ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1990, p. 1611–1614
0066-4804/90/081611-04$02.00/0
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Vol. 34, No. 8

Gene Heterogeneity for Tetracycline Resistance in *Staphylococcus* spp.

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Received 10 October 1989/Accepted 15 May 1990

Nucleotide sequences related to four *tet* genes were studied by hybridization in 183 clinical *Staphylococcus* isolates. *tet(K)* predominated in strains resistant only to tetracycline, while *tet(M)* was responsible for combined tetracycline and minocycline resistance. In strains harboring both genes, they contributed additively. *tet(L)* was detected in only five strains, and no hybridization was observed with *tet(O)*.

Bacterial resistance to tetracycline is common (10, 11). In gram-positive cocci (*Staphylococcus*, *Streptococcus*, and *Enterococcus* spp.), five classes of tetracycline resistance (Tc^r) genes, *tet(K)* (8), *tet(L)* (4), *tet(M)* (4), *tet(N)* (2, 4), and *tet(O)* (20) have been distinguished. The Tet L determinant (10, 14) and probably also the Tet K determinant (11) mediate the active efflux of tetracycline, whereas Tet M and Tet N (3, 10) and probably Tet O (11) confer resistance to tetracycline at the level of protein synthesis. The *tet(M)*, *tet(N)*, and *tet(O)* genes confer resistance to both tetracycline and minocycline, a lipophilic analog of tetracycline, whereas *tet(K)* and *tet(L)* confer resistance to tetracycline only (3, 10, 14, 24).

The nucleotide sequences of *tet(L)* (9) and *tet(M)* (13) from *Streptococcus* spp. and of *tet(K)* (8) from staphylococcal plasmid pT181 have been determined. Analysis of the nucleotide sequence of *tet(O)*, which is responsible for Tc^r in *Campylobacter* spp., led us to postulate that this gene originated in gram-positive bacteria (20). Indeed, we subsequently detected *tet(O)* in *Streptococcus* and *Enterococcus* strains (24). The distribution of *tet(K)* and *tet(L)* seems to vary among gram-positive cocci: *tet(L)* is more widespread in streptococci and enterococci (24) than in staphylococci (17), whereas *tet(K)*, which is rarely encountered in streptococci (24), may be common in staphylococci (11). The presence of *tet(M)* in staphylococci has been reported (10, 11), but its prevalence in staphylococci is not known [the incidence of *tet(M)* in enterococci has been investigated (24)]. The *tet(O)* gene has not been searched for in staphylococci. In France, among Tc^r *Staphylococcus aureus* strains, more than 90% of the methicillin-susceptible isolates are minocycline susceptible (Mc^s), whereas the majority of the methicillin-resistant strains are Mc^r (1). In contrast, the Tc^r coagulase-negative staphylococci, whether susceptible or resistant to methicillin, are Mc^s. We have studied, by DNA-DNA hybridization with intragenic probes, the distribution of *tet(K)*, *tet(L)*, *tet(M)*, and *tet(O)* in tetracycline-resistant clinical isolates of *S. aureus* and in coagulase-negative staphylococci resistant or susceptible to methicillin. Since there is no probe specific for *tet(N)* (2), this resistance determinant was not included in this study.

A total of 99 *S. aureus* clinical isolates and 84 coagulase-negative staphyloccus clinical isolates (37 *S. epidermidis*, 26 *S. haemolyticus*, 4 *S. hyicus*, 4 *S. warneri*, 2 *S. capitis*, 2 *S. saprophyticus*, 3 *S. xylosus*, 2 *S. hominis*, 2 *S. simulans*, and 2 *Staphylococcus* spp.) were collected in four hospitals in Paris in 1987. Coagulase-negative isolates were identified at the species level with API Staph-Ident kits (API-System, La Balme-les-Grottes, France). Strains were selected for resistance to tetracycline, minocycline, or both and screened for resistance to methicillin by the disk-agar diffusion method (Diagnostics Pasteur, Marnes-la-Coquette, France). Strains harboring plasmids pT181 (8), pBC16 (16), pIP1433 (20), and *S. aureus* 80CR5::Tn*1545* (5) were included. *S. aureus* 209P and RN450, 23 other *S. aureus* strains, and 33 coagulase-negative *Staphylococcus* strains from our laboratory collection belonging to the species represented in this study were used as tetracycline-susceptible control strains.

