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Possible Role of Insertion Sequence IS257 in Dissemination and Expression of High- and Low-Level Trimethoprim Resistance in Staphylococci

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The transposon-like structure Tn*4003* and related elements were found to encode high- and low-level trimethoprim resistance (Tp^r) in *Staphylococcus aureus* and coagulase-negative staphylococci. By using transcriptional fusions in *Escherichia coli*, the variation in resistance levels was found to correlate with the transcriptional activity of the region presumed to carry the promoter for the operon containing the Tp^r dihydrofolate reductase gene, *dfrA*, encoded by these elements. The reduced transcriptional activities exhibited by elements encoding low-level Tp^r appear to be a consequence of deletions adjacent to the copy of IS*257* which normally encodes the −35 sequences of these promoters. The data obtained not only support the involvement of IS*257* in the transcription of the proposed *thyE-dfrA-orf-140* operon of Tn*4003* but may also implicate this insertion sequence in the mechanisms resulting in the variation in Tp^r levels observed in staphylococci.

Approximately 50% of the methicillin-resistant *Staphylococcus aureus* strains isolated in Australia during the early 1980s contained plasmid pSK1, which encodes high-level trimethoprim resistance (Tp^r_H; MIC, ≥1,000 μg/ml) (47). The Tp^r determinant on pSK1 is located on the 4.7-kb composite transposon-like structure designated Tn*4003* (see Fig. 1 and reference 40) and is bounded by a single IS*257* element (IS*257*L) at one end and two copies of IS*257* (IS*257*R1 and IS*257*R2) at the other. The two outer IS*257* elements of Tn*4003* on pSK1 are flanked by 8-bp direct repeat sequences, suggestive of transposition of this structure into a precursor of pSK1 (40). Tn*4003* is therefore presumed to be a transposon, although transposition of this element has yet to be demonstrated directly. The central region of Tn*4003* contains three open reading frames apparently organized in a single operon, viz., *thyE* (thymidylate synthetase), *dfrA* (dihydrofolate reductase), and a truncated open reading frame, *orf-140*. The *dfrA* gene encodes the S1 dihydrofolate reductase which is responsible for Tp^r_H (49). The promoter for the *thyE–dfrA–orf-140* operon in Tn*4003* has been suggested to span the junction of IS*257*L and the central region of this putative transposon (40).

In contrast to the Tp^r_H phenotype encoded by pSK1, the Tp^r determinant on the multiresistant conjugative plasmid pJE1, detected in an *S. aureus* strain isolated in Germany (12), confers resistance to only a low level of Tp^r (Tp^r_L; MICs, >50 and ≤300 μg/ml) (7). This determinant is also flanked by three IS*257* elements in an arrangement similar to that in Tn*4003* (7). Plasmid-mediated Tp^r_H and Tp^r_L have also been detected in coagulase-negative staphylococci (16, 47), a group of organisms hypothesized to act as a reservoir of antimicrobial resistance genes for *S. aureus* (14, 28, 46). Furthermore, DNA-DNA hybridization studies have revealed homology between the *dfrA* resistance determinant of Tn*4003* and the determinants of Tp^r_H in coagulase-negative staphylococci (47).

To gain further insights into the relationships between the plasmid-encoded Tp^r determinants found in *S. aureus* and coagulase-negative staphylococci and to establish the basis of the variation in expression levels exhibited by different isolates, we undertook a molecular analysis of selected Tp^r determinants found on plasmids from these organisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The staphylococcal strains and plasmids used in the study and their relevant characteristics are presented in Table 1. The *Escherichia coli* vector pUC118 (48) was used in cloning and subcloning for nucleotide sequencing, and the *E. coli* host strain was DH5α (F^- *endA hsdR17 supE44 thi-1* λ^- *recA1 gyrA96 relA1* ϕ80*dlac*ZΔM15) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The *E. coli* K12 *galK* mutant strain CB1 (22) and the promoter-probe vector pKO500 (38) were used in the measurements of promoter activity; strain CB1 carrying the vector pKL500 (19) in which *galK* is expressed from the *lac* promoter P_{lac} was used as a positive control.

General procedures. MIC determinations and plasmid elimination were performed as described previously (18, 26). In the present study, organisms for which trimethoprim MICs were >50 μg/ml and ≤300 μg/ml were defined as Tp^r_L, whereas those for which trimethoprim MICs were ≥1,000 μg/ml were classified as Tp^r_H isolates. For no isolate studied were MICs found to be between >300 and <1,000 μg/ml.

