Loss of Antibiotic Resistance in *Staphylococcus aureus* Resulting from Growth at High Temperature

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SUMMARY

Fifty strains of *Staphylococcus aureus* resistant to penicillin or tetracycline or both were examined for loss of these two resistances as the result of growth at 43–44°. Twelve strains showed a loss of penicillin resistance under the experimental conditions, and of these twelve, three showed a loss of tetracycline resistance. The two resistances were lost independently; in strains in which both resistances were lost most of the sensitive variants had lost one or other resistance but not both. However, penicillin resistance was lost only in strains that were also tetracycline-resistant and *vice versa*. All strains in which a loss of resistance occurred had similar phage-typing patterns and all belonged to the '52, 52A, 80, 81 complex' of strains. Not all strains in the complex, however, showed a loss of resistance. Both penicillin and tetracycline resistance were transduced into suitable sensitive recipients. The results showed that the heat-sensitivity of the transduced resistance was the same as in the donor in which the transducing phage was propagated. The results are consistent with the hypothesis that these two resistances are carried by two separate plasmids, at least in certain strains of *S. aureus*.

Nine multiply-resistant strains of *Staphylococcus aureus* were examined for loss of resistance to antibiotics other than penicillin and tetracycline as the result of growth at elevated temperatures. These other antibiotics included streptomycin, erythromycin, novobiocin, oleandomycin, neomycin and bacitracin. In no case was any loss of resistance observed.

INTRODUCTION

Penicillin-resistance in most naturally occurring strains of *Staphylococcus aureus* is due to the production of the enzyme penicillinase (Kirby, 1944, 1945). This character was believed to be a stable one until Barber (1949) showed that it was lost at a rather high rate in old cultures maintained in the laboratory under a variety of conditions. Bondi, Kornblum & de Saint Phalle (1958) confirmed this finding and showed that growth of the organism at 45° increased the rate of loss of penicillinase production. Fairbrother, Parker & Eaton (1954) also found that growth at elevated temperatures (44°) increased the rate of loss of penicillin resistance in some but not all strains of staphylococci. May, Houghton & Perret (1964) showed loss of both penicillin and tetracycline resistance during growth of a strain of *S. aureus* at 43–44°. These two resistances were lost independently; the authors concluded that they were probably carried on different plasmids, and that, during growth at elevated temperatures, the plasmids failed to replicate at the same rate as the bacteria and were therefore 'diluted out'.

The experiments reported here were done to see whether this phenomenon occurred in other strains of staphylococci and whether loss of resistance to antibiotics other than penicillin and tetracycline was accelerated by growth at elevated temperatures.

METHODS

Strains of Staphylococcus aureus. Fifty-two strains of *S. aureus* chosen from material sent to this laboratory for routine phage typing and one propagating strain (ps 80, the propagating strain for typing phage 80) were examined. They represented several different phage-typing patterns from fourteen different hospitals, and all except one were resistant to penicillin. The majority were also resistant to tetracycline and nine of the strains were resistant to other antibiotics. Fifty of the strains were examined for loss of resistance to penicillin, or tetracycline, or both during growth at 37° and 43–44°. These fifty strains are listed in Table 1. Six of these 50 strains and 3 additional multiply-resistant strains were examined for loss of resistance to antibiotics other than penicillin and tetracycline. They are listed in Table 4.

A single colony was picked from a blood agar culture of each strain. After growth in broth it was phage-typed and tested for resistance to antibiotics.

Growth at 43–44°. Overnight broth cultures of the strains were diluted 1/4 in fresh nutrient broth and incubated at 37° for 1½ hr. with aeration. Samples (0.04 ml.) of this were then added to each of two tubes containing 5.0 ml. nutrient broth pre-warmed to 37° and to 43–44°, respectively. The tubes were then incubated at the appropriate temperature for 5½ hr without aeration. The cultures were then suitably diluted so that 0.1 ml. contained 100–200 viable units and this amount was spread on nutrient agar plates or on starch agar plates. Plates were incubated overnight at 37° and the resulting colonies counted and then tested for loss of antibiotic resistance.

