Plasmid-Encoded Trimethoprim Resistance in Staphylococci

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High-level (≥1,000 μg/ml) resistance to the antimicrobial agent trimethoprim was found in 17 of 101 (17%) coagulase-negative staphylococci and 5 of 51 (10%) Staphylococcus aureus from a number of different hospitals in the United States. Resistance was plasmid encoded and could be transferred by conjugation in 4 of the 17 (24%) Tp' coagulase-negative staphylococci and 3 of the 5 (60%) Tp' S. aureus. A 1.2-kilobase segment of plasmid DNA from one of the plasmids (pG01) was cloned on a high-copy-number vector in Escherichia coli and expressed high-level Tp' (MIC, 1.025 μg/ml) in the gram-negative host. In situ filter hybridization demonstrated homology between the cloned Tp' gene probe and plasmid DNA from each conjugative Tp' plasmid, a single nonconjugative plasmid from a United States Staphylococcus epidermidis isolate, a nonconjugative plasmid from an Australian methicillin-resistant S. aureus isolate, and chromosomal DNA from three Tp' S. epidermidis isolates that did not contain any plasmid DNA that was homologous with the probe. No homology was seen between the probe and staphylococcal plasmids not mediating Tp', plasmid DNA from 12 Tp' S. epidermidis isolates not transferring Tp' by conjugation, or plasmid-encoded Tp' genes derived from gram-negative bacteria. Plasmid-encoded Tp' appears to be a relatively new gene in staphylococci and, because it can be transferred by conjugation, could become more prevalent in nosocomial isolates.

Resistance among staphylococci to trimethoprim, an inhibitor of bacterial folic acid synthesis, is uncommon; and this antimicrobial agent, alone or in combination with sulfamethoxazole, has been useful for the therapy and prevention of staphylococcal infections. It is particularly efficacious in the treatment of Staphylococcus aureus meningitis (11), in the treatment of infections caused by methicillin-resistant S. aureus isolates that are usually resistant to multiple antistaphylococcal antibiotics (N. Markowitz, L. Saravolatz, D. Pohlod, S. Salo, E. Quinn, M. Somerville, R. del Busto, J. Cardenas, M. Ruthod, and E. Fisher, Progr. Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother. abstr. no. 638, 1983), and in the eradication of the methicillin-resistant S. aureus carrier state among patients and hospital personnel (24). Recently, plasmid-mediated Tp' has been reported in methicillin-resistant S. aureus isolates from Australia (22, 23), but it has not yet been described among staphylococcal isolates from the United States, nor has the nature of the resistance determinant been characterized in any detail. Furthermore, neither plasmid-mediated nor transmissible Tp' has been described in coagulase-negative staphylococci. Multiply resistant coagulase-negative staphylococci found in hospitalized patients are postulated to serve as genetic reservoirs for some antimicrobial resistance determinants that later appear in S. aureus (2, 8). In this report, we describe homologous Tp' genes found on conjugative plasmids in geographically diverse wild-type S. aureus and Staphylococcus epidermidis isolates.

MATERIALS AND METHODS

Isolates. All wild-type staphylococci were isolates of clinical and epidemiological significance obtained during the course of studies conducted between 1978 and 1985. The coagulase-negative staphylococci were from patients with prosthetic valve endocarditis, intravenous catheter-related sepsis, cerebrospinal fluid shunt infections, and median sternotomy wound infections. The isolates came from 12 different geographic areas in the United States and Canada. The species of coagulase-negative staphylococci were determined with the Staph-Ident strip (Analytab Products, Plainview, N.Y.). The S. aureus were either methicillin-susceptible isolates from deep infections or methicillin-resistant isolates from patients or hospital staff colonized or infected during outbreaks. These S. aureus isolates came from six different geographic areas in the United States. In Table 1 is listed and described the plasmids, wild-type isolates, and well-defined bacterial strains used in this study.

