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Suppression of Intrinsic Resistance to Methicillin and Other Penicillins in *Staphylococcus aureus*

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The *p*H of the medium in which staphylococcal susceptibility to penicillins was determined was found to make a profound difference (128- to 8,000-fold) in the expression of "intrinsic" resistance, whereas β-lactamase-mediated resistance was only slightly affected by *p*H; methicillin-resistant staphylococci that are β-lactamase-negative are models of pure intrinsic resistance, and the common β-lactamase-producing organisms (methicillin-susceptible) are examples of pure β-lactamase-mediated resistance. Methicillin-resistant staphylococci were unable to express their resistance at *p*H 5.2. However, growth of methicillin-resistant organisms in acid (*p*H 5.2) medium, followed by susceptibility testing at *p*H 7.4, showed no elimination of the genotype for intrinsic resistance, indicating that the *p*H effect was due to suppression, rather than to elimination of the gene determining the intrinsic resistance. These *p*H changes had little effect on the susceptibility of staphylococci that possessed neither intrinsic resistance nor β-lactamase-mediated resistance. Thus, the suppression of "intrinsic" resistance was highly specific, and probably not the result of a change in ionization of the antibiotic, which would have been expected to affect all cells essentially equally. It is unlikely that foci of inflammation in man become sufficiently acid to suppress methicillin resistance of the staphylococci causing infection and inflammation.

Methicillin-resistant strains of *Staphylococcus aureus* by definition are not inhibited by less than 25 μg of methicillin per ml, and some will grow in the presence of >1,600 μg/ml (13), whereas ordinary strains are usually inhibited by 1.6 to 3.1 μg/ml (14). The mechanism for the resistance is not known, but it is not due to antibiotic inactivation and is therefore considered to be "intrinsic." The most convincing evidence for this is that segregants of β-lactamase-producing strains of methicillin-resistant *S. aureus* that have lost the gene determining β-lactamase production fully retain their methicillin resistance (5, 15). Such segregants also retain some resistance to benzylpenicillin, but much less than they had before the loss of the β-lactamase gene (5). Methicillin-resistant strains are also resistant to all of the other semisynthetic penicillins and cephalosporins currently available (4, 7).

In the course of studying the effect of *p*H on the antibacterial activity of a variety of antibiotics, it was noted that the *p*H effect with penicillins was much greater with methicillin-resistant staphylococci than with those that were susceptible to methicillin. The aim of the present study was to define this phenomenon.

MATERIALS AND METHODS

Organisms. All but 1 of the 19 naturally occurring methicillin-resistant staphylococci studied were isolated in the Medical Bacteriology Department of Boston City Hospital by or under the direct supervision of A. Kathleen Daly or Alice MacDonald. The exception was one (penicillinase-negative) strain (M-R Col^{P-}) isolated in England and obtained from K. G. H. Dyke (5). Five methicillin-susceptible strains were studied of which three produced β-lactamase. A β-lactamase-negative segregant of a methicillin-resistant strain (M-R 1^{P+}) was obtained by applying the Haight-Finland technique (8) to individual colonies (12).

Media, antibiotics, and susceptibility testing. Organisms were grown in Difco brain heart infusion (BHI) broth or on Difco heart infusion (HI) agar. The *p*H of the medium was adjusted to indicated values by the addition of concentrated HCl or NaOH; the final *p*H was measured with a Radiometer (Copenhagen, Denmark) *p*H meter for BHI broth or with *p*Hydrion paper (Micro Essential Laboratory, Brooklyn, N.Y.) for HI agar. All antibiotics used were kindly donated by their suppliers: benzylpenicillin by E. R. Squibb & Sons (New Brunswick, N.J.), methicillin and oxacillin by Bristol Laboratories (Syracuse, N.Y.), cloxacillin by Beecham Laboratories, Division of Beecham-Massengill Inc.

(Clifton, N.J.), and cephalothin, cephaloridine, and cephalexin by Lilly Laboratories for Clinical Research (Indianapolis, Ind.).

For antibiotic susceptibility testing in BHI broth, an inoculum of approximately 10^5 organisms (a 10^{-4} dilution of an overnight BHI culture) per ml of test broth was used. Testing on HI agar was done by the agar-dilution method of Steers, Foltz, and Graves (16); the inoculum was a 10^{-2} dilution (in sterile distilled water) of an overnight culture in BHI broth at *p*H 7.4 or a 42-hr culture at *p*H 5.2 (when indicated). In each instance, the results reported are those recorded after 48 hr of incubation at 37 C.