We tested, by dot blot hybridization, for the presence of nucleotide sequences that were structurally related to *tet(K)*, *tet(L)*, *tet(M)*, and *tet(O)* in tetracycline-resistant *Staphylococcus* strains (Table 1). The DNA probes used were the 870-base-pair (bp) *Hin*cII fragment of pT181 for *tet(K)* (24), the 310-bp *Cla*I-*Hpa*II fragment of pBC16 for *tet(L)* (24), the 850-bp *Cla*I-*Hin*dIII fragment of Tn*1545* for *tet(M)* (13), and the 1458-bp *Hin*dIII-*Nde*I fragment of pIP1433 for *tet(O)* (20). Purified restriction fragments used as probes were labeled with [α-^{32}P]dATP by nick translation (12). DNA fragments cloned in bacteriophage M13mp18 were hybridized with the 15-bp distal primer and labeled by DNA synthesis in the presence of dGTP, dCTP, dTTP, [α-^{32}P]dATP, and DNA polymerase I (Klenow fragment) (6, 23). Dot blot hybridization under stringent conditions was in 50% formamide at 42°C for 24 h and was followed by three washings in 2× SSC–0.1% sodium dodecyl sulfate at room temperature for 15 min and two washings in 0.2× SSC–0.1% sodium dodecyl sulfate at 65°C for 1 h (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization under low-stringency conditions was in 50% formamide at 37°C for 24 h and was followed by one washing in 2× SSC–0.1% sodium dodecyl sulfate at 45°C for 1 h. None of the susceptible strains hybridized with a *tet* probe. All of the Tc^r Mc^s *S. aureus* strains which were susceptible to methicillin hybridized with *tet(K)*, whereas the two methicillin-resistant isolates hybridized with *tet(M)*. All but two Tc^r Mc^s coagulase-negative *Staphylococcus* strains, whether susceptible or

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TABLE 1. Distribution of *tet* gene classes among staphylococci

Hybridization with[a]:	No. of strains[b]							
	S. aureus				Coagulase-negative staphylococci			
	Tc^r Mc^s		Tc^r Mc^r		Tc^r Mc^s		Tc^r Mc^r	
	Met^s (20)	Met^r (2)	Met^s (15)	Met^r (62)	Met^s (46)[c]	Met^r (30)[d]	Met^s (3)[e]	Met^r (5)[f]
tet(K)	20	0	0	0	45	29	0	0
tet(L)	0	0	0	0	1[g]	0	0	0
tet(M)	0	2	8	42	0	0	0	0
tet(K) and *tet(L)*	0	0	0	0	0	1[h]	0	0
tet(K) and *tet(M)*	0	0	5	20	0	0	3	4
tet(L) and *tet(M)*	0	0	2	0	0	0	0	0
tet(K), *tet(L)*, and *tet(M)*	0	0	0	0	0	0	0	1[h]

[a] No hybridization was observed with *tet(O)*. None of the following strains hybridized with the probes: 23 *S. aureus* (21 methicillin susceptible, 2 methicillin resistant), 33 coagulase-negative *Staphylococcus* strains (13 methicillin susceptible [9 *S. epidermidis*, 1 *S. saprophyticus*, 1 *S. sciuri*, 1 *S. capitis*, 1 *Staphylococcus* sp.] and 20 methicillin resistant [11 *S. epidermidis*, 7 *S. haemolyticus*, 2 *Staphylococcus* spp.]), and *S. aureus* RN450 and 209P (tetracycline and minocycline susceptible).

[b] The total number of strains with each phenotype is indicated in parentheses.

[c] 17 *S. epidermidis*, 8 *S. haemolyticus*, 4 *S. hyicus*, 4 *S. warneri*, 3 *S. xylosus*, 2 *S. capitis*, 2 *S. hominis*, 2 *S. simulans*, 2 *S. saprophyticus*, 2 *Staphylococcus* spp.

[d] 16 *S. epidermidis*, 14 *S. haemolyticus*.

[e] 3 *S. epidermidis*.

[f] 4 *S. haemolyticus*, 1 *S. epidermidis*.

[g] *S. haemolyticus*.

[h] *S. epidermidis*.

resistant to methicillin, hybridized with *tet(K)*, which was detected in all of the species studied. One *S. haemolyticus* strain hybridized with *tet(L)*, and one *S. epidermidis* strain hybridized with *tet(K)* and *tet(L)*. The *tet(M)* gene was found in all of the Tc^r Mc^r strains. It was associated with *tet(K)* in one-third of the *S. aureus* strains, and it was always associated with *tet(K)* in the coagulase-negative staphylococci. Two methicillin-susceptible *S. aureus* strains hybridized with *tet(L)* and *tet(M)*, and one methicillin-resistant *S. epidermidis* strain hybridized with *tet(K)*, *tet(L)*, and *tet(M)*. We did not observe hybridization with *tet(O)* under stringent conditions. As expected (13), under low-stringency conditions, all of the strains harboring *tet(M)* hybridized weakly with the *tet(O)* probe.