Susceptibility testing. Susceptibility of isolates to trimethoprim and sulfamethoxazole was determined both on agar and in broth, with and without the addition of 5% lysed horse blood to the medium. Lysed horse blood contains thymidine phosphorylase (Harper-Cawston factor), an enzyme that converts thymidine to thymine (6). The presence of thymidine in the medium bypasses the trimethoprim-induced inhibition of dihydrofolate reductase (DHFR) and causes susceptible isolates to appear to be falsely resistant. An agar dilution susceptibility test was performed on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) by standard methods (25). Broth dilution was performed in microtiter trays with Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) with an inoculum of 105 CFU/ml and by standard methodology (9). Selected isolates determined to be Tp' also were evaluated by measuring timed kill activity in Mueller-Hinton broth-lysed horse blood. Trimethoprim (10 μg/ml) was added to log-phase bacteria and incubated at 37°C, and samples were removed for viable counts hourly for 8 hours.

All selection for resistance during mating and cloning experiments was performed on Mueller-Hinton agar containing 20 μg of trimethoprim per ml. Determination of susceptibility to gentamicin sulfate (Schering Corp., Bloomfield, N.J.) and selection of resistant isolates on agar was performed as described previously (2). Resistance to ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) and alkyltrimethyl ammonium bromide (Sigma) were determined by disc diffusion as described previously (2, 23). The latter

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### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant chromosomal phenotype</th>
<th>Plasmids</th>
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<tr>
<td><strong>S. aureus</strong></td>
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<td></td>
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<tr>
<td>RN450</td>
<td>Nov' Rif'</td>
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<tr>
<td>RN2677</td>
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<tr>
<td>RN27</td>
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<tr>
<td>G1</td>
<td>Mec'</td>
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<tr>
<td>G5</td>
<td>pG005</td>
<td>EthBr' Gm' Qam' Tp' Tra'</td>
</tr>
<tr>
<td>G106</td>
<td>pG0106</td>
<td>EthBr' Gm' Qam' Tp' Tra'</td>
</tr>
<tr>
<td>G105</td>
<td>pG01Δ5A</td>
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</tr>
<tr>
<td>WG525</td>
<td>Mec'</td>
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<tr>
<td></td>
<td>pWG53</td>
<td>EthBr' Gm' Qam' Tp' Tra'</td>
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<td></td>
</tr>
<tr>
<td>G2</td>
<td>Mec' Rif'</td>
<td></td>
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<tr>
<td>G101</td>
<td>Mec'</td>
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<td>pG002</td>
<td>EthBr' Gm' Qam' Tp' Tra'</td>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>SK1592</td>
<td>hsdR4 Gal'</td>
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<tr>
<td>D1204</td>
<td>pOP203(A2')</td>
<td>Te' A2' lac'p0°z' (carried by the F lac exogenote)</td>
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<tr>
<td>P17</td>
<td>pF6E06</td>
<td>Tp'</td>
</tr>
<tr>
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<td>Ap' Te'</td>
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<tr>
<td>RUE10</td>
<td>pER1</td>
<td>Tc'</td>
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**Relevant phenotypes**

- **DNA**
- **lif**
- **lif**
- **tra**
- **tra**
- **tra**
- **tra**
- **tra**
- **tra**

**Size (kb)**

- 50.0
- 51.1
- 51.1
- 41.0
- 15.1 (6.0)
- 7.0
- 7.5
- 16.3
- 15.7
- 7.7
- 20.0
- 4.4
- 11.6

