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Role of β-Lactamase in Expression of Resistance by Methicillin-Resistant *Staphylococcus aureus*

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Of 27 unique clinical isolates of methicillin-resistant *Staphylococcus aureus*, only 4 were homogeneously resistant, and all 4 produced little or no β-lactamase. Among heterogeneously resistant strains, those most resistant to β-lactam antibiotics produced the most β-lactamase. Similar genes may regulate production of the low-affinity penicillin-binding protein and β-lactamase.

Intrinsic resistance to methicillin in *Staphylococcus aureus* is caused by production of a low-affinity penicillin-binding protein (PBP 2′) (11–13, 22, 25). Nearly all such strains also produce β-lactamase (8, 15, 16). Although β-lactamase may be responsible for borderline methicillin or oxacillin resistance in *S. aureus* strains that do not produce PBP 2′ (17), it is not clear whether β-lactamase contributes to the expression of methicillin resistance in intrinsically resistant strains (4, 5).

We quantitated β-lactamase activity and graded heterogeneity of β-lactam resistance in the presence and absence of a β-lactamase inhibitor (clavulanic acid [CLAV]) in clinical strains of methicillin-resistant *S. aureus* from several geographic areas. Our results suggest that β-lactamase does not contribute to methicillin resistance in these organisms and that other genes on the β-lactamase plasmid may affect expression of methicillin resistance by methicillin-resistant *S. aureus*.

We analyzed 27 methicillin-resistant *S. aureus* strains from 13 cities. Antimicrobial susceptibility, bacteriophage type, hemolysin and lipase production, and plasmid content determinations had demonstrated that these isolates were epidemiologically unique strains. For all isolates, methicillin MICs were 16 μg/ml or greater by agar dilution methods (19).

Susceptibility to oxacillin, methicillin, nafcillin, cloxacillin, cefazolin, cephalothin, cefamandole, penicillin, and imipenem was determined by disk diffusion methods (3, 18). Duplicate plates were inoculated and incubated at 35 and 42°C for 24 h. Plates were incubated at 42°C because heterogeneously resistant (HET) strains appear more susceptible to β-lactam antibiotics at high temperatures, whereas homogeneously resistant (HOMO) strains are relatively unaffected by changes in incubation temperatures (1, 13, 21). Zone diameters were recorded, and the growth inside zone margins was graded 1+ to 6+. Isolates with no zones of inhibition around oxacillin, methicillin, nafcillin, cefazolin, cephalothin, or imipenem at 35 or 42°C were defined as HOMO strains. In contrast, all HET strains appeared susceptible to at least cephalothin and imipenem at 42°C. HET strains that showed 5+ to 6+ growth around methicillin, oxacillin, and nafcillin disks at 35°C, with a combined growth score of 16 to 18 for these three antibiotics, were categorized as HET-1 strains. Strains with a combined growth score of <14 for these three antibiotics were defined as HET-2 strains.

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To examine the effect of β-lactamase on the expression of resistance by these strains, additional disk diffusion tests were done after the addition of 5 μg of CLAV to standard β-lactam antibiotic disks. Addition of more than 5 μg of CLAV to blank disks resulted in inhibition of growth by CLAV alone. Plates were incubated at 35°C for 24 h. Zones of complete growth inhibition obtained with standard disks and with disks containing an antimicrobial agent plus CLAV were compared by a matched-pairs signed-ranks test (26).

To determine β-lactamase activity, 1 ml of a standardized suspension (0.5 McFarland standard) of cells harvested from a plate was added to 50 ml of brain heart infusion broth and incubated at 35°C for 24 h. Two 25-ml samples were then diluted in fresh broth or in broth containing methicillin (final concentration, 0.5 μg/ml) and incubated for 24 h at 35°C.

β-Lactamase activity was assayed by adding 10 μl of each uninduced and induced broth whole-cell culture to 2.5 ml of nitrocefin (10^{-4} M) (BBL Microbiology Systems, Cockeysville, Md.). Spectrophotometric readings were taken at 482 nm at 30- to 60-min intervals for 4 h, and a line of best fit was determined by using regression analysis. β-Lactamase activity was expressed as the number of micromoles of nitrocefin hydrolyzed per hour per milligram (dry weight) of the organism. Each organism was tested twice, and an average value was calculated. The mean value obtained with the β-lactamase-negative control strain (*S. aureus* ATCC 25923) was subtracted from the mean value obtained for each study strain. Differences between induced β-lactamase activities were tested by the two-tailed Student *t* test (26).

