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Conjugative Transfer Genes in Staphylococcal Isolates from the United States

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Staphylococcus aureus **and coagulase-negative staphylococcal isolates from various geographic areas in the United States were examined by using a conjugative transfer gene DNA probe in dot-blot hybridization assays. Of 175 *S. aureus* isolates, 47 (27%) hybridized with the probe, while 24 of 208 (11.5%) coagulase-negative staphylococci hybridized. However, among methicillin-resistant *S. aureus* 52% (45 of 89) were probe positive while only 2% (2 of 86) of methicillin-susceptible *S. aureus* were probe positive. In contrast, 12.5% (22 of 176) of methicillin-resistant and 6% (2 of 32) of methicillin-susceptible coagulase-negative staphylococci contained transfer genes. All but one of the staphylococci containing transfer genes were resistant to gentamicin; 91.5% of *S. aureus* and 65% of coagulase-negative staphylococci containing transfer genes transferred gentamicin resistance to a *S. aureus* recipient. Of the 12 isolates that hybridized with the probe but did not transfer resistance, 10 (6 coagulase-negative staphylococci and 4 *S. aureus*) carried both gentamicin resistance and conjugative transfer genes on the same plasmid. Of these 10, 6 contained plasmid target fragments of sizes different from that of the probe, suggesting additions or deletions of DNA essential for transfer, while in 4 no such alterations could be detected. In two coagulase-negative staphylococci the entire transfer region was apparently integrated into the chromosome. Thus, staphylococci carrying conjugative transfer genes are widely disseminated in the United States and are usually found in multiresistant isolates on plasmids that also encode gentamicin resistance.**

Antimicrobial resistance has increased among nosocomial staphylococcal isolates in the United States during the past decade (17). The multiresistant phenotype that includes resistance to methicillin as well as resistance to three or more additional agents has become widespread, particularly among coagulase-negative staphylococci (7, 10, 17). The observation that a number of homologous genes, each mediating resistance to a different antimicrobial agent, have been found in the United States in both *Staphylococcus aureus* and in coagulase-negative staphylococci suggests interspecies transfer of these resistance determinants (5, 7, 8, 21). Two genetic systems capable of mediating cell-to-cell spread of resistance genes, conjugation and conjugative mobilization, have been described among staphylococcal isolates from the United States (14, 22). Large (40- to 60-kb) conjugative plasmids transferring resistance to aminoglycosides, trimethoprim, quarternary ammonium compounds, and penicillin and mobilizing transfer of plasmids encoding resistance to erythromycin, chloramphenicol, and tetracycline among different staphylococcal species have been found in nosocomial staphylococcal isolates associated with hospital outbreaks (2, 6, 8, 14, 18). However, the prevalence of these conjugative plasmids among routine staphylococcal isolates from hospitals in the United States has not been investigated. In the following study, we used a DNA probe to screen *S. aureus* and coagulase-negative staphylococci from multiple geographic sources in the United States for the presence of conjugative transfer genes. We then confirmed conjugative transfer of resistance genes, investigated probe-positive isolates that failed to transfer resistance, and associated the presence or absence of conjugative replicons with specific antimicrobial resistance phenotypes.

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MATERIALS AND METHODS

Bacterial isolates. The staphylococcal isolates examined in this study were selected from those previously described as targets for DNA probe analysis of methicillin resistance (5). Those isolates included both *S. aureus* (118 isolates) and coagulase-negative staphylococci (172 isolates) from the Medical College of Virginia and McGuire Veterans Administration Hospitals in Richmond, Va., methicillin-resistant *S. aureus* from three hospitals in New York City (8 isolates), and both *S. aureus* (49 isolates) and coagulase-negative staphylococci (36 isolates) sent from 26 different hospitals to the Centers for Disease Control over the past 10 years. Species identification of selected coagulase-negative staphylococci was performed by using the API Staphident system (Analytab Products, Plainview, N.Y.) and confirmed in ambiguous cases with biochemical analysis by the method of Kloos and Schleifer (12).