DNA isolation. Rapid DNA isolation techniques and cesium chloride-ethidium bromide density gradient purification procedures have been described previously for the preparation of DNA from cultures of both staphylococci (26) and *E. coli* (37).

DNA-DNA hybridization. To localize the Tp^r determinant on the plasmids studied, uncut or restriction endonuclease-digested DNA fragments were transferred from agarose gels to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Immobilized DNA was hybridized with a probe specific

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TABLE 1. Staphylococcal strains and Tp^r plasmids used in the study

Strain	Tp^r plasmid[a]	Relevant characteristics of strain[b]	Reference
S. aureus			
SK982		Rf^r Nv^r	27
SK2267[c]	pSK1	Rf^r Nv^r Gm^r Km^r Tm^r Eb^r Qa^r Tp^r_H	25
SK4014[c]	pJE1	Rf^r Nv^r Gm^r Km^r Nm^r Pm^r Tm^r Eb^r Qa^r Tra^+ Tp^r_L	12; this study
Coagulase-negative staphylococci			
SK76	pSK818	Gm^r Km^r Nm^r Tm^r Cm^r Pc^r Tc^r Ac^r Eb^r Qa^r Tp^r_H	47
SK275	pSK697	Gm^r Km^r Nm^r Tm^r Cm^r Em^r Su^r Tc^r Ac^r Eb^r Qa^r Tp^r_L	Clinical isolate; this study
SK398	pSK639	Pc^r Ak^r Gm^r Km^r Nm^r Tm^r Ac^r Eb^r Qa^r Tp^r_L	Clinical isolate; this study

[a] The three coagulase-negative staphylococcal isolates also carried other uncharacterized plasmids.
[b] Abbreviations: Ac^r, acriflavine resistance; Ak^r, amikacin resistance; Cm^r, chloramphenicol resistance; Eb^r, ethidium bromide resistance; Em^r, erythromycin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Nm^r, neomycin resistance; Pc^r, penicillin resistance; Pm^r, paromomycin resistance; Qa^r, quaternary ammonium compound resistance; Su^r, sulfonamide resistance; Tc^r, tetracycline resistance; Tm^r, tobramycin resistance; Tp^r_H and Tp^r_L, high- and low-level trimethoprim resistance, respectively, as defined in Materials and Methods; Tra^+, conjugative transfer.
[c] *S. aureus* SK982 carrying the plasmid indicated.

for the *dfrA* gene under high-stringency conditions (41). The probe used was a 863-bp *Eco*RI-*Eco*RV DNA fragment from the central region of Tn*4003* (see Fig. 1) radiolabelled with [α-^{32}P]dATP by nick translation as described by Sambrook et al. (41).

Cloning and nucleotide sequence analyses. Cloning experiments were performed by standard techniques (41). Nucleotide sequences were determined by the procedure of Sanger et al. (42) by using a T7 DNA polymerase sequencing kit (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.); PCR products, however, were sequenced by using a CircumVent thermal cycle dideoxy sequencing kit (New England Biolabs).

Transcript mapping. Total cellular RNA from *S. aureus* was isolated essentially as described by Miller (32) for the isolation of RNA from *E. coli*. This procedure was modified to include lysostaphin in the protoplast lysis buffer at 0.1 mg/ml. The transcriptional start point (tsp) for a transcript initiated upstream of the *dfrA* resistance gene of Tn*4003* was determined by S1 nuclease mapping by the protocols outlined by Sambrook et al. (41).

Galactokinase assay. Galactokinase activity was assayed by the method of McKenny et al. (31).

PCR. PCRs were carried out to synthesize the promoter regions of Tp^r determinants from *S. aureus* and coagulase-negative staphylococci. Oligonucleotide primers specific for the sequences upstream (primer 1) and downstream (primer 2) of the promoter region of Tp^r determinants were 5′-TTTGGATCCG CATATCTTAGATAGTAGCC-3′ (the underlined sequence indicates the *Bam*HI site) and 5′-GTTGAATTCAGGGTCTT GCTGGGAACGATG-3′ (the underlined sequence indicates the *Eco*RI site). Primer 1 corresponds to nucleotides 690 to 709 of the published Tn*4003* sequence (40), whereas primer 2 corresponds to nucleotides 1226 to 1201.