Of the 27 isolates, 4 were HOMO strains. These strains showed no detectable inhibition of growth around disks for eight of the nine β-lactam drugs tested at 35 or 42°C. With cefamandole, zones at 35°C ranged from 12 to 15 mm in diameter and were not affected by incubation temperature. Two of the four strains produced no detectable β-lactamase, and two others produced small amounts of β-lactamase after induction (Table 1).

Twenty-three isolates represented HET strains. Of the 23 strains, 12 produced heavy growth around oxacillin, methicillin, and nafcillin disks at 35°C (HET-1 group) (Fig. 1). HET-1 strains had base-line β-lactamase activities ranging from 0.1 to 1.0 and yielded much higher β-lactamase activities (mean, 5.3) after induction (Table 1).

The remaining 11 HET strains (HET-2) produced less growth around β-lactam-containing disks at 35 and 42°C. The average base-line β-lactamase activity for HET-2 strains was significantly lower than that for HET-1 strains ($P = 0.00002$).

TABLE 1. β-Lactamase activities for 27 study strains of methicillin-resistant *S. aureus*

Group	Strain	β-Lactamase activity[a]	
		Uninduced	Induced
HOMO	MUSC-324	<0.1	<0.1
	MUSC-499	<0.1	<0.1
	UVA-108	0.1	0.4
	SEA-24	0.1	1.3
HET-1	UMC-JOHNSON	0.7	7.0
	UMC-GRIF	0.5	6.0
	STL-5040	0.1	1.2
	MICH-13	0.4	7.1
	NYC-1063	0.5	5.6
	NYC-1281	0.8	5.0
	NYC-797	0.7	4.2
	NYC-1217	0.6	3.8
	NYC-1445	0.7	3.6
	STP-3273	1.0	7.5
	UT-8	0.9	3.9
	MIA-1160	1.0	8.4
HET-2	SEA-25	0.1	1.2
	MICH-30	<0.1	1.1
	HF-S298	0.5	3.5
	MIA-722	0.2	2.7
	HF-3281	0.4	5.3
	SEA-18	0.1	1.8
	ATL-1835	<0.1	1.4
	STL-4171	<0.1	1.2
	LSU-3	0.1	0.7
	ATL-1707	<0.1	2.4
	UT-24	0.1	1.5

[a] Expressed as micromoles of nitrocefin hydrolyzed per hour per milligram (dry weight). Each value represents the average of duplicate determinations.

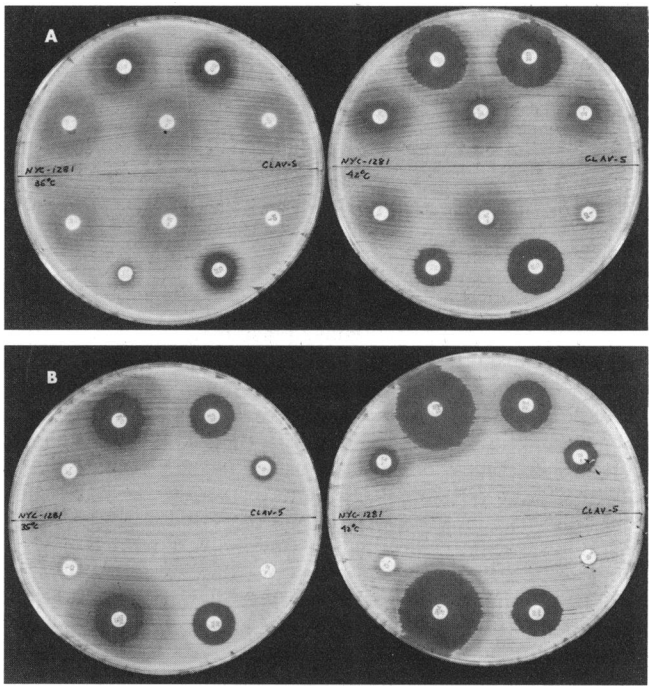

FIG. 1. Susceptibility of strain NYC-1281 to nine β-lactam antibiotics, incubated at 35°C (left plates) and at 42°C (right plates). On the upper half of each plate, 5 μg of CLAV has been added to each antimicrobial disk. Standard antimicrobial disks are present on the lower half of each plate. (A) The antimicrobial disks, left to right, are nafcillin, cefazolin, methicillin, cephalothin, and oxacillin. (B) The antimicrobial disks, left to right, are cloxacillin, imipenem, cefamandole, and penicillin.