The MICs of tetracycline and minocycline against *S. aureus* strains and coagulase-negative staphylococci harboring the different *tet* genes, alone or in various combinations, were determined (Table 2). Resistance to tetracycline and minocycline was defined as an MIC of >4 μg/ml (15). The MICs of both antibiotics against susceptible isolates of *S. aureus* or coagulase-negative staphylococci were similar, with geometric means of 1 and 0.5 μg/ml, respectively. Strains harboring *tet(K)* were resistant to tetracycline and susceptible to minocycline, with only three strains of *S. aureus* being inhibited by 4 μg of minocycline per ml. The activities of the antibiotics were similar against *S. aureus* and coagulase-negative staphylococci with geometric means of ca. 110 and 0.8 μg/ml, respectively. The MICs of tetracycline and minocycline for the single coagulase-negative *Staphylococcus* strain harboring *tet(L)* were 32 and 0.25 μg/ml, respectively. They were increased fourfold (to 128 and 1 μg/ml, respectively) against another coagulase-negative *Staphylococcus* isolate harboring *tet(K)* and *tet(L)*. The *S. aureus* strains harboring *tet(M)* were resistant to both antibiotics (Table 2), except for two strains (Table 1) which were inhibited only by 2 μg of minocycline per ml. There was a twofold difference in the MICs of tetracycline against *S. aureus* strains harboring both *tet(M)* and *tet(K)* and against strains harboring only *tet(M)* (geometric means, 210 and 91 μg/ml, respectively), whereas, as expected, the MICs of minocycline (geometric means, 12.4 and 10.4 μg/ml, respectively) remained unchanged. By contrast, the levels of resistance to tetracycline of coagulase-negative staphylococci harboring *tet(M)* and *tet(K)* were similar to those of strains harboring only *tet(K)*. Coagulase-negative *Staphylococcus* isolates containing either *tet(K)* and *tet(M)* or *tet(K)*, *tet(L)*, and *tet(M)* were resistant to minocycline.

Study of the prevalence of tetracycline resistance determinants in staphylococci indicated that *tet(K)* and *tet(M)*

TABLE 2. MICs of tetracycline and minocycline against staphylococci harboring different *tet* gene classes

tet resistance gene(s)	Geometric mean (range of values) MICs[a] (μg/ml) against:			
	S. aureus		Coagulase-negative staphylococci	
	Tetracycline	Minocycline	Tetracycline	Minocycline
None	1 (0.5–4)	0.4 (0.25–1)	1 (0.25–2)	0.5 (0.12–1)
tet(K)	115 (32–256)	0.8 (0.25–4)	109 (16–256)[b]	0.8 (0.12–2)[b]
tet(M)	91 (32–512)	10.4 (2–16)		
tet(K) and *tet(M)*	210 (128–512)[c]	12.8 (4–16)[c]	112 (32–256)[d]	6.5 (4–16)[d]

[a] The method of Steers et al. (21) was used to determine the MICs of tetracycline and minocycline.

[b] One strain with tetracycline and minocycline MICs of 32 and 0.25 μg/ml, respectively, harbored only *tet(L)*, and one strain with tetracycline and minocycline MICs of 128 and 1 μg/ml, respectively, harbored *tet(K)* and *tet(L)*.

[c] Two strains with tetracycline and minocycline MICs of 256 and 4 μg/ml, respectively, harbored *tet(L)* and *tet(M)*.

[d] One strain with tetracycline and minocycline MICs of 64 and 4 μg/ml, respectively, harbored *tet(K)*, *tet(L)*, and *tet(M)*.

were widespread and that *tet(L)* and *tet(O)* were rare and absent, respectively (Table 1).

There was an excellent correlation between the resistance phenotypes of the strains studied, as determined by the disk-agar diffusion method or by MICs, and the genotypes inferred from DNA-DNA hybridization experiments using specific probes (Table 2). The *tet(K)* gene was present in all of the strains which were resistant to tetracycline and susceptible to minocycline. With the exception of two *S. aureus* strains which were apparently susceptible to minocycline, *tet(M)* was found in all of the strains which exhibited cross resistance to both antibiotics. Curiously, this gene was associated with *tet(K)* in 35% of the *S. aureus* isolates and in all of the coagulase-negative staphylococci.