**Remarks or reference**

- Restriction-deficient Δ1 lysogen of 8325-4 (17)
- 8325-4 lysogenized with 680A
- Wild-type isolate; MCV; one plasmid
- Wild-type isolate, Philadelphia VAH (E. Abrutyn); two plasmids
- Wild-type isolate; MCV; one plasmid
- Transduction deletion (20 kb) of pG01
- Wild-type isolate; Australia (W. Grubb; 23); one plasmid
- Wild-type isolate; MCV (G. Archer [1]; six plasmids)
- Restriction-deficient deficient for transformation of recombinant plasmids (10)
- Host strain for pOP203(A2') with lac repressor; plasmid is positive selection cloning vector (Winter and Gold [26])
- S. aureus HB101 with plasmid encoding type I DHFR (Elwell [5])
- E. coli HB101 with plasmid encoding type II DHFR (Elwell [5])
- SK1592 containing 8.7-kb EcoRI fragment from pG01Δ5A with Tp' gene cloned on pOP203(A2')
- Subclone of pG011 in SK1592; Tp' gene probe
- E. coli HB101 with plasmid containing complete Tn7-encoding type I DHFR (Shipley)
- Cloning vector in SK1592
- B. subtilis thyB and dfrA genes cloned on an E. coli vector (Mayoda [15])

**Antimicrob. Agents Chemother.**

* Abbreviations: bla, beta-lactamase; Em, erythromycin; EthBr, ethidium bromide; Gmr, gentamicin; Kan, kanamycin; Omp, tricarboxylic; Mec, methicillin; Nov, novobiocin, Qam, quaternary ammonium compounds; Rif, rifampin; Sm, sulfanilamide; Tc, tetracycline; Tra, transconjugative transfer.

* Names in parentheses are the individuals who donated the strains and the number is the literature citation. Abbreviations; MCV, Medical College of Virginia Hospital; MGH, Massachusetts General Hospital; Philadelphia VAH, Philadelphia Veterans Administration Hospital; UAB, University of Alabama Hospitals. These are the hospitals from which wild-type isolates were initially recovered.

* The smaller 6.0-kb plasmid in parentheses always transferred with the larger 15.1-kb plasmid and both hybridized with the Tp' gene probe.

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Compound was used to assess resistance to quaternary ammonium compounds (Qam'). Methicillin resistance was defined as growth of 0.1 ml of an overnight broth culture on agar containing 12.5 μg of methicillin sodium per ml after 72 h of incubation at 30°C.

**Filter mating.** Donor and recipient staphylococci were grown overnight at 37°C in brain heart infusion broth (BBL), combined in a syringe, and collected on filters (pore size, 0.45 μm) as described previously (2). Filters were placed upright on nonselective agar plates and incubated for 24 h at 37°C. Bacteria were removed from filters by vortexing and plating on appropriate selective agar.

**Extraction and examination of DNA.** The presence of plasmid DNA in donor and recipient bacteria was confirmed by either of two procedures. Coagulase-negative staphylococci were treated with lysostaphin to damage the cell wall and lysed with sodium dodecyl sulfate. Whole-cell lysates were subsequently examined (4). *S. aureus* was treated with lysostaphin and lysed in a hypotonic buffer; plasmid DNA was separated from chromosomal DNA and cellular debris by centrifugation (3). Plasmid DNA was identified by electrophoresing minilysates through 0.7% agarose, using a vertical slab gel apparatus, and transilluminating ethidium bromide-stained gels with UV light (4).
Purified staphylococcal plasmid DNA was obtained for restriction endonuclease analysis and molecular cloning by cesium chloride-ethidium bromide density gradient centrifugation as described previously (4). Chromosomal DNA was extracted from *Staphylococcus aureus* and coagulase-negative staphylococci by the method of Pattee and Neveln (18). Restriction endonuclease digestion of purified DNA was performed according to the specifications of the manufacturer. Restriction endonuclease maps of plasmids were generated by examining plasmid DNA that was digested with one or two enzymes after the DNA was electrophoresed through agarose that ranged in concentration from 0.7 to 1.2%, usually on a horizontal minigel apparatus (Ann Arbor Plastics, Ann Arbor, Mich.). The location of cleavage sites was confirmed by restriction endonuclease digestion of cloned fragments (see below). Fragments from 100 to 1,000 kilobases (kb) in molecular size were resolved from cloned fragments by electrophoresis through 5% polyacrylamide. The following enzymes were used for mapping and analysis: EcoRI, HindIII, BglII, BamHI, and PstI (all enzymes were from Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Deletions of plasmid DNA were constructed by transductional shortening with generalized transducing phages φ11 or φ80a with strains RN2677 or RN27, respectively, as appropriate lysogenized recipients (2).