RESULTS

Identification of plasmids encoding high- and low-level Tp^r in staphylococci. Clinical coagulase-negative staphylococcal strains isolated from Australian hospitals during the early 1980s were previously screened for Tp^r by Tennent et al. (47). We selected three Tp^r coagulase-negative staphylococcal isolates for further analyses, viz., SK76, which exhibited Tp^r_H, and two strains, SK275 and SK398, that expressed Tp^r_L (Table 1). The *S. aureus* plasmids pSK1 and pJE1 have previously been shown to encode Tp^r_H and Tp^r_L, respectively (7, 29, 45). To ascertain if the Tp^r determinants in the three coagulase-negative staphylococcal isolates are similar to the *dfrA* gene of pSK1 and, if so, to determine if they are chromosomally or plasmid encoded, Southern hybridization analyses were performed. The 863-bp *Eco*RI-*Eco*RV *dfrA*-specific restriction fragment from Tn*4003* (Fig. 1) was used to probe uncut DNA from the coagulase-negative staphylococcal isolates. Plasmids of approximately 13, 11, and 8 kb from SK76, SK275, and SK398, respectively, were found to hybridize with the probe (data not shown). This probe also hybridized to coelectrophoresed pJE1 DNA, confirming that the plasmid encodes a *dfrA* homolog. Plasmid elimination experiments confirmed that the Tp^r associated with SK76, SK275, and SK398 is encoded by the plasmids identified above, now designated pSK818, pSK697, and pSK639, respectively.

On pSK1, *dfrA* is completely contained within an internal 2.7-kb *Bgl*II fragment of Tn*4003*, because each flanking IS*257* element contains a single *Bgl*II site (Fig. 1). Similarly, probing of endonuclease-restricted DNA indicated that the *dfrA* homologs on pJE1, pSK639, pSK697, and pSK818 were also located on *Bgl*II fragments of approximately this size (data not shown). It is therefore likely that the Tp^r conferred by these plasmids is encoded by *dfrA* genes located on Tn*4003*-like elements.

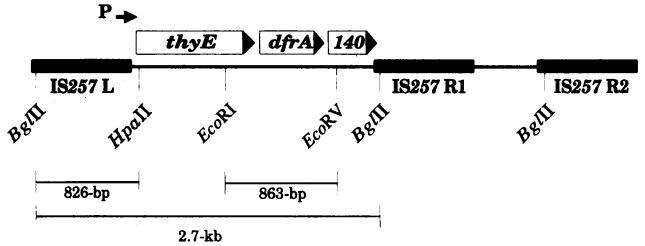

FIG. 1. Genetic and physical map of the 4.7-kb composite transposon-like structure Tn*4003*. Filled boxes represent the directly repeated copies of IS*257*. Open boxes with arrowheads represent the extents and directions of genes *thyE* (thymidylate synthetase), *dfrA* (dihydrofolate reductase), and the open reading frame *orf-140*. The location of the putative promoter (P) for the *thyE–dfrA–orf-140* operon is denoted by an arrow (40). Regions underlined identify restriction fragments (size in kilobases or base pairs) used in hybridization or cloning experiments. Relevant restriction sites are shown.

Expression of staphylococcal Tp^r determinants in *E. coli*. Previous studies have demonstrated that the product of the Tn*4003* *dfrA* gene is synthesized and is functional when expressed in *E. coli* (29). To rule out the possibility that the observed variation in Tp^r levels expressed by *S. aureus* and coagulase-negative staphylococcal isolates is due to diversity in the background of the clinical strains or genetic differences in the *dfrA* homologs themselves, the structural gene responsible for Tp^r from each plasmid (pSK1, pJE1, pSK639, pSK697, and pSK818) was cloned, as a 863-bp *Eco*RI-*Eco*RV fragment (Fig. 1), into the *E. coli* vector pUC118. The cloned fragments lack the putative native promoter and are oriented such that transcription of the *dfrA* gene initiates at the vector promoter, P_{lac}. On the basis of MIC determinations, *E. coli* DH5α cells harboring the resulting recombinant plasmids were all found to exhibit Tp^r_H, including those derived from plasmids normally associated with the low-level resistance phenotype. The basis of the observed phenotypic variation associated with these *dfrA* genes in their native form is therefore probably not due to differences in the *dfrA* structural genes. In support of this notion, nucleotide sequencing (data not shown) has demonstrated that the *dfrA* genes encoded by pJE1 and pSK639 are identical to that encoded by pSK1 (40).

