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CORRELATION OF PENICILLINASE PRODUCTION WITH PHAGE TYPE AND SUSCEPTIBILITY TO ANTIBIOTICS AND HEAVY METALS IN *STAPHYLOCOCCUS AUREUS*

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SUMMARY. One hundred and thirty-nine bacteraemia strains of *Staphylococcus aureus*, representing different combinations of phage type and susceptibility to antibiotics and to cadmium (Cd), arsenate (As) and mercury (Hg), were investigated for penicillinase production. The determination of enzyme activity in induced and uninduced conditions was performed by iodometric titration. The amount of penicillinase produced could be correlated with phage pattern. Epidemically occurring strains of the 94,96 and the 83A complexes produced the largest amount of penicillinase, whereas strains of the 52,52A,80,81 complex were weaker producers. Group-II and group-III strains produced the smallest amount.

Susceptibility to antibiotics and to Cd, As and Hg could not be correlated with enzyme activity, but strains resistant to penicillin plus tetracyclines and strains resistant only to Cd did produce less enzyme than strains with other resistance patterns. The percentage mean values of extracellularity of the enzyme was highest amongst strains of the 94,96 complex and of type 95. Four strains had constitutive production, one being macro-constitutive and three micro-constitutive. All four strains represented rare combinations of the above properties but were susceptible to fusidic acid. The importance of penicillinase production by epidemically occurring strains is discussed.

INTRODUCTION

Resistance to penicillin G in *Staphylococcus aureus* depends mainly upon the production of a penicillinase enzyme, and the amount of enzyme produced varies from

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strain to strain. Richmond *et al.* (1964) investigated 181 selected strains and found that the active production of penicillinase closely correlated with multiple antibiotic resistance and resistance to mercury salts. An exception to this rule was that five strains belonging to the 52,52A,80,81 complex and resistant only to penicillin showed high penicillinase activity. The authors suggested that the ability to produce much penicillinase was a characteristic of "hospital staphylococci" and might be an essential prerequisite for the acquisition of resistance to other antibiotics.

From 1964 until now, the staphylococcal flora in Denmark (Rosendal *et al.*, 1976; Rosendal and Bentzon, in press) and in other countries (Shanson, 1981; Boyce and Causey, 1982) has changed in regard to phage type and susceptibility to antibiotics. Rosdahl and Rosendal (1980) found that these changes were connected with changes in resistance to heavy metals. Resistance to mercury was most often found in multi-resistant strains but rarely in strains resistant only to penicillin.

The present investigation was performed to record the connection between the above three properties and the amount of penicillinase produced by the individual strain. The material has been supplemented with recently isolated representatives of the "newer" epidemically occurring strains—type 95 and members of the 94,96 complex (Rosendal and Bentzon, in press)—to determine whether the suggestion by Richmond *et al.* (1964) about "hospital staphylococci" would also be valid for strains from the latest period.

MATERIAL AND METHODS

From 139 patients, 139 bacteraemia strains of *S. aureus*, were investigated—112 strains isolated during 1957–1974 and examined previously (Rosdahl and Rosendal, 1980) and 27 strains isolated during 1978–1980. Phage typing was performed (Blair and Williams, 1961; Rosendal *et al.*, 1976); all non-typable strains and strains referred to more than one serological group (Parker, 1962) were omitted, but several representatives of the three phage complexes 52,52A,80,81; 83A and 94,96 and phage type 95, which have had epidemic significance in Denmark, were included in the study. The strains were selected according to phage type and antibiotic and heavy-metal susceptibility pattern (see below) in such a way that all possible combinations of the most common patterns of these three characteristics were represented.

During the year of isolation, all the strains were examined for susceptibility to penicillin (P), streptomycin (S), tetracyclines (T), erythromycin, methicillin and gentamicin by a 20-h pre-diffusion method (Thomsen, 1962, 1964). Strains of the following susceptibility patterns were selected: P, PS, PT, which signify that the strains were resistant to the indicated antibiotic(s) and sensitive to those omitted. PST $\pm$ signifies that the strains might be resistant to other antibiotics as well.

