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Comparative Inhibition of Methicillin-resistant Strains of *Staphylococcus aureus* by Lysostaphin and Other Antibiotics

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Sixteen methicillin-resistant strains of *Staphylococcus aureus* obtained from Europe were found to be sensitive to the lytic activity of lysotaphin. With only minor exceptions, the strains were found to be sensitive to novobiocin, erythromycin, fusidic acid, and lincomycin, and slightly less sensitive to vancomycin and chloramphenicol. All strains were resistant to tetracycline, penicillinase-sensitive penicillins (benzylpenicillin, ampicillin, and propicillin), penicillinase-resistant penicillins (methicillin, nafcillin, ancillin, oxacillin, cloxacillin, and dicloxacillin), and two cephalosporin antibiotics (cephalothin and cephaloridine).

The occurrence of clinical staphylococci resistant to methicillin and other newly synthesized semisynthetic penicillins has been reported with increasing frequency (1, 2, 4-6, 10, 11). Whereas the clinical significance of coagulase-positive staphylococci resistant to methicillin appears to be minor at this time (6), the more widespread occurrence of methicillin-resistant coagulase-negative staphylococci may be a cause for concern (7). After spiramycin and novobiocin were used in a hospital ward, resistance among strains of *Staphylococcus epidermidis* was observed; later, this resistance pattern was also observed among *S. aureus* strains (8). It has been suggested that the appearance of strains of *S. epidermidis* resistant to the new penicillins should alert us to the possibility of a subsequent significant occurrence of *S. aureus* strains resistant to the same penicillins (7).

We have shown that lysostaphin is at least four to eight times more potent than the recently synthesized penicillins in inhibiting growth of *S. aureus* (13). In view of this activity against potent penicillinase-producing strains, it appeared desirable to extend our studies to include strains of *S. aureus* highly resistant to methicillin. In addition to lysostaphin, penicillins containing the four prosthetic groups (benzyl, phenoxy, dimethoxyphenyl, and isoxazolyl), two cephalosporin compounds, and several other potent antistaphylococcal antibiotics were compared for their growth inhibiting properties against sixteen methicillin-resistant strains obtained from Europe.

MATERIALS AND METHODS

Cultures. All of the resistant *S. aureus* strains used in this study were kindly supplied by R. Sutherland, Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, England. For convenience, the cultures are designated by their BRL numbers. The sources of the individual cultures are shown in Table 1, and additional information on these cultures has been reported by Sutherland and Rolinson (11). Two control methicillin-sensitive strains of *S. aureus*, strain FDA 209P (a nonpenicillinase producer) and strain 4180 (a bacteriophage 80/81 clinical isolate and potent penicillinase producer), were also used.

Antibiotic sensitivity tests. Minimal inhibitory concentrations (MIC) for the antibiotics were determined by serial dilution of the antibiotics in 2-ml volumes of Penassay Broth (Difco). An inoculum of one drop (approximately 0.05 ml) of an overnight broth culture was added to the tubes and resulted in the addition of 0.8×10^7 to 10^7 viable cells per tube. The inoculated tubes containing various concentrations of antibiotics were incubated at 37 C and were examined for visible growth after 48 hr.

Viable cell counts. To further compare the antibiotic resistance of these methicillin-resistant isolates, a viable count-agar plate technique was employed. Plates of Trypticase Soy Agar (BBL) containing specified antibiotic concentrations were inoculated with cell populations of 10^6 to 1.2×10^6 viable cells in 0.1 ml of 0.145 M NaCl, and the cells were uniformly distributed on the agar surfaces. Individual colonies were enumerated after 72 to 96 hr of incubation at 37 C.

Antibiotics. The antibiotics used were obtained from the following suppliers: penicillin G (benzylpenicillin), E. R. Squibb & Sons, New York, N.Y.; ampicillin (α-aminobenzylpenicillin, sodium), methicillin (di-

TABLE 1. *Methicillin-resistant strains of S. aureus and their sources*

BRL no.	Source	Strain no.
1484A	Colindale (England)	13137
1591	Poland	P89
1735	Queen Mary's Hospital, Carshalton (England)	QMHI
1736		QMHII
1754		6467
1756		8054
1776	Birmingham (England)	BRL 1776
1778	Copenhagen, Denmark	5982
1782	Hammersmith Hospital (England)	M11
1783		M15
1786		M25
1804	Paddington Hospital (England)	19223/62
1806		24414/62
1807		1702/63
1808		1529/63
1811	Central Middlesex Hospital (England)	1244/62