In contrast to the uniform Tp^r_H expressed by the various *dfrA* genes transcribed by P_{lac} of pUC118, *E. coli* DH5α harboring recombinant plasmids containing the internal *Bgl*II fragment from each plasmid (Fig. 1), which encodes *dfrA* and its putative natural promoter cloned in opposite orientation to P_{lac}, were found to exhibit levels of resistance comparable to those displayed by the staphylococcal isolates from which the determinants were originally derived (data not shown). Since the cloned 863-bp *Eco*RI-*Eco*RV fragments presumably contain all of the sequences associated with *dfrA* translation, these findings taken together suggest that the observed Tp^r_H and Tp^r_L in *E. coli*, and probably in the original staphylococcal isolates also, results largely from transcriptional rather than translational differences.

Transcriptional start point for *thyE–dfrA–orf-140* on Tn*4003*. As a prelude to examining the transcriptional activities of the various *dfrA* promoters from the plasmids under study, S1 nuclease mapping was used to confirm the location of the promoter believed to be responsible for transcription of the *dfrA* gene within Tn*4003*. An 826-bp *Bgl*II-*Hpa*II fragment (Fig. 1) from Tn*4003* was 5′ end-labelled at the *Hpa*II site with [γ-^{32}P]dATP and hybridized to RNA isolated from plasmid-free *S. aureus* SK982 or the pSK1-containing SK982 derivative SK2267. After hybridization, the nonprotected ends of the labelled DNA were digested with S1 nuclease and the resulting products were fractionated on a 5% polyacrylamide–8 M urea DNA sequencing gel. A dideoxy chain termination sequencing reaction performed on a single-stranded M13 template was coelectrophoresed as a size standard. The resulting autoradiograph is shown in Fig. 2. A short ladder of bands corresponding to protected fragments centered at around 121 nucleotides is apparent only in samples which had been hybridized to RNA from cells harboring pSK1 (Fig. 2, lane 1). The most intense band in the ladder corresponded to a tsp at position 843 of the published sequence of Tn*4003* (40). Promoter sequences corresponding to this tsp include an outwardly directed −35 sequence in the terminal inverted repeat of IS*257*L and a −10 sequence within the adjacent central region of Tn*4003* upstream of *thyE* (Fig. 3 and 4); these sequences coincide with the proposed *dfrA* promoter (40).

Differences in levels of Tp^r are due to variations in promoter regions of *dfrA*. As described above, the variation in the levels of Tp^r conferred by the plasmids pSK1, pJE1, pSK639, pSK697, and pSK818 appears to be due to differences at the level of transcription. For this reason, nucleotide sequencing of the regions of pJE1, pSK639, pSK697, and pSK818 analogous to the promoter region of Tn*4003* was undertaken. The nucleotide sequence data demonstrated that the promoter sequences from plasmids pSK1 and pSK818, which both encode Tp^r_H, were identical and that the putative promoter regions from plasmids associated with Tp^r_L (pJE1, pSK639, and pSK697) differed not only from those encoding Tp^r_H but also from each other. All of the sequence differences detected represent deletions extending various distances from the rightward terminus of IS*257*L into the central region of the Tn*4003*-like elements. pJE1, pSK639, and pSK697 were found to have suffered deletions of 11, 32, and 286 bp, respectively (Fig. 4). Examination of the resulting sequences revealed that these deletions appear to have resulted in the pairing of the outward facing −35 promoter sequence located within IS*257*L with alternative −10 sequences (Fig. 3). In pJE1, for example, a −10-like sequence, TAATAT, is evident 18 bp downstream of the −35 sequence (Fig. 3). These putative alternative −10 regions differ from those of Tn*4003* in sequence and/or distance from the −35 sequence (Fig. 3). The PCR amplification products of each promoter region were derived from the original staphylococcal plasmids by using primers 1 and 2 (Materials and Methods). DNA sequencing of these PCR products confirmed that the deletions identified are actually present on these staphylococcal plasmids (pJE1, pSK639, and pSK697) rather than artifacts in our recombinant plasmids caused by cloning these segments into *E. coli*.