DNA probe analysis. The probe used to identify conjugative transfer gene sequences was the 6.2-kb *Eco*RI C fragment of pG01. This fragment is within the 14.5-kb conjugative transfer locus and contains sequences essential for plasmid transfer (22). A detailed map of this region has previously been published (22). The fragment was cloned in *Escherichia coli* on the Bluescript II vector (Stratagene, La Jolla, Calif.), and both vector and insert were included in probe DNA. The vector was shown not to hybridize with staphylococcal DNA. Crude lysates of each isolate were probed by using dot-blot hybridization with previously described techniques (5). The probe was labeled with a nonradioactive indicator (digoxigenin-dUTP, anti-digoxigenin

complexed with alkaline phosphatase; Genius, Boehringer Mannheim, Indianapolis, Ind.) for screening, and equivocal results were confirmed with a radioactive label (^{32}P) as previously described (5).

Bacterial mating. All probe-positive isolates were filter mated with novobiocin and rifampin-resistant recipient *S. aureus* RN422ONR by using a screening method in which donor and recipient were pelleted together in Eppendorf tubes and pipetted onto a 0.45-μm-pore-size nitrocellulose filter (22). Probe-positive isolates that yielded no transconjugants were mated again by using the more sensitive technique of forcing donor and recipient mixtures onto a filter with a syringe (3). Conjugative plasmid transfer was confirmed by selection of colonies on agar containing gentamicin, novobiocin, and rifampin (5 μg/ml each; all antibiotics were from Sigma, St. Louis, Mo.). Transfer of only gentamicin or trimethoprim resistance was sought. Cotransfer of unselected antimicrobial resistance phenotypes was not analyzed, nor was transfer of resistance from isolates that were probe negative.

DNA extraction and analysis. Plasmid DNA was extracted from transconjugants or wild-type isolates by a method using cetyltrimethylammonium bromide precipitation (24), or whole-cell lysates were generated as previously described (4); both were then examined by agarose gel electrophoresis. For more detailed mapping plasmid DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation. Chromosomal DNA was prepared by a variation of the Marmur technique (8). Purified plasmid or chromosomal DNA that had been cleaved with appropriate restriction endonucleases and transferred to nitrocellulose by the Southern technique was also used as a target for hybridization with the pG01 *Eco*RI C probe and, in selected isolates, a probe containing the opposite end of the pG01 conjugative transfer region (*Hin*dIII E, 4.6 kb [22]). Hybridization was performed under conditions of high stringency that included inclusion of 50% formamide in the hybridization solution, incubation of the hybridization mixture with target DNA overnight at 42°C, and washing at both low-salt concentration (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and at high temperature (55°C). These conditions have been previously described (7). All other techniques and conditions have either been previously described (8) or were performed by the method of Sambrook et al. (16).

Antimicrobial agent susceptibility testing. Susceptibility was determined by using a multipoint inoculator to transfer 0.001 ml from microtiter wells, each well containing approximately 10^7 CFU of bacteria per ml, to Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) containing the test antimicrobial agent. The antimicrobial agents tested and the concentration of each in agar were as follows (all antimicrobial agents were from Sigma): gentamicin (Gm), 5 and 100 μg/ml; kanamycin (Km), 10 and 500 μg/ml; amikacin (Ak), 8 μg/ml; neomycin (Nm), 10 and 500 μg/ml; streptomycin (Sm), 10 μg/ml; spectinomycin (Sp), 50 μg/ml; chloramphenicol (Cm), 16 μg/ml; clindamycin (Cl), 10 μg/ml; erythromycin (Em), 5 μg/ml; trimethoprim (Tp), 25 and 500 μg/ml; tetracycline (Tc), 5 μg/ml; ethidium bromide (Eb), 20 μg/ml; and cadmium nitrate (Cd), 5×10^{-5} M. Penicillinase production was determined by using the starch iodide method (11) with both uninduced cells and cells induced by growth on 0.1 μg of methicillin per ml. Phenotypic methicillin resistance was determined by the spread-plate method, and the presence of the *mecA* gene was detected with a DNA probe, both as previously described (5).