**Construction of plasmid maps.** Physical plasmid maps were constructed by restriction endonuclease digestion of the original plasmids and cloned fragments as described above. The location of phenotypic markers was determined as follows. The gentamicin resistance genes were located by transposon inactivation and analysis of deletion derivatives as reported previously (1). We could not reliably detect expression of gentamicin resistance on cloned fragments in *Escherichia coli* SK1592, however. Genes encoding resistance to ethidium bromide and alkytrimethyl ammonium bromide were located by analysis of plasmids with deletions. The gene encoding penicillin G resistance was located on cloned fragments by detecting beta-lactamase production in *E. coli*. Finally, the genes for conjugative transfer were localized to a region of the plasmid by analysis of deleted plasmids and plasmids containing transposon insertions that were conjugation defective.

**Cloning of staphylococcal DNA.** The gene(s) for Tp'' was cloned from staphylococcal plasmid DNA onto a multicyclic *E. coli* vector. The vector, pOP203(A'''), engineered by Winter and Gold (26), incorporates the A₂ gene from φ8 phage on a ColE1-derived replicon. The A₂ gene product, under control of the lac promoter-operator, produces a maturation protein that results in lysis of the bacterial cell unless it is interrupted by a fragment of foreign DNA cloned into one of several unique sites (e.g., EcoRI, BglIII, Sp6I, or XhoI). By using this positive selection vector, all EcoRI fragments of staphylococcal plasmid DNA could be cloned for both fine structure mapping and analysis of phenotypic markers. Ligation was performed according to the specifications of the ligase manufacturer (International Biotechnologies, Inc., New Haven, Conn.) with 0.1 U of T4 DNA ligase per reaction. After overnight incubation at 4°C, recombinant DNA was transformed into appropriate *E. coli* recipients that were made competent by treating with CaCl₂ by standard techniques (13). After 3 h of incubation of transformed cells at 37°C in L broth, the cells were plated on media containing isopropyl-β-D-thiogalactopyranoside to induce the lac promoter-operator and either 20 μg of tetracycline per ml, to select for the presence of the vector plasmid or tetracycline plus 20 μg of trimethoprim per ml, to directly select for the cloned gene. Clones were screened by a minilysis procedure, and DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation with chloramphenicol amplification (13). Subcloning to other *E. coli*-based vectors was accomplished by the same cloning procedure.

**In situ filter hybridization.** DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (21). Gels were depurinated in 0.25 M HCl for 8 min before they were denatured in 0.5 M NaOH–1.5 M NaCl. Filters were baked for 3 h in vacuo at 80°C and prehybridized with denatured salmon sperm DNA.

Probe DNA was from one of two sources. Staphylococcal DNA was probed with cloned staphylococcal DNA plus its *E. coli* vector, while *E. coli* DNA was probed with a restriction fragment from a staphylococcal plasmid. The staphylococcal plasmid restriction fragment was excised from an agarose gel and electroeluted from agarose into buffer with a D-GEL apparatus (EpiGene, Baltimore, Md.). All probes were radiolabeled with [³²P]dCTP by nick translation according to the specifications of the manufacturer (New England Nuclear Corp., Boston, Mass.). Probe DNA was denatured prior to hybridization by heating for 5 min at 60°C after the addition of 50% formamide.

Hybridization was performed under conditions of high and low stringency, whenever appropriate. For conditions of high stringency, probe and immobilized target DNA were hybridized for 24 h at 42°C in 50% formamide–Denhardt reagent, washed first in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM phosphate buffer, 1 mM EDTA) and then in 0.1× SSPE, both of which were carried out at room temperature. For conditions of low stringency, the concentration of formamide was reduced to 25% (14). Hybridized filters were exposed to x-ray film at −70°C for a sufficient amount of time to produce an acceptable autoradiograph.