The tetracycline resistance levels of *S. aureus* strains harboring *tet(K)* or *tet(M)* were similar. They were increased twofold in strains harboring both resistance determinants, indicating that the two genes contribute, in an additive fashion, to the degree of resistance to tetracycline. This was not the case for coagulase-negative staphylococci, for which the MICs of tetracycline against strains containing *tet(K)* or *tet(M)* and *tet(K)* were similar. The reason(s) for this differential gene expression depending upon the bacterial host remains unknown.

In France, among methicillin-susceptible *S. aureus* strains, Tc^r Mc^s is the most prevalent (10 to 15%) resistance phenotype (1); the 20 Tc^r Mc^s *S. aureus* strains harboring *tet(K)* alone were susceptible to methicillin (Table 2). Among methicillin-resistant *S. aureus* strains, the incidence of the Tc^r Mc^r phenotype was 96%; it was only 6% in their methicillin-susceptible counterparts (1). The *tet(M)* gene was detected in all of the Tc^r Mc^r strains and also in two methicillin-resistant *S. aureus* strains that appeared to be Mc^s. The *tet(K)* gene was never found alone in the methicillin-resistant *S. aureus* strains (Table 1). Whether they were methicillin susceptible or resistant, one-third of the Tc^r Mc^r *S. aureus* strains were found to harbor *tet(K)* and *tet(M)* (Table 1). The use of specific probes allowed the differentiation of two genotypes, *tet(M)* and *tet(K) tet(M)* among methicillin- and tetracycline-resistant *S. aureus* strains. This approach provides a useful epidemiological tool to trace strains responsible for nosocomial infections (22).

Among coagulase-negative staphylococci, tetracycline resistance, like other antibiotic resistances, is more common in *S. epidermidis* and *S. haemolyticus* than in the other species (18, 19). However, *tet(K)* was detected in all the Tc^r Mc^s strains belonging to the various species studied (Table 1). As opposed to the situation with *S. aureus*, this gene was frequently alone in both methicillin-resistant and -susceptible strains. The incidence of tetracycline resistance in coagulase-negative staphylococci in France is similar for methicillin-resistant (49%) and methicillin-susceptible (42%) strains (R. Bismuth, unpublished results). In contrast to *tet(K)*, the *tet(M)* gene was found rarely and only in two species, *S. epidermidis* and *S. haemolyticus*. This resistance determinant was always associated with *tet(K)*, and the incidence of the Tc^r Mc^r phenotype was 1 and 4% in methicillin-susceptible and -resistant French isolates of coagulase-negative staphylococci, respectively (R. Bismuth, unpublished results).

The *tet(K)* gene, which was detected in a single *Streptococcus* strain and not at all in *Enterococcus* spp. (24), was widely distributed among staphylococci (Table 1). By contrast, *tet(L)*, which is common in streptococci and enterococci (24), was detected only in two strains of *S. aureus* and in three coagulase-negative *Staphylococcus* strains. Nevertheless, the finding of *tet(L)* in *Staphylococcus* spp., as well as the finding of *tet(K)* in *Enterococcus* spp., confirms the occurrence of genetic exchanges between gram-positive cocci under natural conditions.

The *tet(M)* gene is often responsible for tetracycline resistance in gram-positive cocci. It was detected in 100% of methicillin-resistant *S. aureus* strains, 80% of group B and nongroupable *Enterococcus* strains, and 60% of group A, C, and G *Streptococcus* strains (24) but also in 30% of methicillin-susceptible *S. aureus* strains and 5% of coagulase-negative *Staphylococcus* strains (Table 1). Dissemination of this resistance determinant could be due to the fact that *tet(M)* is carried by broad-host-range conjugative transposons (5, 7). This gene was detected alone or associated with *tet(K)* in staphylococci and with *tet(L)* in streptococci and enterococci (24). The *tet(O)* determinant, which has a common ancestor with *tet(M)* (20, 24), was not found in staphylococci but is present in streptococci and enterococci (24).

The four classes of *tet* genes screened account for tetracycline resistance in all the staphylococcal isolates studied, whereas 12% of streptococci and enterococci did not hybridize to the same set of probes (24). It appears, therefore, that there is a lesser degree of *tet* gene heterogeneity in staphylococci than in streptococci and enterococci. This observation could indicate that the latter two genera, rather than staphylococci, act as a reservoir of genes for other gram-positive and gram-negative bacteria.

We thank T. Lambert, R. Leclercq, and A. Philippon for the gifts of bacterial strains.

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