Statistical analysis. The differences in antimicrobial resistance profiles between selected groups of isolates were determined by the chi-square and Fisher exact tests.

TABLE 1. Staphylococcal isolates hybridizing with the conjugative transfer gene probe[a]

Source[b]	No. of isolates hybridizing/ total no. tested (%)		No. of hybridizing MR staphylococci/total no. of MR staphylococci (%)	
	CoNS	SA	CoNS	SA
MCV	11/90 (12)	23/43 (53)	11/76 (14)	22/29 (76)
VAH	8/82 (10)	12/75 (16)	6/72 (8)	11/28 (39)
MRSA		4/8 (50)		4/8 (50)
CDC	5/36 (14)	8/49 (16)	5/28 (18)	8/22 (36)
Total	24/208 (11.5)	47/175 (27)	22/176 (12.5)	45/87 (52)

[a] MR, methicillin resistant; CoNS, coagulase-negative staphylococci; SA, *S. aureus*.

[b] MCV, Medical College of Virginia Hospital; VAH, McGuire Veterans Administration Hospital; MRSA, Methicillin-resistant *S. aureus* from New York City; CDC, Centers for Disease Control.

RESULTS

Hybridization. The number of isolates hybridizing with the conjugative transfer gene probe is shown in Table 1. The 24 probe-positive coagulase-negative staphylococci were of the following species: *S. epidermidis* (13 isolates), *S. haemolyticus* (4 isolates), *S. hominis* (3 isolates), *S. simulans* (1 isolate), *S. warneri* (1 isolate), *S. saprophyticus* (1 isolate), and *S. capitis* (1 isolate). Species were not determined for all probe-negative coagulase-negative staphylococci.

Mating. All of the probe-positive isolates were mated with selection for gentamicin, the antimicrobial resistance marker most commonly found on conjugative plasmids that contain transfer sequences. Of the 71 isolates hybridizing with the probe, 59 (83%) transferred gentamicin resistance to a *S. aureus* recipient. The single probe-positive isolate that was gentamicin susceptible, a *S. epidermidis* isolate, transferred high-level trimethoprim resistance to a *S. aureus* recipient. Four of the 12 isolates not transferring the resistance marker were *S. aureus*, and 8 were coagulase-negative staphylococci. These 12 isolates were further examined.

Analysis of transfer-negative, probe-positive isolates. All 12 isolates that hybridized with the transfer gene probe but did not transfer in filter matings were resistant to gentamicin, and all contained DNA sequences hybridizing with the 6′ AAC/2″ APH aminoglycoside resistance gene cloned from conjugative plasmid pG01 (7). Ten of the 12 isolates contained plasmid DNA that hybridized with the *Eco*RI C conjugative transfer gene probe, and in each isolate the same plasmid also hybridized with the gentamicin resistance gene probe. Six of the 10 isolates that had plasmid DNA fragments hybridizing with the probe had *Eco*RI fragments of a different size than that of the probe (range, 4.0 to 12.0 kb); all six were in coagulase-negative staphylococci. The other four, all in *S. aureus*, had *Eco*RI, *Bgl*II, and *Hin*dIII fragments of the same size as those of pG01. Two coagulase-negative staphylococci had no plasmid DNA detected by using agarose gel electrophoresis with two different lysis techniques. In addition, neither displayed plasmid DNA components in cesium chloride-ethidium bromide density gradients. *Eco*RI, *Bgl*II, and *Hin*dIII fragments of the same size as those composing the entire conjugative transfer region of pG01 were present in total cellular DNA from each