The proportion of methicillin-resistant organisms in a given strain was determined by comparing the colony count on drug-free HI agar with that obtained on HI agar containing 50 μg of methicillin or cloxacillin per ml; in each instance, plates were incubated for 48 hr at 37 C; further incubation, to 96 hr, did not change results.

RESULTS

The effect of *p*H on the intrinsic resistance of *S. aureus* to some penicillins and cephalosporins as determined in BHI broth is shown in Table 1. Note that there was essentially no effect of *p*H on the susceptibility of strain M-S Oxford to any of the β-lactam antibiotics listed in Table 1; M-S Oxford possesses neither the β-lactamase gene nor a mechanism for intrinsic resistance. In contrast, the strains with "pure intrinsic resistance" (no β-lactamase) showed a profound *p*H effect; neither strain M-R Col $^{P-}$ nor M-R 1^{P-} could express resistance to benzylpenicillin, methicillin, or cloxacillin at *p*H 5.2 even after 96 hr of incubation, and this diminution of resistance at *p*H 5.2 represented a 128- to >4,000-fold change in susceptibility.

There was little effect of *p*H on the susceptibility of any of these organisms to cephaloridine (Table 1).

Those organisms possessing the penicillinase gene (M-S 1^{P+} and M-R 1^{P+}) could express resistance to benzylpenicillin in acid medium; the minimal inhibiting concentration (MIC) of

TABLE 1. *Effect of pH of medium (BHI) on methicillin resistance of Staphylococcus aureus*

Antibiotic	*S. aureus* strain[a]	MIC (μg/ml) when tested at		Ratio[b]
		*p*H 7.4	*p*H 5.2	
Methicillin	M-S 1^{P+}	6.2	3.1	2
	M-S Oxford^{P-}	3.1	1.6	2
	M-R 1^{P+}	1,600	12.5	128
	M-R 1^{P-}	1,600	12.5	128
	M-R Col^{P-}	1,600	12.5	128
Cloxacillin	M-S 1^{P+}	1.6	0.2	8
	M-S Oxford^{P-}	0.4	0.1	4
	M-R 1^{P+}	1,600	0.4	4,000
	M-R 1^{P-}	1,600	0.4	>4,000
	M-R Col^{P-}	1,600	0.4	>4,000
Benzylpenicillin	M-S 1^{P+}	1,600	400	4
	M-S Oxford^{P-}	0.05	0.05	1
	M-R 1^{P+}	800	50	16
	M-R 1^{P-}	50	0.1	500
	M-R Col^{P-}	50	0.05	1,000
Cephaloridine	M-S 1^{P+}	6.2	6.2	1
	M-S Oxford^{P-}	0.1	0.05	2
	M-R 1^{P+}	50	25	2
	M-R 1^{P-}	25	12.5	2
	M-R Col^{P-}	50	6.2	8
Cephalexin	M-S 1^{P+}	19.3	2.4	8
	M-S Oxford^{P-}	4.8	1.2	4
	M-R 1^{P+}	>300	9.6	≥64
	M-R 1^{P-}	>300	9.6	≥64
	M-R Col^{P-}	>300	9.6	≥64

[a] M-S, methicillin-susceptible; M-R, methicillin-resistant; P^+, β-lactamase-producing organism; P^-, no β-lactamase produced. All tests at 37 C, 48 hr of incubation, by the broth dilution technique (Difco brain heart infusion) with inocula of about 10^5 organisms per ml.

[b] Ratio of the MIC at *p*H 7.4 to the MIC at *p*H 5.2.

TABLE 2. *Effect of pH of HI agar on methicillin resistance of Staphylococcus aureus*