To establish that the variation in promoter sequences resulted in differential transcription of the *dfrA* gene, homologous segments representing each sequence type were amplified by PCR and cloned upstream of the promoterless *galK* gene of

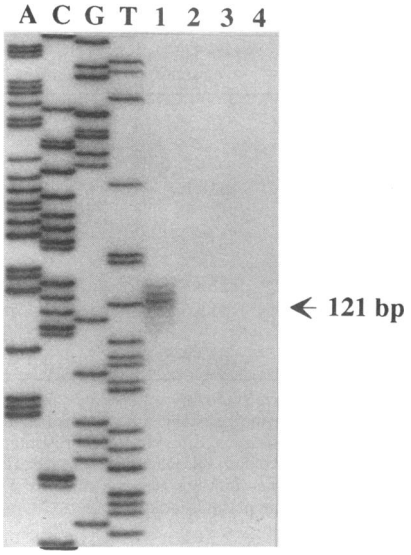

FIG. 2. S1 nuclease mapping of the Tp^r determinant tsp. Autoradiograph showing S1 nuclease protected fragments fractionated on a 5% polyacrylamide–8 M urea sequencing gel. A sequence ladder (lanes A, C, G, and T) derived from an M13 template was coelectrophoresed to enable size determination. An end-labelled 826-bp *Bgl*II-*Hpa*II fragment of Tn*4003* (Fig. 1), which includes most of IS*257*L and the junction of this element with the central region of the transposon, was hybridized to RNA isolated from the pSK1-carrying host SK2267 (lane 1), SK982 (lane 2), and no RNA (lane 3), respectively. Lane 4 contained untreated end-labelled fragment. The size (in base pairs) of the predominant fragment protected in lane 1 is indicated on the right.