methoxyphenylpenicillin, sodium), and oxacillin (5-methyl-3-phenyl-4-isoxazolylpenicillin, sodium), Bristol Laboratories, Syracuse, N.Y.; propicillin (α-phenoxypropylpenicillin), vancomycin, erythromycin A, and cephalothin [7-(thiophene-2-acetamido) cephalosporanic acid], Eli Lilly & Co., Indianapolis, Ind.; ancillin (2-biphenylylpenicillin, sodium), Smith Kline & French Laboratories, Philadelphia, Pa.; cloxacillin (3-*o*-chlorophenyl-5-methyl-4-isoxazolylpenicillin) and dicloxacillin [3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolylpenicillin], Ayerst Laboratories, Rouses Point, N.Y.; nafcillin [6-(2-ethoxy-1-naphthamido)penicillin], Wyeth Laboratories, Philadelphia, Pa.; cephaloridine (7-[2-thienyl) acetamido]-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine), Glaxo Laboratories, Ltd., Greenford, England; lincomycin and novobiocin (sodium), The Upjohn Co., Kalamazoo, Mich.; chloramphenicol, Parke Davis & Co., Detroit, Mich.; tetracycline hyclochloride, American Cyanamid Co., New York, N.Y.; fusidic acid (sodium), Leo Pharmaceutical Products, Ballerup, Denmark; lysostaphin (low molecular weight protein), Mead Johnson & Co., Evansville, Ind.

RESULTS AND DISCUSSION

The high level of resistance of the cultures to penicillin-like antibiotics is clearly shown in Table 2. In contrast, growth of all 16 strains was inhibited by lysostaphin concentrations of 0.78 μg/ml or less. All of the cultures seemed to be potent penicillinase producers, as evidenced by the confluent growth observed on agar plates containing 100 μg of benzylpenicillin, ampicillin, or propicillin per ml. Of the six penicilinase-resistant semisynthetic penicillins, nafcillin, ancillin, and possibly dicloxacillin appeared to inhibit growth more effectively than the others in the group. At a concentration of 100 μg/ml, cephaloridine completely inhibited growth of all of the strains, whereas only three strains were completely inhibited by cephaloridine at a concentration of 25 μg/ml. At this antibiotic level, the remaining strains showed colony counts of 100 to 300 per plate in two instances and confluent growth in the other instances. Uniformly heavy growth was found with all cultures on plates containing 10 μg of cephaloridine per ml (Table 3).

All strains tested were sensitive to 2 μg of lincomycin, fusidic acid, or novobiocin per ml, with the exception of strain 1806, which required 10 μg of novobiocin per ml for complete inhbition by the plate method. Although most of the cultures were sensitive to 0.5 μg of erythromycin per ml, strain 1783 required 10 μg of antibiotic per ml for inhibition, and strain 1591 was resistant to 100 μg of antibiotic per ml. The cultures were uniformly sensitive to 10 μg of vancomycin or chloramphenicol per ml, with the exception of strain 1786, which required >100 μg of chloramphenicol per ml for inhibition. All strains were resistant to tetracycline (Table 4).

Although colony counts of at least 20 per plate were found with four of the strains at a lysostaphin concentraion of 0.5 μg/ml, only sporadic colonies appeared on plates containing 2 and 10 μg of lysostaphin per ml (Table 5). The three isolates which appeared on the plates containing 10 μg of lysostaphin per ml with strains 1782 and 1806 were inhibited by 6.25 μg of lysostaphin per ml in the tube dilution assay.

We found clear differences in the degree of

TABLE 2. *Comparative sensitivity of 16 methicillin-resistant strains of S. aureus to lysostaphin and penicillin-like antibiotics*

Antibiotic	Minimal inhibitory concn		
	0.20–0.78 μg/ml	50–100 μg/ml	>100 μg/ml
Lysostaphin	16	0	0
Cephalothin	0	16	0
Cephaloridine	0	6	10
Nafcillin	0	6	10
Ancillin	0	0	16
Methicillin	0	0	16
Oxacillin	0	0	16
Dicloxacillin	0	0	16
Cloxacillin	0	0	16
Propicillin	0	0	16
Ampicillin	0	0	16
Benzylpenicillin	0	0	16

methicillin-resistance among the cultures (Fig. 1). Growth of strain 1754 was uniformly heavy and confluent. Discrete colonies were visible although uniformly dense on plates seeded with strain 1736. Although colony counts of more than 500 were obtained with strain 1735, the colonies tended to be evenly distributed on the plate surfaces. Finally, viable-cell counts of less than 50 were found with strain 1808. In contrast, the levels of growth obtained on lysostaphin-containing plates were approximately equivalent with these four cultures (Fig. 2). Interestingly, the lysostaphin concentration was very low (0.1 μg/ml) and only 0.1% of the methicillin concentration.

