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MAGNESIUM-ION SUSCEPTIBILITY OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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METHICILLIN-RESISTANT *Staphylococcus aureus* differs from methicillin-sensitive staphylococci in several other characteristics. Methicillin-resistant strains more often produce enterotoxin B (Hallander and Laurell, 1971), fail to produce a tween-splitting enzyme (Jevons, Coe and Parker, 1963), possess temperature-sensitive autolytic activity (Seligman, 1970), and differ from methicillin-sensitive strains in their mobility curves determined by electrophoresis of whole cells at varying pH (Hill and James, 1972). Penicillinase production (Dyke, Jevons and Parker, 1966), resistance to streptomycin and tetracycline (Dornbusch, Hallander and Löfquist, 1969; Parker and Hewitt, 1970; Hallander and Laurell, 1971; Rountree and Vickery, 1973), and resistance to sulphonamides (Dornbusch *et al.*, 1969; Seligman, 1973), are found in almost all naturally occurring methicillin-resistant strains but in a variable proportion of methicillin-sensitive strains.

This report describes magnesium-ion susceptibility as another characteristic of methicillin-resistant *S. aureus*.

MATERIALS AND METHODS

Strains. Methicillin-resistant *S. aureus* were obtained from the sources indicated in table I. Methicillin-sensitive derivatives of methicillin-resistant strain no. 13136 were isolated by Al Salihi and James (1972). The methicillin-resistant transductant no. 451-2 was obtained by Cohen and Sweeney (1970) with strain Villaluz as donor and no. 8325 ($\alpha\omega$) as recipient.

The methicillin-sensitive strains were clinical isolates from Kings County Hospital, NY (24 strains), or were obtained from the following sources: Dr J. L. Strominger, Madison, Wis. (two strains); Dr F. Kaiser, Zurich, (two strains); and one strain each from the American Type Culture Collection, Rockville Md; the Center for Disease Control, Atlanta, Ga; Dr R. P. Novick, NY; Dr A. Bernheimer, NY; Dr F. Rozgonyi, Debrecen; Dr H. Sheinfeld, San Francisco, Calif.; and the author, Los Angeles, Calif.

Penicillinase-negative variants are referred to by the strain designation followed by the letter N.

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Media. Cultures were grown on Trypticase Soy Agar (TSA, Baltimore Biological Laboratories), or in Trypticase Soy Broth (TSB, Baltimore Biological Laboratories).

Inoculation of strains on to test agars. Overnight cultures were diluted 10^{-3} in saline and inoculated with a replica-plating apparatus (Steers *et al.*, 1959). The inoculum size was 500–1000 organisms.

Magnesium-ion susceptibility. A series of agars containing 0.1M increments of magnesium-ion from 0.1 to 1.0M was prepared by adding filter-sterilised $MgCl_2$ to liquefied TSA at 50°C. All agars were incubated overnight at 37°C before inoculation. After incubation, the agars were examined daily for a least three days. The results are reported as the lowest Mg^{++} concentration giving no growth, no more than one colony or a faint film of growth after one day's incubation.

TABLE I
Sources of methicillin-resistant strains of Staphylococcus aureus

Contributors and area of isolation of strains		Number of strains	
		contributed	epidemiologically distinct
Dr F. H. Kaiser	Zurich, Switzerland	8	7
Dr K. Dornbusch and			
Dr H. Ericsson	Stockholm, Sweden	8	3
Dr G. Pulverer	Cologne, Germany	6	5
Dr M. P. Jevons and			
Dr E. Asheshov	London, England	6	5
Dr K. Dornbusch	Uppsala, Sweden	2	1
Dr K. Siboni	Odense, Denmark	4	3
Dr Y. A. Chabbert	Paris, France	4	4
Dr E. Yourassowsky	Brussels, Belgium	4	3
Author	New York, NY, USA	3	1
Dr W. M. M. Kirby	Seattle, Wash., USA	2	2
Dr F. Rosgonyi	Debrecen, Hungary	1	1
Dr K. R. Ericksen	Copenhagen, Denmark	1	1
Author	Los Angeles, Calif., US	1	1*
Dr J. Borowsky	Gdańsk, Poland	1	1
Totals		51	38

* This strain was isolated from a burned patient recently arrived from South Vietnam.

Calcium-ion susceptibility. Calcium-ion susceptibility was determined in a manner similar to magnesium-ion susceptibility except that 0.6M $CaCl_2$ was the lowest test concentration.

Methicillin resistance. All methicillin-resistant strains inoculated as above with the replica-plating device grew within two days on TSA containing 5% NaCl and methicillin 10 µg per ml.

Tween-splitting reaction. The test agar contained 1% Tween 80 and 0.05M $CaCl_2$. Sierra (1957) had employed 0.01% $CaCl_2$. Incubation was for two days at 37°C. A positive test consisted of a precipitate in the medium around the inoculum.