Susceptibility to cadmium acetate (Cd), sodium arsenate (As) and mercuric chloride (Hg) was determined as described by Rosdahl and Rosendal (1980). Strains with susceptibility pattern 0 (susceptible to all three metals), Cd (resistant to Cd but susceptible to As and Hg), Cd, As (resistant to Cd and As) and Cd, As, Hg (resistant to all three metals) were selected for this survey.

Before tests for penicillinase production, all strains were re-tested for resistance to Cd, As, and Hg as described above, and for susceptibility to the above mentioned antibiotics, and to fusidic acid (F), which was tested by a diffusion method with Biodisk PDM (Ericsson, Högman and Wickman, 1954). Only strains having their original susceptibility pattern were included.

The properties of all 139 strains are shown in table I. To illustrate special problems, the original 55 strains were later supplemented with 84 strains: 19 of the 52,52A, 80,81 complex, 12 of group II, 8 of group III, 12 of the 83A complex, 17 of the 94,96 complex and 10 of type 95. In some cases, however, it was not possible to find representatives of all phage groups with the special combination of properties, either among 2956 strains originally investigated (Rosdahl and Rosendal, 1980), or among strains examined later.

Penicillinase assay was by the iodometric method of Perret (1954). One penicillinase unit is defined as the activity that destroys 1.0 μ mol of benzyl penicillin/h at 30°C and pH 5.9. Penicillinase concentrations are expressed as penicillinase units/mg dry wt of bacteria. The bacteria to be tested were inoculated into 10 ml of 1% (v/v) CY medium (Richmond, 1963) containing 0.4% (w/v) glucose (Richmond *et al.*, 1964) and shaken overnight at 35°C. The next day, 100 ml of warmed CY broth with glucose was seeded with some of the above culture to give a density of 0.1 mg dry wt of bacteria/ml. This culture was shaken for 1 h at 35°C and then induced with methicillin 0.5 μ g/ml of culture. Shaking was continued for a further 3.5 h. The penicillinase activity in the whole culture and in the supernate was determined. The basal level of enzyme activity was measured in similar conditions without methicillin; only the activity in the whole culture was determined. Induction ratio was defined as the amount of enzyme produced in the whole culture after induction, divided by the amount produced without induction.

The percentage of extracellularity was calculated as the concentration of enzyme in the supernate $\times$ 100 divided by the concentration of enzyme in the whole culture. The accuracy of the iodometric titration is within the limits of normal volumetric techniques. If the penicillinase production of a strain is measured under well controlled conditions, as described above, the maximum variation from day to day or from culture to culture of the same strain is within the limits of $\pm 20\%$.

Loss of penicillinase plasmids was studied as described by Asheshov (1966) by cultivation at elevated temperature and prolonged storage.

RESULTS

Amount of enzyme produced

In table I the test strains are divided according to phage pattern and susceptibility to antibiotics and heavy metals; the mean values of the induced penicillinase produced by the strains are shown.

Correlation with phage type. As seen in table II, strains of phage group II produced the least penicillinase, just less than those of group III. Production by strains of the 52,52A, 80,81 complex was of the same order as by the other strains in group I. Type 95 strains had an intermediate level of production, whereas strains of the 94,96 and 83A complexes produced the greatest amount of enzyme.

From the figure, however, it can be seen that production by the various strains within the groups/complexes was not uniform, except for type-95 strains, even though the results as a whole, as shown in table II, were confirmed. Within the two complexes with the greatest production, only two out of 19 strains (94,96) and one out of 20 strains (83A) produced 0–50 units in comparison with 9 out of 21 group-II strains and 8 out of 22 group-III strains. However, one group-II strain and one of the 52,52A,80,81 strains did produce ≥ 200 units/mg, a production otherwise reached by only 8 of the 94,96 strains and 5 of the 83A strains.

Correlation with antibiotic susceptibility. Antibiotic susceptibility could not be correlated with the amount of penicillinase produced (table I). The mean values of the amount of penicillinase produced by all strains within the three antibiotic patterns P, PS and PST $\pm$ did not differ by much, but strains with the pattern PT produced somewhat less than did strains with the other patterns. Strains with the same antibiotic-susceptibility pattern did not have a uniform enzyme production. Even among strains resistant to PT, high-concentration penicillinase producers from the 94,96 and the 83A complexes were found.