Similarly, the mean induced β-lactamase activity for HET-2 strains (2.1) was significantly lower than that for HET-1 strains ($P = 0.0003$) but was significantly higher than that for HOMO strains ($P = 0.037$).

Addition of CLAV to the antibiotic disks had no appreciable effect on zones observed with HOMO strains and yielded no significant increase in the zones of complete growth inhibition for eight of the nine β-lactam drugs when HET-1 strains were tested (Fig. 1; Table 2). With HET-1 isolates, disks containing penicillin plus CLAV yielded significantly larger zones of inhibition than did standard penicillin disks ($P < 0.0005$). With HET-2 strains, the addition of CLAV yielded larger zones of complete growth inhibition for cefazolin ($P > 0.05$), cefamandole ($P = 0.02$), and penicillin ($P = 0.008$).

Most methicillin-resistant *S. aureus* strains produce β-lactamase and are heterogeneously resistant to methicillin (6, 8–13, 22, 24). A few β-lactamase-negative strains that have been described were highly resistant to methicillin, and several of these strains were shown to produce a low-affinity PBP constitutively (4, 8, 13, 22, 24).

Naturally occurring HOMO strains have been reported infrequently (1, 20, 21, 23). Of our 27 unique clinical isolates, 4 were classified as HOMO strains. Two of the four strains examined produced no detectable β-lactamase, and the other two produced only small amounts of β-lactamase after induction. CLAV did not affect the levels of β-lactam resistance of these organisms.

In contrast, all of our HET strains produced β-lactamase. HET-1 strains produced significantly more β-lactamase than did strains that were phenotypically less resistant (HET-2). However, inhibition of β-lactamase by CLAV affected expression of resistance only for those antibiotics that are relatively susceptible to staphylococcal β-lactamase (penicillin, cefazolin, and cefamandole). McDougal and Thornsberry (17) also found that CLAV did not affect methicillin, oxacillin, and cephalothin MICs in methicillin-resistant *S. aureus*. Thus, it appears likely that the level of methicillin resistance in these strains is primarily a function of the amount of low-affinity PBP produced, rather than the amount of β-lactamase present.

TABLE 2. Average zone of complete growth inhibition[a]

Group	Zone of complete growth inhibition (avg diam, in mm) by:																	
	Z	Z-C	MA	MA-C	K	K-C	N	N-C	O	O-C	ME	ME-C	P	P-C	C	C-C	I	I-C
HOMO ($n = 4$)	6	6	12.7	13	7	7.5	6	6	6	6	6	6	6.5	7	6	6	6.2	6.7
HET-1 ($n = 12$)	6.6	6.8	16.7	16.7	11.7	11.9	6	6	6	6	6	6	7	8.9	6	6	18.3	17.7
HET-2 ($n = 11$)	12.2	14.2	20	21.6	19.6	19.6	6	6	6	6.1	6.2	6.5	7.9	11.2	12.1	10.6	24.9	24.8

[a] Abbreviations: Z, cefazolin; -C, with CLAV; MA, cefamandole; K, cephalothin; N, nafcillin; O, oxacillin; ME, methicillin; P, penicillin; C, cloxacillin; I, imipenem. Plates were incubated at 35°C for 24 h.

The fact that β-lactam antibiotics can induce both β-lactamase (9, 14) and PBP 2′ (6, 22, 24) and the observation that mutations in plasmid-mediated penicillinase repressor genes may affect the level of methicillin resistance in methicillin-resistant *S. aureus* suggest that these proteins may be regulated by similar mechanisms (7, 13). Also, Beck et al. (2) have cloned a 3.5-kilobase *Bgl*II fragment that appears to be part of the chromosomal methicillin resistance determinant (*mec*). The cloned fragment hybridized with chromosomal DNA from methicillin-resistant *S. aureus* and with fragments derived from β-lactamase plasmids, suggesting that sequences involved with production of PBP 2′ may be present on some penicillinase plasmids.

These reported observations and the data presented above suggest that the β-lactamase plasmids found in HET strains of methicillin-resistant *S. aureus* may carry a gene or genes that regulate the production of both PBP 2′ and β-lactamase. Thus, plasmid-mediated repressors of the genes that determine PBP 2′ may result in heterogeneous resistance to methicillin by inhibiting production of PBP 2′. Conversely, β-lactamase-negative strains, lacking these repressor genes, may produce PBP 2′ constitutively. This hypothesis may explain why almost all β-lactamase-positive clinical isolates of methicillin-resistant *S. aureus* are heterogeneously resistant to methicillin.

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