The autoradiographs shown in Fig. 3 and 4 are representative of hybridization experiments. Each target was probed at least three separate times. In each experiment new gels were run and probes were freshly radiolabeled.

**RESULTS**

**Trimethoprim resistance in wild-type isolates and resistance transfer.** A total of 51 *S. aureus*, 21 (41%) of which were methicillin resistant, and 101 coagulase-negative staphylococci, 80 (80%) of which were methicillin resistant, were analyzed for trimethoprim resistance by agar dilution, with the agar containing 10 μg of trimethoprim per ml and 5% lysed horse blood. Resistance to trimethoprim was found in 5 of 51 (10%) *S. aureus* and 17 of 101 (17%) coagulase-negative staphylococcal isolates. All 17 of the *Tp''* coagulase-negative staphylococci were identified as *S. epidermidis*. Of the 5 (10%) *Tp''* *S. aureus* isolates, three were methicillin resistant and two were methicillin susceptible. Three of the five (60%) *Tp''* *S. aureus* (one methicillin resistant; two methicillin susceptible) transferred *Tp''* to *S. aureus* recipient RN2677 (Table 3) after filter mating. Two of the three conjugally proficient *S. aureus* isolates has been recovered during epidemiological investigations. Isolate G5 was responsible for a hospital-wide methicillin-resistant *S. aureus* outbreak at the Philadelphia Veterans Administration Hospital, while isolate G106 was a methicillin-susceptible *S. aureus* isolate that caused an outbreak of infections in the Medical College of Virginia neonatal intensive care unit. Conjugal transfer of *Tp''* was demonstrated in 4 of the 17 (24%) *Tp''* *S. epidermidis* isolates. *S. epidermidis* isolates...
that transferred Tp' to RN2677 were isolates that caused infective endocarditis, three on prosthetic valves and one on a native valve, and were recovered at three different hospitals (Table 1).

Five of the wild-type isolates (two S. aureus; three S. epidermidis) transferred a single 40- to 50-kb plasmid that encoded resistance to various combinations of the following compounds: gentamicin (tobramycin, kanamycin), penicillin G, and ethidium bromide-quaternary ammonium compounds (Table 1). S. aureus isolate G5 transferred to all transconjugants screened by gel electrophoresis a single 55-kb plasmid encoding all of the resistance markers. In 60% of transconjugants a second 30-kb cryptic plasmid was mobilized as well. S. epidermidis isolate G104 transferred two plasmids with molecular sizes of 15 and 6 kb to all Tp' transconjugants. A third 50-kb plasmid in the donor, encoding resistance to gentamicin (tobramycin, kanamycin) and ethidium bromide-quaternary ammonium compounds, transferred independently of the other two plasmids. This was ascertained by the fact that only this plasmid was found in transconjugants that exhibited these phenotypic markers. No other marker besides Tp' was identified in transconjugants containing only the two small plasmids. To confirm interspecies transfer of these conjugative plasmids, S. aureus transconjugants were mated with a plasmid-free wild-type S. epidermidis isolate (G111); all conjugative plasmids transferred from S. aureus to S. epidermidis.

All Tp' transconjugants were resistant to >1,000 μg/mL of the drug per mL but were sulfamethoxazole sensitive at the same MIC as the recipient S. aureus strain (12.5 μg/mL). Tp' transconjugants therefore were susceptible to the combination of trimethoprim-sulfamethoxazole at the sulfamethoxazole MIC (12.5 μg/mL), while the recipient without the plasmid was susceptible at the trimethoprim MIC (1 μg/mL). The trimethoprim susceptibility of staphylococcal isolates that were resistant to the drug but that did not transfer resistance was similar to that of isolates containing transmissible resistance (MIC, >1,000 μg/mL). Plasmid-mediated resistance of staphylococci to the bactericidal effect of trimethoprim was tested by performing experiments with transconjugants containing either pG01 or pG05 to obtain killing curves. The recipient, RN2677, was reduced in viable count from 10⁷ to 10⁴ CFU/mL with 10 μg/mL of trimethoprim per mL over 8 h, while the transconjugants grew as rapidly in broth with trimethoprim as without it. 