Enterotoxin production was determined by Dr Merlin S. Bergdoll, of the Food Research Institute, University of Wisconsin.

Selection of penicillinase-negative variants. Overnight cultures inoculated with isolated colonies were diluted 3×10^{-6} and plated on to TSA in 0.05-ml linear streaks. After 1 day's incubation penicillinase-negative variants were identified by the N-phenyl-1-naphylamine-azo-*o*-carboxybenzene (PNCB) test (Novick and Richmond, 1965). The colonies were covered with alkalinised PNCB in N-N dimethylformamide for 10 min., excess fluid was poured off, and the colonies were flooded with a solution containing benzylpenicillin 35 mg

per ml. PNCB, a pH indicator insoluble in acid, precipitates over colonies or portions of colonies producing penicillinase. Colonies or sectors of colonies on which PNCB did not precipitate were streaked on to TSA.

Penicillinase production. TSA and TSA with methicillin 0.5 µg per ml were inoculated with 10^{-3} diluted overnight cultures by means of the Steers replica-plating apparatus. After overnight incubation the resulting growth was treated with PNCB and penicillin (see above).

Phage typing was done in the laboratory of Dr Richard Novick, Health Research Institute of the City of New York.

Coagulase production. From an overnight culture, 0.1 ml was added to 0.5 ml of horse plasma and incubated for 24 hours.

Acidification of mannitol. Salt mannitol agar was inoculated with the replica-plating apparatus and incubated overnight.

RESULTS

All strains were coagulase positive. All methicillin-resistant strains produced penicillinase and used mannitol. Three methicillin-sensitive, penicillinase-producing strains and one methicillin-sensitive, penicillinase-negative strain failed to use mannitol.

TABLE II
Magnesium-ion susceptibility of S. aureus strains

Methicillin susceptibility and penicillinase production	Number of strains							
	examined	suppressed by Mg^{++} at concentration (molar) of						
		0.2	0.3	0.4	0.5	0.6	0.7	0.8
Methicillin resistant	38	33	1	2	1	1	0	0
Methicillin sensitive, penicillinase positive	17	0	1	7	2	4	2	1
	12	0	3	4	2	2	1	0
penicillinase negative								

Of the 51 methicillin-resistant strains tested, 38 were epidemiologically distinct in that they were isolated in different cities (table I) or had a different phage-typing pattern, different degrees of magnesium resistance, or produced different enterotoxins. By these criteria, 29 of the 35 methicillin-sensitive strains were epidemiologically distinct. Thirty-three (87%) of 38 methicillin-resistant strains but none of 29 methicillin-sensitive strains were suppressed by 0.2M $MgCl_2$ (table II).

Production of enterotoxin B was found in 30 (79%) of 38 methicillin-resistant strains and in 6 (21%) of 29 methicillin-sensitive strains (table III). Lack of a tween-splitting enzyme was a characteristic of 32 (84%) of 38 methicillin-resistant and 5 (17%) of 29 methicillin-sensitive strains. Of the 38 methicillin-resistant strains, 24 had the combined characteristics of susceptibility to 0.2M Mg^{++} , inability to split Tween 80, and ability to produce enterotoxin B (table IV).

Some of the methicillin-resistant strains were as resistant as methicillin-sensitive strains to magnesium (table II). Additional evidence that susceptibility to magnesium ion was not invariably associated with methicillin resistance was

TABLE III
Characteristics of methicillin-resistant and methicillin-sensitive S. aureus strains

Methicillin susceptibility	Number of strains	Percentage of strains						
		suppressed by Mg ⁺⁺ 0.2M	Tween-splitting negative	producing enterotoxin*				
				A	B	C	D	nil
Methicillin resistant	38	87	84	5	79	0	0	16
Methicillin sensitive	29	0	17	7	21	10	7	62

* Two methicillin-sensitive strains each produced two types of enterotoxin.

obtained in a comparison of genetically-related strains (table V). Although a methicillin-resistant transductant (no. 451-2) was more sensitive to magnesium than was the methicillin-sensitive recipient strain (no. 8325 $\alpha\omega$), both penicillinase-negative variants had the same susceptibility to magnesium ion.

TABLE IV
Characteristics of 38 epidemiologically distinct strains of methicillin-resistant S. aureus

Number of strains	Suppressed by magnesium at concentration (molar) of	Tween-splitting reaction	Enterotoxin production
24	0.2	—	B
1	0.2	—	A
4	0.2	—	nil
3	0.2	+	B
1	0.2	+	nil
1	0.3	—	B
1	0.4	—	B
1	0.4	—	A
1	0.5	+	nil
1	0.6	+	B

+ = Reaction positive; — = reaction negative.