TABLE 3. *Effect of penicillin-like antibiotics on growth of methicillin-resistant staphylococci*

Strain no.	Viable counts/plate[a]						
	Methicillin (100 μg/ml)	Nafcillin (100 μg/ml)	Ancillin (100 μg/ml)	Oxacillin (100 μg/ml)	Cloxacillin (100 μg/ml)	Dicloxacillin (100 μg/ml)	Cephaloridine (100 μg/ml)
1484A	164	4	28	497	500	295	500
1591	>500	122	340	>500	>500	>500	0
1735	>500	67	>500	438	468	320	0
1736	>500	>500	>500	>500	>500	>500	>500
1754	>500	>500	>500	>500	>500	>500	>500
1756	>500	3	284	>500	>500	>500	>500
1776	492	7	57	>500	>500	>500	>500
1778	>500	12	67	>500	>500	103	>500
1782	>500	22	98	307	251	158	120
1783	221	18	51	244	272	62	>500
1786	405	10	47	161	162	105	270
1804	500	18	132	196	197	90	0
1806	18	0	3	144	126	61	>500
1807	343	17	52	159	165	126	>500
1808	10	0	3	74	91	21	>500
1811	>500	7	178	368	323	152	>500

[a] An inoculum level of 10^6 to 1.2×10^6 viable cells/plate in a volume of 0.1 ml was used, and growth was scored after 4 days at 37 C. Confluent growth (>500 colonies/plate) was observed on plates containing 100 μg of benzylpenicillin, ampicillin, or propicillin per ml.

TABLE 4. *Effect of various antibiotics on growth of methicillin-resistant staphylococci*

Strain no.	Viable counts/plate[a]						
	Novobiocin (0.5 μg/ml)	Erythromycin (0.5 μg/ml)	Fusidic acid (0.5 μg/ml)	Lincomycin (2.0 μg/ml)	Vancomycin (10 μg/ml)	Chloramphenicol (10 μg/ml)	Tetracycline (100 μg/ml)
1484A	3	0	20	0	0	0	>500
1591	21	>500	12	0	0	0	>500
1735	0	0	329	0	0	0	59
1736	3	1	>500	0	0	0	404
1754	7	0	>500	0	0	0	360
1756	3	0	7	0	0	0	>500
1776	5	0	9	0	0	0	>500
1778	1	0	1	0	0	0	>500
1782	0	0	2	0	0	0	>500
1783	1	>500	18	1	0	0	>500
1786	37	0	9	0	0	>500	>500
1804	2	0	9	0	0	0	>500
1806	>500	0	23	0	0	0	>500
1807	0	0	5	0	0	0	>500
1808	0	0	5	0	0	0	>500
1811	6	0	8	0	0	0	>500

[a] An inoculum level of 10^6 to 1.2×10^6 viable cells/plate in a volume of 0.1 ml was used, and growth was scored after 4 days at 37 C.

TABLE 5. *Effect of lysostaphin on ability of staphylococci to form colonies*

Strain no.	Viable counts/plate[a] with lysostaphin concn of		
	10 μg/ml	2.0 μg/ml	0.5 μg/ml
1484A	0	0	71
1591	0	1	4
1735	0	0	8
1736	0	0	8
1754	0	1	6
1756	0	2	13
1776	0	3	1
1778	0	5	2
1782	2	1	3
1783	0	1	4
1786	0	0	5
1804	0	0	30
1806	1	1	7
1807	0	0	10
1808	0	0	2
1811	0	6	13
FDA 209P	0	0	0
Clinical isolate 4180[b]	0	0	0

[a] All platings consisted of 10^6 to 1.2×10^6 viable cells/plate in a volume of 0.1 ml, and growth was scored after 4 days at 37 C.

[b] Bacteriophage 80/81 type.

Although there is no agreement among investigators as to whether methicillin-resistant strains of *S. aureus* inactivate methicillin, it has been suggested that staphylococcal penicillinase plays a major role in the slow destruction of methicillin and other relatively penicillinase-resistant penicillins (3, 5, 6). Our data (Table 6) also support the concept of marked methicillin destruction with actively growing cultures of certain *S. aureus* methicillin-resistant strains. Much of the methicillin inactivation appears to be intracellular. In addition to the biological assay procedure that we used (Table 6), an iodine titrimetric method (9) also failed to detect significant quantities of extracellular β-lactamase activity for methicillin.