TABLE 2. Antimicrobial resistance profiles of study isolates

Bacterium[a] (no. of isolates)	Antimicrobial resistance phenotype (% of isolates)[b]															
	Gm^5	Km^{10}	Tm^5	Nm^{10}	Ak	Sm	Tp^{25}	Eb	Em	Cl	Sp	Cm	Tc	Cd	Bla	Bla^i
S. aureus																
$MRTra^+$ (45)	100	100	100	64	9	9	18	20	89	89	91	2	16	93	91	100
$MSTra^+$ (2)	100	100	100	0	0	0	0	50	0	0	50	0	100	100	100	100
$MRTra^-$ (42)	40	95	88	78.5	26	12	9.5	9.5	88	74	95	12	24	88	78.5	93
$MSTra^-$ (86)	5	7	5	5	3	5	3	1	16	10	19	1	8	92	82	92
Coagulase-negative staphylococcus																
$MRTra^+$ (24)	96	92	96	17	17	25	54	50	67	54	71	8	42	96	58	78
$MSTra^+$ (2)	100	100	50	0	0	50	50	100	50	0	100	0	50	100	50	100
$MRTra^-$ (153)	50	71	56	39	30	15	49	42	75	62	57	24	34	95	39	88
$MSTra^-$ (29)	11	25	7	4	4	7	14	14	25	21	43	4	25	82	43	68

[a] MR, methicillin resistant; MS, methicillin susceptible; Tra, hybridization with conjugative transfer (*tra*) gene probe.
[b] Superscript number is the low drug concentration (in micrograms per milliliter) when both a low and a high concentration were tested. Bla, β-lactamase; Bla^i, induced β-lactamase.

isolate. Thus, the transfer region seemed to be intact but integrated into the chromosome in both isolates.

Antimicrobial susceptibility. Examination of staphylococcal antimicrobial resistance profiles, as seen in Table 2, showed that all but one of the isolates hybridizing with the conjugative transfer probe were resistant to 5 μg of gentamicin per ml, and 93% were methicillin resistant. The multiresistant nature of the *tra*-positive isolates was confirmed by finding all resistant to kanamycin and tobramycin, 94% resistant to heavy metals (arsenate, cadmium, and mercuric nitrate), 83% resistant to spectinomycin, and 72% resistant to erythromycin and clindamycin; 89% produced β-lactamase after induction. Surprisingly, only 31% were resistant to 25 μg of trimethoprim per ml and 32% were resistant to 25 μg of ethidium bromide per ml, two markers found on conjugative plasmids (18, 22). Transfer-sequence-positive *S. aureus* were significantly ($P < 0.01$) more resistant to gentamicin, trimethoprim, ethidium bromide, neomycin, spectinomycin, erythromycin, and clindamycin than the *S. aureus* not hybridizing with the probe. However, when isolates were grouped according to their susceptibilities to methicillin the only difference in overall antimicrobial resistance between transfer-positive and -negative *S. aureus* isolates was to gentamicin (Table 2). The only resistance phenotype that differed significantly between probe-positive and probe-negative coagulase-negative staphylococci, regardless of methicillin susceptibility, was gentamicin resistance. Among the 68 gentamicin-resistant *S. aureus*, 69% (47 of 68) contained conjugative transfer gene sequences and 63% (43 of 68) were able to transfer gentamicin resistance to recipients on filters. In contrast, only 23% (24 of 104) of gentamicin-resistant coagulase-negative staphylococci hybridized with the conjugative transfer gene probe.

High-level resistance to gentamicin (>100 μg/ml) was seen in only 7 of 175 (4%) *S. aureus* isolates but was seen in 18% of coagulase-negative staphylococci. Likewise, high-level trimethoprim (>500 μg/ml) and ethidium bromide (>100 μg/ml) resistance was seen in only 4 and 2% of *S. aureus* isolates, respectively, while 9% of coagulase-negative-staphylococci were highly trimethoprim resistant and 24% were highly ethidium bromide resistant. The only high-level resistance phenotype associated with the presence of conjugative transfer gene sequences was trimethoprim resistance in *S. aureus*. All high-level trimethoprim resistance in this species was found in seven isolates capable of conjugative transfer of gentamicin resistance; high-level trimethoprim resistance was cotransferred in each case.