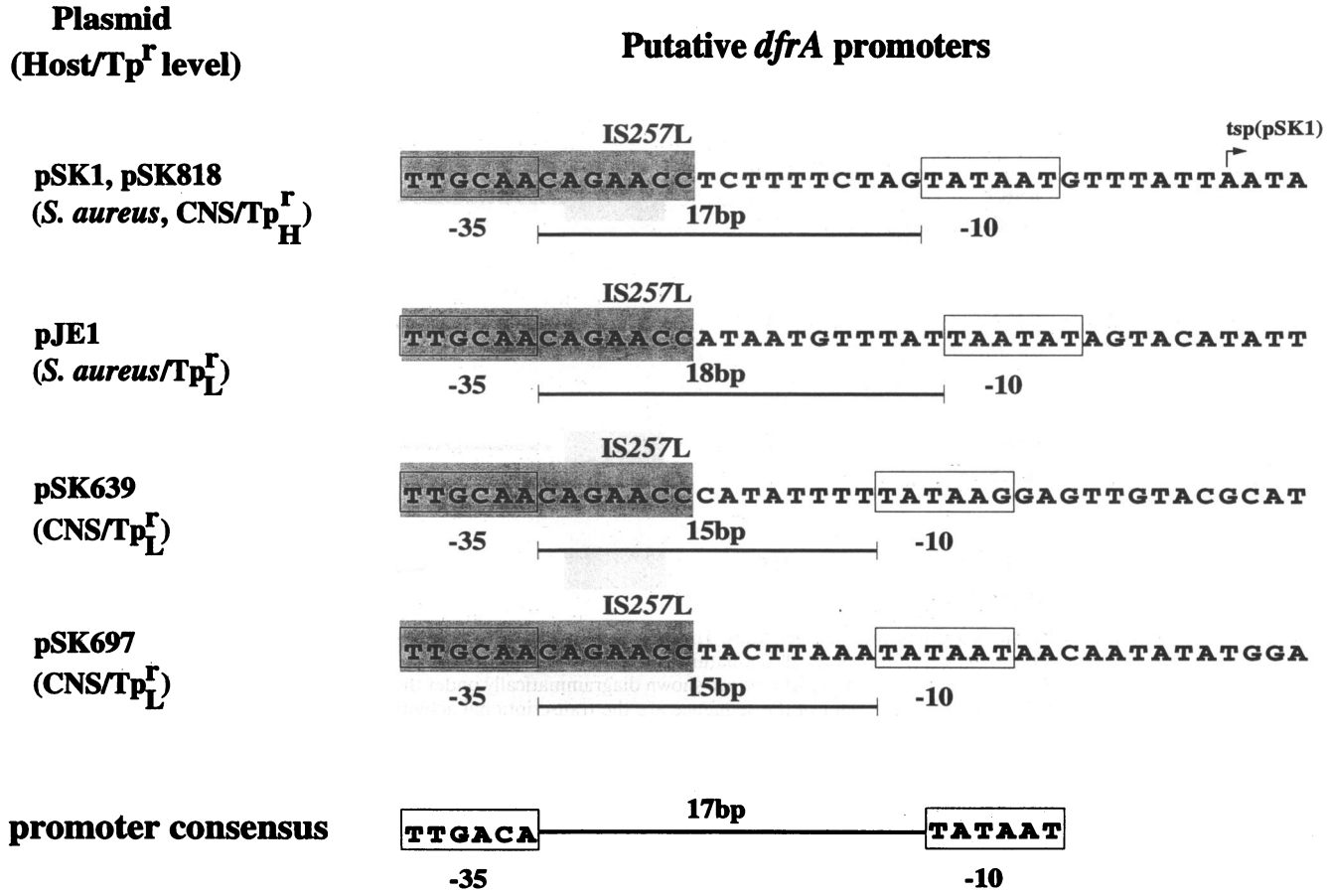

FIG. 3. Putative promoters of the Tp^r *dfrA* determinants. Predicted promoter regions are aligned with respect to the right-hand end of IS*257*L, which is shaded. Sequence homologies, corresponding to −35 and −10 promoter consensus sequences (shown at bottom), are boxed. The respective plasmid names are shown at the left along with their natural host organism and associated Tp^r phenotype. The spacing between the −35 and −10 promoter consensus sequences is indicated below each putative promoter. The tsp mapped for the Tn*4003 thyE–dfrA–orf-140* operon on pSK1 is indicated. CNS, coagulase-negative staphylococci.

*Bam*HI-*Eco*RI-digested pKO500 promoter-probe vector DNA (38). Because of the deletions present in the promoter regions of the Tp^r_L plasmids, the fragments amplified and cloned from pSK1, pJE1, pSK639, and pSK697 were 544, 533, 512, and 258 bp in size, respectively. Transformation of these recombinant plasmids, designated pSK4759, pSK4760, pSK4761, and pSK4763, respectively (Fig. 4), into the *galK* mutant host *E. coli* CB1 and subsequent galactokinase (GalK) assays enabled the determination of the relative promoter activities encoded within the cloned segments. The GalK activities obtained for each clone are shown in Fig. 4, alongside a diagrammatic representation of the staphylococcal DNA present in each plasmid. The activity measured for the pSK1-derived promoter region was found to be approximately fivefold greater than that of any of the equivalent regions derived from Tp^r_L-encoding plasmids. Although these experiments were not performed in the natural host, these findings strongly support the contention that the Tp^r_L phenotypes of pJE1, pSK639, and pSK697 reflect reduced transcriptional activity.

DISCUSSION

We established that Tp^r_H and Tp^r_L can be mediated by Tn*4003*-like elements located on plasmids in both *S. aureus* and coagulase-negative staphylococci, consistent with the notion that such elements have moved between these groups of organisms. Furthermore, plasmid-encoded Tn*4003*-like elements have now been detected in staphylococcal isolates from Australia (7), Europe (4), and North America (16). The spread of antibiotic resistance genes and plasmids among staphylococci is believed to be mediated by mechanisms such as conjugative transfer (13, 33) and mobilization (36); with regard to Tp^r, Tn*4003*-like elements have been detected on conjugative plasmids such as pGO1 (8) and pJE1 (7).

When examined, Tp^r in staphylococci has been found to be attributable exclusively to an IS*257*-associated *dfrA* determinant, implicating this element in the initial capture of a chromosomal gene encoding a Tp^r dihydrofolate reductase (DHFR). The genetic organization of the sequences flanking *dfrA* on Tn*4003*-like elements indicate that this determinant is not derived from the Tp^s DHFR *S. aureus* chromosomal gene *dfrB* (9, 40) but, rather, points to an origin in the *Bacillus* lineage (40). IS*257*, also referred to as IS*431* (2), is additionally found in staphylococci in association with plasmid and/or chromosomal genes encoding resistance to various antimicrobial agents, such as aminoglycosides (5, 6), quaternary ammonium compounds (5), tetracycline, methicillin, and heavy metals (17, 30), virginiamycin (11), and mupirocin (10, 34). Interestingly, copies of IS*257* also flank the *tra* regions of conjugative *S. aureus* plasmids (7, 13, 33). Clearly, IS*257* has