Because plasmids mediating penicillinase production may also carry resistance to inorganic salts (Novick and Roth, 1968), the increased magnesium sensitivity of the penicillinase-negative variants compared with their penicillinase-producing parents suggests that some degree of magnesium resistance may be associated with the penicillinase plasmid in 8325 ($\alpha\omega$). Novick and Roth (1968) did not detect any resistance to magnesium sulphate in this plasmid; however the magnesium sulphate concentration used in their screening test was insufficient to inhibit strain no. 8325.

TABLE V
Comparison of genetically related strains of S. aureus

Strain	Relationship	Methicillin susceptibility	Penicillinase production	Suppressed by magnesium at concentration (molar) of	Tween-splitting reaction	Enterotoxin production	Phage-typing pattern at RTD (or at 1000 RTD)
Villaluz 8325(ααα) 45102 8325N 4510-2N	donor	R	+	0.3	—	B	53/85
	recipient	S	+	0.8	+	nil	6/42E/47/53/54/75/83A/85/81
	transductant	R	+	0.6	+	nil	not typable (47/53/75/85)
	penicillinase-negative variant	S	—	0.4	+	nil	6/47/53/54/75/83A/85/81
	penicillinase-negative variant	R	—	0.4	+	nil	not typable (47/53/75/85)
13136 13136 Met S	parent	R	+	0.2	—	B	53/77
	methicillin-sensitive variant	S	—	0.2	—	B	53/77
13137 13137N 13137N Mg R	parent	R	+	0.2	—	B	42E/47/53/54/75/77/84/85
	penicillinase-negative variant	R	—	0.2	—	B	42E/47/53/54/75/77/84/85
	magnesium-resistant mutant	R	—	0.4	...	...	...

R = resistant; S = sensitive; + = positive; — = negative; ... = not tested.

A methicillin-sensitive, penicillinase-negative variant of strain no. 13136 and a methicillin-resistant, penicillinase-negative variant of strain no. 13137 each had the magnesium-ion susceptibility of their parental strains (table V). A magnesium-resistant mutant of strain no. 13137N remained methicillin resistant.

In contrast to the results of Dornbusch (1971) with strains DU4916, enterotoxin-B production was neither co-transduced from strain Villaluz nor co-eliminated with methicillin resistance in strain no. 13136.

Susceptibility to calcium had a similar distribution in methicillin-sensitive and -resistant strains (table VI). Results with magnesium sulphate were similar to those with magnesium chloride.

TABLE VI
Calcium-ion susceptibility of S. aureus strains

Methicillin susceptibility and penicillinase production	Number of strains			
	examined	suppressed by Ca^{++} at concentration (molar) of		
		≤ 0.6	0.7	0.8
Methicillin resistant	37	11	26	0
Methicillin sensitive, penicillinase positive penicillinase negative	16	4	12	0
	11	4	7	0

DISCUSSION

Relative susceptibility to magnesium ions is a characteristic of many methicillin-resistant strains of *S. aureus*. The studies with calcium chloride and magnesium sulphate indicated that the magnesium ion and not the chloride ion was responsible for the suppressive effect observed with magnesium chloride.

The occurrence of methicillin-resistant, magnesium-resistant strains, failure of magnesium susceptibility to be co-transduced with methicillin resistance, failure of variants losing methicillin resistance to acquire magnesium resistance, and failure of magnesium-resistant mutants of a methicillin-resistant strain to become methicillin sensitive indicate that methicillin resistance and magnesium sensitivity are biochemically distinct and genetically unlinked in the strains studied.

The biochemical mechanism and the biological significance of methicillin resistance are poorly understood. The strains studied were isolated from many parts of the world over a span of more than a decade, yet 63% of the methicillin-resistant strains were unusually susceptible to magnesium ion, produced enterotoxin B and failed to produce tween-80 splitting enzyme. The association of these four unusual and apparently unrelated characteristics suggests that these strains have an undiscovered ecological role. Accordingly methicillin resistance may confer some survival advantage in addition to resistance to

methicillin and the other β -lactam antibiotics. Dyke *et al.* (1966), noting that methicillin-resistant strains were isolated from several parts of the world before methicillin was used, have also postulated that methicillin resistance may confer some other advantage. Knowledge of the nature of this advantage may help to explain the marked geographical differences in the prevalence of methicillin resistance.

SUMMARY

The susceptibility to magnesium ion of 38 epidemiologically distinct strains of methicillin-resistant *Staphylococcus aureus* was investigated. Eighty-seven per cent were inhibited by 0.2M Mg^{++} . None of 29 naturally-occurring methicillin-sensitive strains were inhibited by less than 0.3M Mg^{++} . Methicillin-resistant *S. aureus* strains were not especially susceptible to calcium ions. Neither a biochemical association nor genetic linkage was demonstrated between susceptibility to magnesium ion and resistance to methicillin. Susceptibility to magnesium ion, production of enterotoxin B, and inability to split Tween 80 were three unusual characteristics biochemically unrelated to methicillin resistance which were concomitantly present in 63% of methicillin-resistant *S. aureus*.

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