The correlation between phage pattern and enzyme production was also found within the various antibiotic-susceptibility groups. There were, however, a few

TABLE I

Inducible penicillinase production in 139 strains of S. aureus, related to phage pattern and susceptibility to antibiotics and cadmium, arsenate and mercury

Resistance to Cd,As,Hg	Phage pattern	Mean value of inducible penicillinase production (units/mg of bacteria) (and number of strains) with antibiotic-resistance pattern				
		P	PS	PT	PST±	Any
0	52,52A,80,81	24 (1)	...	...	...	24 (1)
	Group I	132 (1)	...	...	...	132 (1)
	Group II	60 (3)	3 (1)	37 (3)	42 (3)	42 (10)
	Group III	12 (1)	162 (1)	70 (1)	70 (1)	79 (4)
	83A	56 (1)	...	...	...	56 (1)
	94,96	257 (4)	198 (9)	110 (2)	...	202 (15)
	95	110 (10)	...	...	...	110 (10)
Cd	Mean	120 (21)	177 (11)	67 (6)	49 (4)	121 (42)
	52,52A,80,81	77 (1)	...	...	...	77 (1)
	Group I	86 (1)	...	30 (1)	...	58 (2)
	Group II	80 (3)	...	...	...	80 (3)
	Group III	83 (1)	3 (1)	71 (1)	45 (1)	51 (4)
	83A	...	...	22 (1)	...	22 (1)
	94,96	158 (3)	...	...	194 (1)	167 (4)
Cd,As	Mean	107 (9)	3 (1)	41 (3)	120 (2)	88 (15)
	52,52A,80,81	147 (5)	132 (2)	...	91 (2)	131 (9)
	Group I	97 (5)	37 (1)	114 (1)	...	91 (7)
	Group II	38 (2)	111 (3)	22 (1)	60 (1)	70 (7)
	Group III	31 (1)	24 (1)	...	69 (1)	41 (3)
	83A	71 (2)	121 (1)	...	153 (6)	131 (9)
	Mean	98 (15)	98 (8)	68 (2)	123 (10)	103 (35)
Cd,As,Hg	52,52A,80,81	108 (7)	69 (10)	88 (1)	59 (2)	83 (20)
	Group I	107 (3)	26 (1)	90 (1)	162 (1)	100 (6)
	Group II	35 (1)	...	...	...	35 (1)
	Group III	167 (1)	...	127 (1)	57 (9)	73 (11)
	83A	326 (1)	340 (1)	...	173 (7)	209 (9)
	Mean	124 (13)	88 (12)	102 (3)	105 (19)	106 (47)
	Any	113 (58)	119 (32)	69 (14)	105 (35)	108 (139)

Cd = resistance to cadmium, As = resistance to arsenate, Hg = resistance to mercury, 0 = susceptible to all these metals.

P = resistant to penicillin only; PS = resistant to penicillin and streptomycin only; PT = resistant to penicillin and tetracycline only; PST± = resistant to penicillin, streptomycin and tetracycline, with or without resistance to other antibiotics.

exceptions—mainly in groups in which only a few strains represented the combination of characters in question—but it was obvious that P-resistant strains of the 52,52A,80,81 complex and of group I produced more penicillinase (a mean of 109 units/mg) than did other strains of the same types resistant to PS (mean, 73 units/mg).

Correlation with susceptibility to heavy metals. Among the strains as a whole (table I), correlation between resistance to Cd, As, Hg, and penicillinase production could not be demonstrated, even though the mean value for penicillinase production obtained with all Cd-resistant strains was lower than the values obtained with the other strains. For strains sensitive to all three metals, the mean value was 121 units/mg; for strains resistant to Cd, 88 units/mg; for those resistant to Cd,As, 103 units/mg; and for strains resistant to all three metals, 106 units/mg.