Cloning Tp' genes. Two plasmids, both from S. aureus, were chosen for further study. Restriction endonuclease maps of these two plasmids, pG01 and pG05 (Table 1), were mostly different, but there were some cleavage fragments of the same size common to the two plasmids that were in the same position relative to identified phenotypic markers (Fig. 1). Analysis of transduction-derived deletion derivatives and resistance mediated by cloned fragments (see below) confirmed different relative locations for the trimethoprim genes on the two plasmids. EcoRI digests of pG05 and a deletion derivative of pG01 (pG01Δ5A) were cloned into pOP203(A₂'), transformed into restriction-deficient E. coli recipient SK1592 (10), and selected on plates containing tetracycline and trimethoprim. All Tp' E. coli transformants contained either a 4.2-kb EcoRI fragment from pG05 or an 8.7-kb EcoRI fragment from pG01Δ5A. This 8.7-kb fragment was a deletion of the largest (15-kb) EcoRI fragment from pG01. It still contained the Gm' gene, but Qam' was deleted. All E. coli transformants were resistant to trimethoprim to the same degree as were staphylococci (MIC, >1,000 μg/mL).

The A₂ gene in the cloning vector is under the control of the lac promoter-operator, and therefore, the expression of the cloned trimethoprim gene may have been influenced by this strong promoter. To investigate this possibility two
experiments were performed. First, pG012, the clone containing the 4.2-kb fragment from pG05, was transformed into D1204, an E. coli strain containing a mutation of an F lac plasmid (lacP) so that it hyperproduces lac repressor (26). Tp' was expressed at the same level as it was in SK1592, in which the promoter was fully induced. Second, the gene was subcloned onto pBR322 and retained full expression of Tp'.

We therefore conclude that the cloned Tp' gene(s) contain their own promoter and that this staphylococcal promoter is functional in these E. coli hosts.

The clone containing the 8.7-kb fragment from pG01Δ5A (pG011) was reduced in size by excision of cloned DNA until a 1.2-kb EcoRI-BglII fragment remained that retained full Tp' expression on both pOP203(A2+) and pBR322. This construct, pG015, was used as the probe for investigation of DNA homology from Tp' staphylococci. The strategy for cloning is depicted in Fig. 2.

**Homology of the cloned probe with other DNA.** The entire vector plasmid plus the cloned staphylococcal Tp' gene(s) (pG015) were used to probe staphylococcal DNA. There was no homology of pOP203(A2+) DNA alone with any of the target staphylococcal DNA. The 4.2-kb pG05 EcoRI fragment containing the Tp' gene(s) was used to probe DNA of gram-negative origin or cloned on other E. coli vectors. Representative hybridization experiments are shown in Fig. 3 and 4. The pG015 probe gave strong hybridization signals with EcoRI restriction fragments of pG01 and its deletion derivative (pG01Δ5A), pG05, pG0101, pG0103, and pWG53 (a nonconjugative Tp' plasmid from an Australian methicillin-resistant S. aureus isolate [23]) but not with pG02, a conjugative Tp' plasmid with ~80% homology with pG01 (1). EcoRI restriction fragments from pG0102 and pG0106 were also homologous with the probe (data not shown), as was undigested plasmid DNA from pG0104 and pG0105 (Fig. 4). There was no homology of the 4.2-kb pG05 probe with any plasmids bearing genes encoding either type I or type II DHFR that are commonly found in members of the family Enterobacteriaceae (data not shown). Fainter signals were seen with some fragments of all plasmids (Fig. 3). These signals were due to homology of plasmid sequences with the 3' end of the probe, beyond the DNA encoding Tp' (J. Coughter, manuscript in preparation).