As anticipated, we found the methicillin-resistant strains to be refractory to five other relatively penicillinase-resistant penicillins and two cephalosporin-type antibiotics. Other investigators (5, 6, 11) have shown similar cross-resistance to cloxacillin, oxacillin, and cephalothin among methicillin-resistant strains of *S. aureus*. With only minor exceptions, the strains as a group were found to be sensitive to novobiocin, erythromycin, fusidic acid, and lincomycin, and slightly less sensitive to vancomycin and chloramphenicol. As reported by others (11), all strains were resistant to tetracycline.

Regardless of the mechanisms underlying their

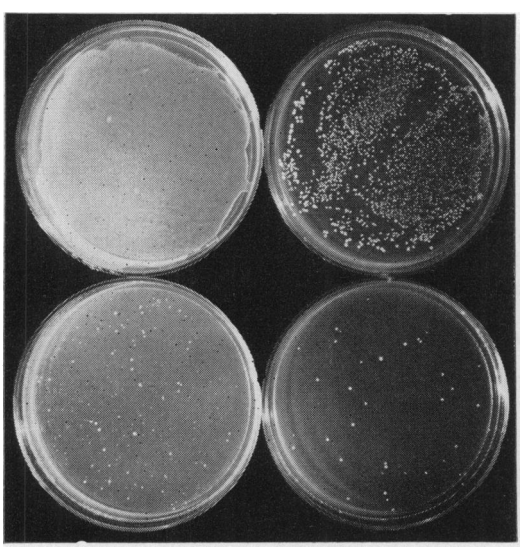

FIG. 1. *Growth of four methicillin-resistant strains of S. aureus (1754 and 1736, upper and lower left, respectively; 1735 and 1808, upper right and lower right, respectively) on agar plates containing 100 μg of methicillin per ml.*

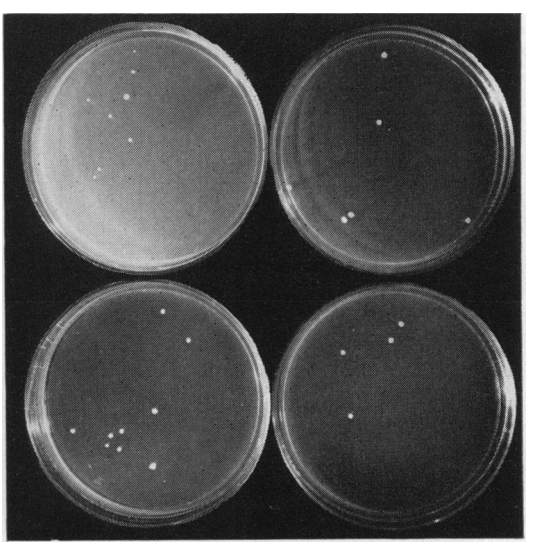

FIG. 2. *Growth of four methicillin-resistant strains of S. aureus (1754 and 1736, upper and lower left, respectively; 1735 and 1808, upper right and lower right, respectively) on agar plates containing 0.1 μg of lysostaphin per ml.*

antibiotic resistance patterns, no differentiation could be made, based on lysostaphin susceptibility, between these methicillin-resistant strains and other coagulase-positive staphylococci (12).

TABLE 6. *Residual methicillin concentrations after growth of five methicillin-resistant strains of S. aureus in the presence of this antibiotic*[a]

Strain	Methicillin concn (μg/ml)		Recovery	Methicillin concn after 48 hr	Recovery[b]
	Initial	After 21 hr			
			%	μg/ml	%
1735	20	10.5	75	<1	<8
	40	40	100	<1	<3
	80	64	96	2.9	5
	160	128	100	26.5	22
1736	20	7.9	56	<1	<8
	40	43	100	<1	<3
	80	85	100	<1	<2
	160	132	100	2.8	2
1754	20	2.1	15	<1	<8
	40	4.5	15	<1	<3
	80	16.5	25	<1	<2
	160	33	26	<1	<1
1756	20	<1	<7	<1	<8
	40	<1	<3	<1	<3
	80	<1	<1	<1	<2
	160	<1	<1	<1	<1
1811	20	<1	<7	<1	<8
	40	2.0	6	<1	<3
	80	6.5	10	<1	<2
	150	9.6	8	<1	<1
Noninoculated control medium	20	14		12.8	
	40	31		35.5	
	80	67		62	
	160	127		119	

[a] Cultures were grown in Penassay Broth (Difco) at 28 C in 50-ml Erlenmeyer flasks with aseptic additions of methicillin (Seitz sterilization) and final volumes of 10 ml/flask. Methicillin concentrations were determined microbiologically by an agar plate-disc method employing *Sarcina lutea* ATCC 9341.

[b] Based on noninoculated control values.

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