUCL287-like plasmids representing the other two. Comparative sequence analyses reveal that genes encoding products sharing statistically significant amino acid sequence similarity to pSK1 Orf245 are represented on plasmids possessing yet more classes of repli-

cation initiation genes. Across the entire family of these proteins, sequence conservation is restricted to two linear segments, as shown in Fig. 4; viz., an N-terminal region confined to the first half of a predicted helix-turn-helix DNA-binding domain present in each protein and a segment located within the C-terminal halves of the proteins. Another theta-replicating plasmid, pIP404 from *Clostridium perfringens* (15), which encodes a replication initiation gene related to those of the broad-host-range plasmids pAMβ1 and pIP501, also possesses a gene encoding an Orf245 homolog ($Z = 4.4$). As on pUCL22 and pUCL287, the pIP404 *orf245*-like gene is encoded downstream of the *rep* gene, but rather than having an overlapping organization, in this case the two genes are separated by 757 nt of noncoding sequence (Fig. 4) which includes the probable origin of replication. Notably, our analysis has also identified *orf245*-like genes on members of two distinct classes of plasmids that replicate via an RC mechanism. The streptococcal plasmid pSSU1 and the *Lactobacillus* plasmid pLH2 (38) belong to the pE194/pMV158 family of RC plasmids. On pSSU1, the *rep* and *orf245*-like genes are separated by a small open reading frame that slightly overlaps them (Fig. 4), whereas on pLH2 these genes are separated by almost 2 kb. Finally, another streptococcal plasmid, pVA380-1, which belongs to the pC194/pUB110 RC plasmid family, contains an *orf245* homolog immediately downstream of its replication initiation gene (Fig. 4); interestingly, this gene has been assigned a role in the mobilization of pVA380-1 (24).

Despite the prevalence of *rep* gene-associated *orf245* homologs, only the plasmid maintenance assays of the pSK1 minireplicons described here and the findings of Benachour et al. (3) concerning the stabilities and copy numbers of pUCL287 derivatives have implied a role for these genes in plasmid replication. The association of *orf245* homologs with a range of seemingly distinct types of plasmid replication initiation genes is both intriguing and perplexing. Such a distribution implies a significant role for this gene, a notion supported experimentally for pUCL287 and now pSK1. However, for each of the six replicon types represented in Fig. 4, there are examples of naturally occurring plasmids which lack an *orf245*-like gene. Such a "patchy" distribution is consistent with plasmids variously acquiring and losing a gene of this type, possibly indicating that *orf245*-like genes play a functionally and evolutionarily significant role only under certain conditions. For example, it is conceivable that such a gene is required for plasmid survival in some hosts and/or environments but not in others. The precise role of *orf245* and related genes has yet to be elucidated, although the effect on plasmid copy number observed when *repB287* was removed from pUCL287 (3) suggests that at least in this case the gene influences replication directly rather than by enhancing plasmid maintenance as a stand-alone segregation mechanism. We are pursuing the working hypothesis that *orf245* exerts a regulatory effect on *rep* transcription. It is hoped that such studies will ultimately provide insights into the interaction among plasmids, bacterial hosts, and their environment.

The sequence similarity detected between the pSK1 replication region and those of the β-lactamase-heavy-metal resistance plasmids and pSK41-like conjugative plasmids establishes that all three recognized groups of large staphylococcal multiresistance plasmids utilize evolutionarily related thetamode replication systems. It is sobering to consider the impact of this single replicon type on the worldwide development of antimicrobial-resistant staphylococcal strains.

ADDENDUM IN PROOF

The recently published nucleotide sequence of the pIP630 replication region (J. Allignet and N. El Solh, Plasmid **42:**134–138, 1999) has confirmed our suggestion that this plasmid belongs to the same plasmid replication complex as pSK1 and pI9789::Tn*552*.

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