To investigate the possibility that the plasmid-borne staphylococcal Tp' gene had evolved from normal gram-positive DHFR genes by point mutation or gene duplication, we probed chromosomal DNA from Tp' S. aureus RN450 and from G11, a plasmid-free, Tp' S. epidermidis isolate, and plasmid DNA containing cloned genes from Bacillus subtilis encoding thymidine kinase and DHFR (15). There was no homology of the probe with DNA from any of these sources.

Finally, homology of probe DNA with DNA from the Tp' S. epidermidis isolates that did not transfer resistance by filter mating was investigated. First, gradient-purified plasmid DNA from 10 of the 13 S. epidermidis isolates was examined; plasmid DNA from G104 and G5 was included on the gel as a positive control. Only one of the 10 Tp' isolates exhibiting nontransmissible resistance had any plasmid DNA that hybridized with the pG015 probe, while both transmissible plasmid controls gave a positive signal (Fig. 4). In addition, EcoRI-digested chromosomal DNA from five Tp' isolates exhibiting nontransmissible Tp' were also probed, and hybridization occurred with chromosomal DNA from three of these isolates (data not shown).

**DISCUSSION**

The plasmid-encoded Tp' genes described in this report are medically important for several reasons. First, plasmid-associated Tp' has not previously been described among
Archibald and (on the right) the autoradiographs of the same restriction fragments after they were transferred to nitrocellulose and hybridized with the 32P-labeled 1.2-kb Tp probe (B). The numbers to the left are the molecular sizes (in kilobases) of four of the eight EcoRI restriction fragments of pGO1 (lane 1). The plasmids in each lane and the major fragments hybridizing with the probe are as follows: lanes 1, pG01, the largest fragment; lanes 2, pG01A, the largest fragment; lanes 3, pG05, the sixth largest fragment; lanes 4, pG02, no hybridization; lanes 5, pG0101, the third largest fragment; lanes 6, pG0103, the fifth largest fragment; lanes 7, pG53, the third largest fragment. Weaker major hybridization signals in some lanes compared with those in others was due to inefficient transfer to nitrocellulose or a smaller quantity of DNA in the gel. Minor hybridization signals in some lanes, in addition to the major signal, were due to DNA in the probe outside the trimethoprim gene that had homology with repeated plasmid DNA sequences (J. Coughter, manuscript in preparation).

Staphylococci isolated from hospitals in this country and has only recently been identified in Australia (22, 23). Even though Tp' S. aureus were relatively uncommon among a large number of clinical isolates that we screened (10%), the percentage of resistant isolates was higher than the 1.0 to 2.1% resistance frequency reported in earlier susceptibility surveys (12, 16, 20). In a recent survey from England (19), it was reported that while 16% of clinically significant S. epidermidis isolates from that country were highly Tp' (MIC ≥500 μg/ml), only 4% of epidemiologically independent isolates from northern Europe and North America were Tp'. This latter figure is in contrast to the 17% Tp' frequency found among S. epidermidis isolates in our study. Thus, high-level Tp' among both coagulase-positive and coagulase-negative staphylococci seems to be increasing in the United States. Because at least 30% of the Tp' in coagulase-negative staphylococci (5 of 17 isolates) and 60% in S. aureus (3 of 5 isolates) was plasmid-mediated, it can be suggested that the apparent increase in Tp' resistance in staphylococci may be due to the emergence of these plasmids. Second, seven of the eight Tp' plasmids were conjugative and could transfer interspecifically between S. epidermidis and S. aureus. This has obvious epidemiological consequences and implies that Tp' may increase in hospitals in which conjugative staphylococcal plasmids have been identified and transmissible resistance to other antimicrobial agents, particularly aminoglycosides, has become widespread (1, 2, 8). Five of the seven conjugative Tp' plasmids in our study also encoded gentamicin resistance. Two of the wild-type S. aureus isolates from which the Tp' plasmids were obtained (G5 and G106) were each representative of multiple isolates responsible for widespread outbreaks in the Philadelphia Veterans Administration Hospital and the Medical College of Virginia neonatal intensive care unit, respectively. The outbreak isolates in Philadelphia were multiresistant methicillin-resistant S. aureus, illustrating the ability of new, plasmid-specific resistance genes to compound the multiresistant phenotype and limit therapeutic options. Finally, the largest number of Tp' plasmids was found in S. epidermidis. This further argues for the propensity of this species to function as a reservoir for antibiotic resistance genes (2, 8).