Antibiotic	*S. aureus* strain	MIC (μg/ml) when tested at			Ratios[a]	
		*p*H 7.4	*p*H 5.5	*p*H 5.2 ± 1	*p*H 7.4 to 5.5	*p*H 7.4 to 5.2 ± 1
Methicillin	M-S 2^{P+}	6.2	6.2	3.1	1	2
	M-S 3^{P+}	3.1	6.2	1.6	0.5	2
	M-S 4^{P+}	6.2	6.2	12.5	1	0.5
	M-S Oxford^{P-}	1.6	6.2	3.1	0.25	0.5
	M-S 209 P^{P-}	3.1	3.1	1.6	1	2
	M-R 1^{P+}	>800	200	3.1	>4	>250
	M-R 2^{P+}	>800	200	3.1	>4	>250
	M-R 3^{P+}	>800	200	3.1	>4	>250
	M-R 4^{P+}	>800	200	3.1	>4	>250
	M-R 5^{P+}	>800	200	3.1	>4	>250
	M-R 6^{P+}	>800	200	6.2	>4	>125
	M-R 7^{P+}	>800	400	6.2	>2	>125
	M-R 8^{P+}	800	400	6.2	2	125
	M-R 9^{P+}	400	50	3.1	8	125
	M-R 10^{P+}	800	200	3.1	4	250
	M-R 11^{P+}	400	25	3.1	32	125
	M-R 12^{P+}	800	200	25	4	32
	M-R 13^{P+}	800	200	3.1	4	250
	M-R 14^{P+}	400	25	3.1	16	125
	M-R 15^{P+}	200	50	3.1	4	64
	M-R 16^{P+}	800	50	3.1	16	250
	M-R 17^{P+}	800	25	3.1	32	250
	M-R 18^{P+}	800	25	3.1	32	250
	M-R Col^{P+}	>800	400	12.5	>2	>64
Cloxacillin	M-S 2^{P+}	1.6	3.1	0.8	0.5	2
	M-S 3^{P+}	1.8	0.4	0.4	2	2
	M-S 4^{P+}	1.6	3.1	0.8	0.5	2
	M-S Oxford^{P-}	1.6	6.2	0.8	0.25	2
	M-S 209 P^{P-}	0.8	0.4	0.4	2	2
	M-R 1^{P+}	>800	400	0.8	>2	>1,000
	M-R 2^{P+}	>800	400	3.1	>2	>250
	M-R 3^{P+}	>800	800	0.8	>1	>1,000
	M-R 4^{P+}	>800	400	3.1	>2	>250
	M-R 5^{P+}	>800	400	1.6	>2	>500
	M-R 6^{P+}	>800	800	3.1	>1	>250
	M-R 7^{P+}	>800	800	1.6	>1	>500
	M-R 8^{P+}	200	200	1.6	1	125
	M-R 9^{P+}	800	100	0.8	8	1,000
	M-R 10^{P+}	400	50	0.8	8	500
	M-R 11^{P+}	12.5	6.2	0.8	2	16
	M-R 12^{P+}	800	200	0.8	4	1,000
	M-R 13^{P+}	800	400	0.8	2	1,000
	M-R 14^{P+}	400	12.5	0.8	32	500
	M-R 15^{P+}	800	50	0.8	16	1,000
	M-R 16^{P+}	800	50	0.8	16	1,000
	M-R 17^{P+}	800	6.2	0.8	125	1,000
	M-R 18^{P+}	400	12.5	0.8	31	500
	M-R Col^{P-}	>800	800	3.1	>1	>250

each was reduced only one- to fourfold at *p*H 5.2. However, in tests with methicillin and cloxacillin, the resistant strain (M-R 1^{P+}) showed a profound reduction in MIC (128- and 4,000-fold), and M-S 1^{P+} showed only a minimal change, comparable to M-S Oxford.

Susceptibility tests were also performed in HI agar with all 24 strains against the following β-lactam antibiotics: methicillin, cloxacillin, cephalothin, and cephaloridine (Table 2). The inability of methicillin-resistant strains to exhibit resistance at *p*H 5.2 was again evident, and the MIC