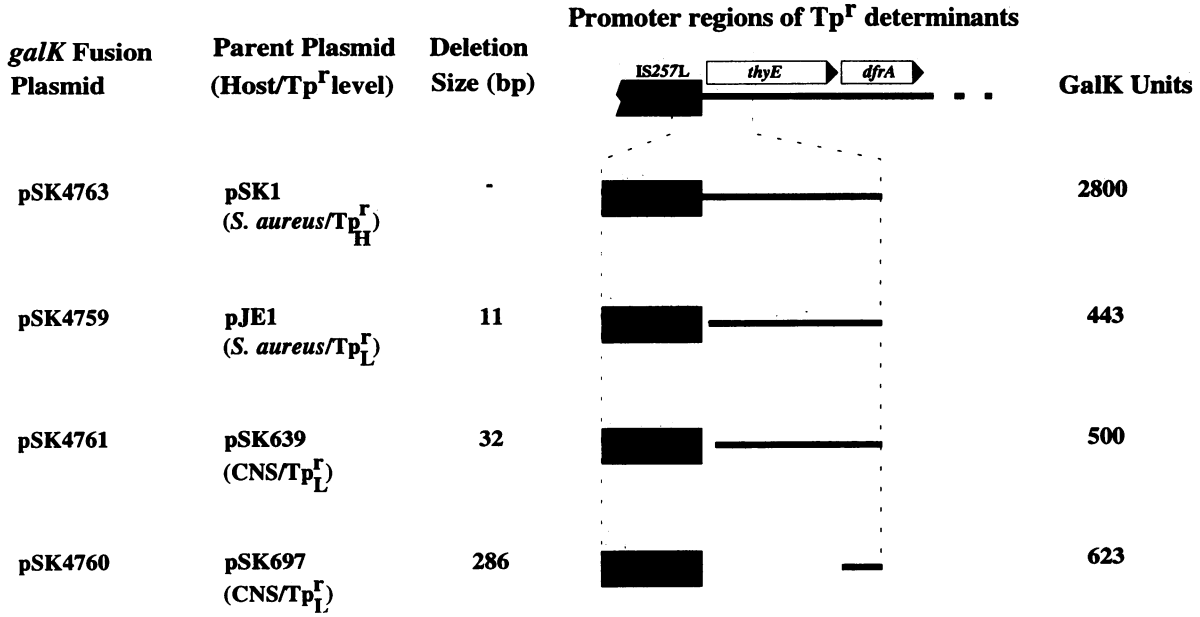

FIG. 4. *galK* transcriptional fusion plasmids and their activities. The name of each promoter region-pKO500 fusion plasmid is shown on the left, as is the respective parent plasmid from which it is derived, the natural host organism, the associated Tp^r phenotype, and the size of the deletion immediately flanking IS*257*L. The segments cloned into pKO500 are shown diagrammatically under the relevant portion of the Tn*4003* genetic map as described in the legend to Fig. 1. Shown at the right of the sequence are the transcriptional activities associated with each promoter region, as indicated by the measured galactokinase activities expressed by cells harboring the fusion plasmids. Galactokinase (GalK) units are expressed as nanomoles of galactose phosphorylated per minute per milliliter of cells at an optical density at 650 nm of 1.0. The sequence of the promoter region of pSK818 is identical to that of pSK1, and its activity was therefore not tested. Results are averages of at least three independent experiments. For comparison, the activities obtained for pKO500 (no promoter) and pKL500 (P_{lac}) were 46 and 1,700 galactokinase units, respectively.

played a significant role in the evolution and spread of staphylococcal antimicrobial resistance in general.

In addition to mediating the dissemination of Tp^r, the data presented here also highlight the involvement of IS*257* in the expression of this resistance and in the generation of flanking deletions that lead to the variations in observed resistance levels. Transcriptional analyses indicate that the promoter for the expression of the *dfrA* resistance gene in Tn*4003* involves IS*257* and the central-region sequences immediately adjacent to it. Furthermore, the variation in the levels of Tp^r encoded by Tn*4003*-like elements described here appears to be a result of sequence deletions adjacent to IS*257* in these elements, thereby implicating IS*257* in the generation of these deletions. The Tp^r phenotypes resulting from the deletions provide the first physical evidence that the putative promoter proposed by Rouch et al. (40), which we have now mapped, is indeed responsible for transcription of the *thyE–dfrA–orf-140* operon on Tn*4003*.