We explored the origin of the plasmid-encoded Tp' genes by probing various DNAs with the gene. A fragment from each of the transmissible Tp' plasmids hybridized with the probe, and the homologous fragments were in various locations on their respective plasmid maps (Fig. 1 and 3). While this suggests that the resistance gene may be on a transpos-
able element, attempts to demonstrate transposition so far have been unsuccessful (unpublished data). The possibility that the resistance genes originated in the staphylococcal chromosome was suggested by hybridization of the probe with chromosomal fragments in three Tp' S. epidermidis isolates with no plasmid DNA homologous with the probe. These chromosomal genes are unlikely to represent mutations of normal DHFR genes because there was no hybridization of the probe with chromosomal DNA from Tp' S. aureus or S. epidermidis isolates. The nature of the chromosomal Tp' genes are currently under investigation.

Plasmid-mediated Tp' in gram-negative bacteria is due to the production of functional DHFR that has markedly decreased binding affinity for trimethoprim (5). The MICs of E. coli strains containing Tp' plasmids producing either type I or type II DHFR are very high, at least as high as the >1,000 μg/ml MIC exhibited by the staphylococcal genes that were cloned on a high-copy-number vector in E. coli (5). However, there was no homology between the cloned type I and type II genes from E. coli and our staphylococcal gene probe. A third type of DHFR, found in gram-negative bacteria (7), was not investigated with our probe, but its MICs (64 μg/ml) are lower than those seen for resistant staphylococcal isolates containing a Tp' plasmid or E. coli isolates containing the cloned staphylococcal gene. The Tp' genes in staphylococci therefore are not likely to be closely related to known plasmid-encoded Tp' genes from gram-negative bacteria.

There are two aspects of the data discussed above that deserve further comment. First, although the hybridization studies that we performed were done under conditions of both high and low stringency, short sequences of identical DNA may exist between staphylococcal and gram-negative Tp' genes and not show homology by in situ filter hybridization. Only determination of the exact DNA sequence will reveal similarities between the two genes. Second, the similarity of the MICs for Tp' staphylococci containing the plasmid-encoded resistance gene and E. coli containing the cloned gene does not necessarily indicate a common evolutionary origin for the gene in gram-negative and -positive species. The similarity may be due to the production of a large amount of gene product on a high-copy-number cloning vector in E. coli versus production of a smaller amount of gene product on a large, lower copy number plasmid in staphylococci. However, this does not diminish the potential usefulness of this resistance gene as a selection marker for shuttle vectors that can be used in both E. coli and staphylococci.

The plasmid-mediated Tp' gene appears to be relatively new in staphylococci. However, like transmissible aminoglycoside resistance plasmids in staphylococci, these genes appear to have arisen in a number of different geographic locations at close to the same time. Homologous Tp' genes that were resident in staphylococci isolated within the last 5 years were detected on plasmids from Australia and five different hospitals in the United States; none of our staphylococcal isolates received before 1980 contained the plasmid-associated gene (unpublished data). The study of this gene therefore gives us the unique opportunity to investigate the origins and dissemination of a new staphylococcal antibiotic resistance gene and the genetic construction of the multiresistant phenotype.

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