TABLE 2—*Continued*

Antibiotic	*S. aureus* strain	MIC (μg/ml) when tested at			Ratios[a]	
		*p*H 7.4	*p*H 5.5	*p*H 5.2 ± 1	*p*H 7.4 to 5.5	*p*H 7.4 to 5.2 ± 1
Cephalothin	M-S 2^{P+}	25	25	12.5	1	2
	M-S 3^{P+}	1.6	1.6	1.6	1	1
	M-S 4^{P+}	12.5	12.5	12.5	1	1
	M-S Oxford^{P-}	0.8	3.1	6.2	0.25	0.125
	M-S 209 P^{P-}	0.8	1.6	0.8	0.5	1
	M-R 1^{P+}	200	25	12.5	8	16
	M-R 2^{P+}	200	25	12.5	8	16
	M-R 3^{P+}	200	50	12.5	4	16
	M-R 4^{P+}	200	50	12.5	4	16
	M-R 5^{P+}	200	25	12.5	8	16
	M-R 6^{P+}	200	25	12.5	8	16
	M-R 7^{P+}	>200	50	12.5	>4	>16
	M-R 8^{P+}	100	50	12.5	2	4
	M-R 9^{P+}	100	25	12.5	4	8
	M-R 10^{P+}	100	12.5	12.5	8	8
	M-R 11^{P+}	50	12.5	12.5	2	2
	M-R 12^{P+}	100	50	50	2	2
	M-R 13^{P+}	100	100	50	1	2
	M-R 14^{P+}	100	50	12.5	2	8
	M-R 15^{P+}	50	12.5	12.5	4	4
	M-R 16^{P+}	50	12.5	12.5	4	4
	M-R 17^{P+}	100	12.5	12.5	8	8
	M-R 18^{P+}	100	50	12.5	2	8
	M-R Col^{P-}	>200	50	12.5	>4	>16
Cephaloridine	M-S 2^{P+}	100	200	800	0.5	0.125
	M-S 3^{P+}	6.2	25	50	0.25	0.125
	M-S 4^{P+}	50	200	800	0.25	0.06
	M-S Oxford^{P-}	1.6	3.1	3.1	0.5	0.5
	M-S 209 P^{P-}	0.4	0.2	0.1	2	4
	M-R 1^{P+}	50	200	400	0.25	0.125
	M-R 2^{P+}	100	400	800	0.25	0.125
	M-R 3^{P+}	100	200	800	0.5	0.125
	M-R 4^{P+}	100	400	800	0.25	0.125
	M-R 5^{P+}	100	200	800	0.5	0.125
	M-R 6^{P+}	50	200	800	0.25	0.06
	M-R 7^{P+}	100	400	800	0.25	0.125
	M-R 8^{P+}	200	400	800	0.5	0.25
	M-R 9^{P+}	50	200	400	0.25	0.125
	M-R 10^{P+}	50	200	800	0.25	0.06
	M-R 11^{P+}	25	100	400	0.25	0.06
	M-R 12^{P+}	200	400	>800	0.5	<0.25
	M-R 13^{P+}	200	400	>800	0.5	<0.25
	M-R 14^{P+}	200	400	>800	0.5	<0.25
	M-R 15^{P+}	50	200	400	0.25	0.125
	M-R 16^{P+}	50	200	400	0.25	0.125
	M-R 17^{P+}	100	200	800	0.5	0.125
	M-R 18^{P+}	100	400	800	0.25	0.125
	M-R Col^{P-}	50	50	50	1	1

[a] Ratios of the MIC at *p*H 7.4 to the MIC at *p*H 5.5 and at *p*H 5.2 ± 1.

values were comparable to those observed in liquid. It was noted that resistance to cephalothin was also significantly diminished at *p*H 5.2, but that cephaloridine remained an exception, as it was in liquid medium, in that the lower *p*H had only a minimal effect on its activity. For each of the five methicillin-susceptible strains, the MIC of methicillin at *p*H 7.4 was one-half to two times the

MIC at *p*H 5.2, irrespective of the presence of a β-lactamase gene. For most of the 19 M-R strains studied, tests at *p*H 5.5 yielded results fairly similar to those obtained at *p*H 7.4 with significantly less reversal of intrinsic resistance; however, 3 M-R strains showed a 32-fold change at *p*H 5.5, compared with 125- to 250-fold declines in MIC at *p*H 5.2 (when compared with MIC values at *p*H 7.4).

Organisms grown at *p*H 5.2 and then tested at *p*H 7.4 for the proportion of colony-forming units (CFU) capable of growing in the presence of 50 μg of cloxacillin per ml yielded essentially the same proportion as when grown at *p*H 7.4 (Table 3). In contrast, no CFU appeared on cloxacillin plates at *p*H 5.2 whether grown at *p*H 7.4 or 5.2 immediately before scoring.

Similarly, when 50 μg of methicillin per ml was incorporated into the agar, no resistant organisms appeared if the medium was at *p*H 5.2 (even if incubated 96 hr), whereas 34% of the inoculum grew on the *p*H 7.4 plates that contained 50 μg of methicillin per ml. A comparison of CFU from a given liquid culture of M-R cells detected on drug-free nutrient agar at *p*H 5.2 and 7.4 showed significantly fewer CFU at *p*H 5.2 (usually 50% or less) than at *p*H 7.4.