The generation of adjacent deletions is a property exhibited by a number of transposable elements (15). The mechanistic basis of the sequence deletions adjacent to IS*257* evident in the Tn*4003*-like elements encoding Tp^r_L is unknown. However, several models have been proposed to account for such deletions flanking various elements, viz., intramolecular cointegrate resolution (1, 43), nonreplicative transposition involving element ends located on sister episomes (24, 39), and aberrant transposition (21, 24, 39). In any event, DNA strand scission at the boundary of the IS*257* terminal inverted repeat, mediated by IS*257* transposase, is likely to represent a stage of the process. Examination of sequences around the deletion endpoints failed to identify any obvious commonality, other than the fact that each is followed by a sequence resembling a −10 promoter sequence. This probably reflects the high A+T content of staphylococcal DNA rather than any deletion-associated specificity. Reinforcing the potential significance of IS*257*-mediated deletions, Inglis et al. (20) and Nahvi et al. (35) have found that chromosomal deletions (greater than 60 and 1 kb, respectively) also appear to be associated with copies of IS*257*.

Several insertion sequence elements, including IS*1*, IS*2*, IS*4*, IS*5*, IS*6*, IS*26*, IS*30*, and IS*140*, carry potential outwardly directed −35 promoter sequences within their ends and so may promote expression of flanking genes by the formation of hybrid promoters (15, 23). The mechanism by which the elements present on pJE1, pSK639, and pSK697 have presumably derived from Tn*4003* appears to resemble the evolution proposed for the antibiotic resistance (IAB) operon of pBWH77 from *Klebsiella pneumoniae* (23). The IAB operon contains the *aphA7* and *blaS2A* genes encoding aminoglycoside-3′,5″-phosphotransferase-I and β-lactamase (SHV-2A), respectively. Similar to our findings, a deletion of sequences flanking an insertion sequence has apparently resulted in the formation of a modified promoter, of which the −35 sequence is encoded by IS*26*. However, in contrast to the situation with the IAB operon, which represents a modification of an existing promoter (with the original −10 sequence and tsp presumably being used), the deletions which have occurred on pJE1, pSK639, and pSK697 appear to have resulted in the utilization of alternative −10 sequences. Furthermore, the sequence alterations in this case have resulted in lower rather than higher levels of transcription. Although the deletions detected in pJE1, pSK639, and pSK697 appear to have resulted in the formation of new hybrid promoters for *dfrA* (Fig. 3), the possibility that the low-level Tp^r expressed by cells harboring these plasmids may be due to transcription initiation at other promoters, either within IS*257* or the *thyE* gene, cannot be

ruled out at this time. However, the *galK* fusion data indicate that the promoter(s) responsible is likely to be located within the fragments that we cloned into pKO500.

Rouch et al. (40) suggested that the *thyE* gene present on Tn*4003* may facilitate nonantimicrobial selection for the transposon, because it was found previously that *thy* mutations, which result in a moderate level of resistance to trimethoprim, could be selected by that agent (3, 44). It is therefore intriguing that two of the plasmids reported here represent natural *thyE* deletion mutants; the transcript from pSK639 would not contain the ribosome-binding site for this gene, and the deletion in pSK697 extends into the *thyE* open reading frame itself. The significance of the fact that these mutant types were detected only in coagulase-negative staphylococcal isolates so far is unknown. Although the *thyE* gene has been shown previously to be nonessential for Tp^r (29), the importance of its translation on the level of expression of the downstream *dfrA* gene cannot be determined from the results of the experiments reported here. It is therefore possible that the lower levels of resistance expressed by pSK639 and pSK697 may be due to a combination of both lower transcriptional and translational efficiencies. However, the fact that these plasmids do express Tp^r, albeit at comparatively low levels, suggests that translation of *dfrA* is not tightly coupled to that of the upstream *thyE* gene.

The differences in the Tn*4003*-like elements described here give an insight into their evolution, and perhaps into the evolution of composite transposons in general. It is tempting to speculate that flanking deletions of the type described here, mediated by IS*257*, result not only in the evolution of more compact composite transposons but also provide a means of modulating the expression of the downstream genes carried by that element. In the cases of pJE1, pSK639, and pSK697, it is possible that elements expressing Tp^r_L may have become established in the population of their respective isolates as a consequence of a selective advantage over a higher-level-encoding progenitor (i.e., Tn*4003*), perhaps because of a lower metabolic overhead, while still encoding a sufficient level of trimethoprim resistance for survival in a clinical environment.

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