Correlation with combinations of the three properties. It was not possible to analyse

TABLE II

Inducible penicillinase production by 139 strains of S. aureus related to phage pattern and antibiotic susceptibility

Phage pattern	Mean value of inducible penicillinase production (units/mg of bacteria) (and number of strains) with antibiotic-resistance pattern				
	P	PS	PT	PST ±	Any
52,52A,80,81	114 (14)	80 (12)	88 (1)	75 (4)	95 (31)
Group I	103 (10)	32 (2)	78 (3)	162 (1)	93 (16)
Group II	59 (9)	84 (4)	33 (4)	47 (4)	57 (21)
Group III	73 (4)	63 (3)	89 (3)	58 (12)	66 (22)
83A	131 (4)	231 (2)	22 (1)	164 (13)	157 (20)
94,96	214 (7)	198 (9)	123 (2)	194 (1)	196 (19)
95	110 (10)	...	...	...	110 (10)

Abbreviations as in table I.

all the combinations (table I), because the various susceptibility groups were unevenly represented among the phage patterns. There were, however, some groups for which a comparison was possible. Among the P-resistant 94,96 strains, those sensitive to all metals produced more enzyme than those resistant only to Cd.

P and PS-resistant Hg-resistant strains of the 52,52A,80,81 complex produced less penicillinase than the Hg-sensitive strains, which mainly were Cd,As resistant. Strains of the 83A complex resistant to Cd,As,Hg produced more penicillinase (209 units/mg) than those resistant to Cd,As (131 units/mg), but the difference was less obvious among

FIGURE.—Penicillinase production by 139 strains of *S. aureus* divided into phage groups/complexes. The strains are divided according to whether they are resistant to mercury (Hg^R) or susceptible (Hg^S).

the strains with the antibiotic-susceptibility pattern PST $\pm$ (173 and 153 units/mg respectively).

Hg resistance could not be correlated with high levels of penicillinase production, *e.g.*, Hg-resistant strains were not found among those of the 94,96 complex that produced the most enzyme (figure). If these strains were excluded from the calculation, there was, however, greater penicillinase production among Hg-resistant, P-resistant strains (106 units/mg) than among the corresponding Hg-sensitive strains (83 units/mg).

Correlation with strains of epidemic occurrence. The strains that in the past decades have spread in Denmark belong to the 52,52A,80,81 and the 83A complexes (1957–1970) (Rosendal *et al.*, 1976) and in more recent years (1975–1980) to the 94,96 complex and type 95 (Rosendal and Bentzon, *in press*). Their percentage occurrence in the years in question and their percentage distribution over the three resistance patterns, P, PS, PST $\pm$, which comprise >90% of these strains, are shown in table III. The various groups of strains each had their characteristic pattern of antibiotic resistance: 78% of the 52,52A,80,81 strains were resistant to PS, 89% of the 83A strains were resistant to PST $\pm$ and 88–89% of the latest emerging strains (94,96 and 95) were resistant only to P.

In table IV, enzyme production by representatives of these strains with the most common resistance pattern in their group is compared with that of other, non-epidemic strains with the same antibiotic resistance patterns. The 52,52A,80,81 strains produced less enzyme than the 20 non-epidemic strains resistant to PS, but the 83A

TABLE III

Occurrence and antibiotic-resistance patterns of Danish epidemic bacteraemia strains

Period	Phage type/complex	Number of epidemic strains (and percentage of total)	Percentage resistant to		
			P	PS	PST $\pm$
1957–1960	52,52A,80,81	194 (39.0)	9.5	78.3	4.2
1961–1970	83A	1236 (36.8)	7.6	0.9	88.7
1975–1980	94,96	512 (14.8)	88.8	1.7	0.3
1976–1980	95	184 (6.3)	87.5	1.0	0

Abbreviations as in table I.

TABLE IV

Penicillinase production by epidemic strains compared with production by other strains with identical antibiotic-resistance patterns

Phage type/complex (and resistance pattern)	Mean induced penicillinase production (units/mg of bacteria)
52,52A,80,81 (PS)	80
Not 52,52A,80,81 complex (PS)	143
83A (PST $\pm$)	164
Not 83A complex (PST $\pm$)	70
94,96 (P)	214
95 (P)	110
Not 94,96 or 95 (P)	97

Abbreviations as in table I; epidemic strains are in **bold type**.

strains, the 94,96 strains and the 95 strains, produced more enzyme than other strains with corresponding antibiotic-resistance pattern.