DISCUSSION

The *p*H effect on staphylococcal susceptibility to penicillins is clearly not due to acid inactivation of antibiotic, for that would have raised, rather than lowered, the MIC values and would have affected all strains in a similar way. Change in ionization of the antibiotic is a well-known cause for large changes in antibiotic activity associated with changes of *p*H of the medium (1, 2, 11); however, that would be an unlikely explanation for the very selective effect reported. The *p*K of benzylpenicillin is 2.7 (17), and that of other semisynthetic penicillins is similar. Thus, much more of that antibiotic would be un-ionized at *p*H 5.2 than at *p*H 7.4, and, on the basis of ionization, greater activity would have been expected against all organisms at the lower *p*H.

TABLE 3. *Effect of pH of growth medium on proportion of M-R Col^{P-} cells resistant to cloxacillin or methicillin*[a]

*p*H of growth medium	Antibiotic in medium[b]	Proportion of cells growing when tested at	
		*p*H 5.2	*p*H 7.4
5.2	Cloxacillin	0[c]	0.64
	Methicillin	—	—
7.4	Cloxacillin	0	0.73
	Methicillin	0	0.34

[a] Plates were scored after 48 hr at 37 C; plates containing methicillin were also scored after 96 hr and yielded identical results.

[b] The concentration of both antibiotics was 50 μg/ml.

[c] Zero indicates that no cells were found with an inoculum of 10^9 colony-forming units; therefore, $<10^{-9}$ would be more precise.

Although the selective *p*H effect on antibiotic susceptibility noted suggests a direct action of *p*H on the methicillin-resistant cells (possibly change in ionization of a receptor or inhibition of an active resistance mechanism) rather than on the antibiotic, the possibility still exists that a change in ionization of the antibiotic is what is important. If this were the case, it would suggest that ionization of the penicillin was relatively unimportant for access to the receptor on the methicillin-susceptible strains but critically important for activity against methicillin-resistant strains.

The fact that growth of cells in medium at *p*H 5.2 before antibiotic susceptibility testing at *p*H 7.4 failed to suppress antibiotic resistance indicates (i) that the gene determining methicillin resistance was not eliminated during growth at acid *p*H and (ii) that suppression of intrinsic resistance is readily reversed (also supported by fact that cells grown at *p*H 7.4 appeared to be methicillin-susceptible if tested at *p*H 5.2). Membrane lipids produced by staphylococci grown in acid medium have been shown to differ from those obtained from cells grown at neutral *p*H (9), and such a structural change could be the basis for the change in antibiotic susceptibility. However, this would seem to be a less plausible explanation than a characteristic that could be more readily changed, e.g., state of ionization. Thus, changes in ionization (of antibiotic or of a wall component) or acid suppression of an as yet unidentified resistance mechanism would seem to be more attractive explanations.

Another possible explanation for the *p*H suppression of methicillin resistance which must be considered is that the acid medium itself prevents methicillin-resistant cells from growing, whether or not antibiotic is present: the only cells then growing would be the methicillin-susceptible portion of the original heterogeneous population. This would account for the absence of methicillin resistance at *p*H 5.2 and also for the lower colony counts on drug-free medium at *p*H 5.2. However, this postulated explanation would not account for the fact that resistance to cephaloridine can be expressed at *p*H 5.2. Hence, it cannot be concluded that the acid medium by itself is selectively inhibiting growth of the resistant cells.

It has previously been reported that methicillin

resistance of *S. aureus* cannot be expressed at high temperatures (3, 10), and the possibility that the expression of the resistance mechanism is easily inhibited, by both heat and acid, is attractive.

It seems unlikely that acid suppression of methicillin resistance in *S. aureus* would be clinically important. A fall in *p*H to 5.5 would only minimally lower the inhibitory concentration of methicillin for most resistant staphylococci. However, a few of the methicillin-resistant strains did show 32-fold changes in MIC at *p*H 5.5, and in infections caused by such strains *p*H suppression of methicillin resistance may be clinically important. Although methicillin resistance could not be expressed in an inflammatory lesion with a *p*H of 5.2, we are not aware of this degree of acidity occurring frequently in infected foci.

The lowest *p*H of 33 pneumonic pleural fluids reported by Finland was 5.4; 2 were *p*H 5.6 but 30 of the 33 were *p*H 6.3 or higher (6).

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