DISCUSSION

In our study we found a high prevalence of conjugative plasmids among multiresistant *S. aureus* isolates from different geographic areas in the United States. Although some areas (e.g., Medical College of Virginia hospital) had a higher prevalence of isolates with conjugative plasmids than others, the overall prevalence was high enough to conclude that there has been widespread dissemination of these elements among methicillin-resistant *S. aureus* throughout the country. Furthermore, since 63% of all gentamicin-resistant *S. aureus* examined transferred the resistance phenotype by conjugation and the resistant isolates were distributed among different geographic areas, one could reasonably conclude that conjugative plasmids have been important in the dissemination of gentamicin-resistance among *S. aureus* in this country. These data are in contrast to findings among methicillin-resistant staphylococci in eastern Australia and Ireland. In Melbourne (19) and Dublin (20) both methicillin- and gentamicin-resistant *S. aureus* are present as a high percentage of total *S. aureus* isolates in local hospitals, yet conjugative plasmids are uncommon. In these areas, gentamicin resistance seems to be encoded on a transposon and the gene is found on a variety of nonconjugative plasmids and in the chromosome.

The low prevalence of conjugative gene sequences among multiresistant (methicillin-resistant) coagulase-negative staphylococci (12.5 versus 52% of multiresistant *S. aureus*) and the frequency with which conjugative transfer genes among these species were rearranged to abolish transfer (8 of 24 [33%] versus 4 of 47 [8.5%] in *S. aureus*) were surprising findings. It has been postulated that coagulase-negative staphylococci serve as a genetic reservoir for resistance genes and mobile elements that eventually find their way into *S. aureus*. However, the results of this study do not contradict that hypothesis. As normal flora colonizing superficial skin sites, coagulase-negative staphylococci are undoubtedly subject to different environmental pressures than *S. aureus* (1). The prevalence of a particular gene or element would not have to be high among this huge population of organisms for it to serve as a reservoir for genes capable of dissemination. The finding of transfer genes among seven

different species of coagulase-negative staphylococci is further evidence for their wide interspecies dissemination. In addition, other gene transfer mechanisms, including conjugative elements unrelated to pG01, may also be operative among coagulase-negative staphylococci.

It is interesting to contrast some of our findings with those of Gillespie et al. (9), who performed a phenotypic survey of Australian multiresistant *S. aureus* isolates. High-level trimethoprim resistance was found to be mediated only by the plasmid-encoded *dfrA* gene in Australian *S. aureus* isolates. We found similar results in our study among *S. aureus* isolates carrying trimethoprim resistance on conjugative plasmids. However, their suggestion that the combination of low-level resistance to ethidium bromide, gentamicin, and neomycin predicts carriage of conjugative plasmids did not prove to be true for our isolates. This combination was equally common among *S. aureus* isolates that either did or did not contain conjugative transfer gene sequences, and only 64 and 20% of transfer-gene-containing isolates were resistant to neomycin and ethidium bromide, respectively.

A secondary goal of our study was to look for the origins of conjugative plasmids among a wide range of staphylococci of diverse phenotype. However, all of the isolates that hybridized with the probe contained either complete, functioning transfer regions or complete regions that were nonfunctional due to insertion, deletion, integration, or other unknown mechanisms. One could postulate that since conjugative plasmid transfer and gentamicin resistance appeared among staphylococci in epidemic form at about the same time (2, 3, 6, 13, 18, 25), conjugative plasmids had existed for some time but awaited the association with a selectable marker for their presence to be detected and environmentally amplified. Our failure to detect transfer sequences on all but one plasmid that did not also carry gentamicin resistance makes that possibility less likely. However, since only eight staphylococci in our collection (all methicillin-susceptible *S. aureus*) were isolated before 1979, a survey of a larger number of earlier isolates may reveal an original staphylococcal source for transfer genes. Another possibility is that staphylococcal transfer genes were acquired from a different bacterial genus. Staphylococcal conjugative plasmids contain six or more directly repeated copies of an insertion sequence-like element (IS*431*/*257*) that may have been the genetic glue responsible for initial construction of these replicons (22, 23). The fact that these insertionlike elements have considerable homology to gram-negative elements of the IS*15* family (15) lends support to the notion that some associated staphylococcal conjugative transfer genes may be related to those in gram-negative bacteria as well. DNA and deduced amino acid sequence analysis of the transfer region may be more revealing in this regard.

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