The Hg-resistant PST $\pm$ strains of the 83A complex had a mean penicillinase production of 173 units/mg compared with 66 units/mg for other strains with the same resistance pattern. Hg-susceptible strains, PST $\pm$ and of the 83A complex had a mean production of 153 units/mg compared with 75 units/mg for other strains of the same resistance pattern.

Percentage of extracellularity

The mean percentage of extracellularity (see *Methods*) in the whole set of test strains was 39%, ranging from 2 to 77%. Strains of the 94,96 complex and type 95 had, however, values of 47% and 50%, *i.e.*, above the average figure. Otherwise correlation could not be found between extracellularity and phage type, or susceptibility to antibiotics and to heavy metals.

Induction ratio

The induction ratio (see *Methods*) varied in the present material from 1 to 374. Correlation could not be demonstrated between this property and phage type or susceptibility to antibiotics and to heavy metals.

A ratio *c.* 1 signifies that the enzyme production is constitutive and not inducible. Among the 139 strains, 4 strains with constitutive production were found (table V).

TABLE V
Properties of the four strains with constitutive penicillinase production

Strain no.	Resistance to Cd,As,Hg	Resistance to antibiotics	Phage type	Induced penicillinase production (units/mg of bacteria)	Percentage extra-cellularity	Induction ratio
E5942	Cd-As-Hg	PS	84	340	43	1.0
E7057	0	PT	94,96	2.9	52	1.4
E7957	0	PS	3A	3.3	33	1.2
E10933	0	PT	3A/3C/55*	1.6	12	0.9

Abbreviations as in table I.

* Typed at 100 $\times$ RTD.

All these strains had rare combinations of phage type and susceptibility to antibiotics and to heavy metals. In three of the strains, production was micro-constitutive and in one, type 84, macro-constitutive. Resistance to P, S, Cd, As, and Hg was preserved when the latter strain was exposed to conditions normally favouring loss of plasmid(s) (Asheshov, 1966).

DISCUSSION

From these results it can be concluded that there was a connection between the amount of penicillinase produced by the individual strain and its phage-typing pattern. In agreement with the results of Richmond *et al.* (1964), strains belonging to group II were weak producers and members of the 83A complex strong producers of penicillinase. Our results, however, did not show any correlation between enzyme production, multiple antibiotic resistance, and resistance to mercury.

The discrepancies between the results of Richmond *et al.* (1964) and ourselves are due mainly to the different periods of isolation of the strains. The antibiotic-susceptibility patterns of the recently spreading strains of the 94,96 complex (table III) show that high enzyme production does not always lead to multiple antibiotic resistance (*cf.*, Richmond *et al.*, 1964), but it may have done so for the 83A strains because two non-multiresistant strains of the 83A complex in our series were very strong penicillinase producers (table I).

Identification of the bacteriological properties that favour epidemic spread of a strain is a complex problem because changing environmental conditions, including antibiotic policy, also have an important influence on the susceptibility of the strains. Strains of three of the four epidemic complexes investigated produced more penicillinase than other strains with the same antibiotic-susceptibility pattern. The multiresistant 83A strains had their peak incidence when antibiotics such as S and T were in use in Danish hospitals, whereas the 94,96 strains emerged when the use of these antibiotics had become restricted (Rosendal *et al.*, 1977). Type 95, which appears to produce only moderate amounts of enzyme, has the advantage of a high percentage of extracellularity, also found among 94,96 strains. The epidemically occurring strains of the 52,52A, 80,81 complex, however, do not produce large amounts of penicillinase (table IV). They may, in the late 1950s, have been protected by their resistance to S, but early in the 1960s they were outnumbered by strains that were resistant to T as well (Rosendal and Jessen, 1964).

Constitutive penicillinase production has, until now, been found only among strains resistant to fusidic acid (Lacey and Rosdahl, 1974), and Osowiecki, Mlynarczyk and Mlynarczyk (1981) claimed that such a production occurred only among strains harbouring the PF plasmid. All four strains with constitutive production in our series, however, were susceptible to fusidic acid, but they also showed rare combinations of phage type and resistance patterns to antibiotics and to heavy metals. This might indicate mutation in the gene for penicillinase induction as a result of the insertion of other resistance genes by transposition.

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