Testing for antibiotic resistance. Two methods were used to screen colonies for penicillin resistance: (a) Suitably diluted cultures were plated on starch agar plates and screened for penicillinase production by a modification of Perret's iodometric method (Perret, 1954). (b) Cultures were plated on nutrient agar plates and the colonies replicated on to agar plates containing benzylpenicillin 0.2 µg./ml.

Resistance to other antibiotics was tested by replicating colonies grown on nutrient agar plates to agar plates containing the following concentrations of antibiotics: streptomycin calcium chloride, 100 µg./ml. (73.5 units/ml.); novobiocin (sodium salt), 1.0 µg./ml. (0.895 unit/ml.); tetracycline HCl, 5.0 µg./ml. (5 units/ml.); erythromycin base, 2.0 µg./ml. (1.8 unit/ml.); oleandomycin phosphate, 2.0 µg./ml.; neomycin sulphate, 5.0 µg./ml. (3.4 units/ml.); bacitracin, 5.0 units/ml.

Colonies which failed to appear on the appropriate antibiotic plate or appeared to be penicillinase-negative on starch plates were subcultured to nutrient agar plates. They were phage-typed to ensure that they corresponded to the parent culture and the loss of antibiotic resistance was confirmed by restreaking on the appropriate antibiotic-containing agar plate.

Transduction of antibiotic resistance. Penicillinase production and tetracycline resistance were transduced to sensitive recipients by using typing phage 80 as transducing phage. The phage was grown on an appropriate resistant donor strain by

Table 1. *Effect of growth at 43–44° on resistance to penicillin and tetracycline in strains of Staphylococcus aureus*

Strain no.	Phage type	Resistance to P and T	% losing resistance during growth					
			At 37°			At 43–44°		
			No. colonies screened	P ^s	T ^s	No. colonies screened	P ^s	T ^s
1	80	PrTr	199	—	—	56	23.2	—
2	52/52 A/80		219	—	—	118	4.2	9.3
3	52/52 A/80		200	—	—	52	15.3	7.7
4	80/81		201	—	—	148	8.1	—
5	52/52 A/80/81		361	—	—	144	4.1	—
6	52/52 A/80/81		241	—	—	72	4.1	—
7	52/52 A/80/81		321	—	—	345	1.1	—
8	52/52 A/80		364	—	—	167	1.7	—
9	52/52 A/80/81		777	—	—	1078	3.5	—
10	52/52 A/80		490	—	—	380	4.0	—
11	80/81		554	—	—	468	7.5	—
12	52/52 A/80		455	—	—	260	2.3	0.4
13	29/+		230	—	—	128	—	—
14	52 A/79		407	—	1.0	224	—	1.2
15	29		512	0.4	—	900	0.2	—
16	52		340	—	—	696	—	—
17	52 A/79	PrT ^s	309	—	.	314	—	.
18	29		343	—	.	191	—	.
19	52/52 A/80/81		275	—	.	684	—	.
20	80/81		366	—	.	248	—	.
21	29/52/52 A/80		350	—	.	79	—	.
22	81		240	—	.	192	—	.
23	52/80		326	—	.	248	—	.
24	81		470	—	.	462	—	.
25	29/52/52 A/80/77	PrT ^s	139	—	.	205	—	.
26	80/81	P ^s T ^s	525	.	—	521	.	—
27	55/71	PrTr	215	—	0.5	720	—	0.7
28	3 C	PrT ^s	350	—	.	180	—	.
29	3 C/55	PrT ^s	616	—	.	1680	—	.
30	83 A	PrTr	211	—	—	237	—	—
31	58/75/77		183	—	—	143	—	—
32	7/47/54/75/77/83 A		169	—	—	122	—	—
33	83 A		290	—	—	360	—	—
34	7/47/54		207	—	—	197	—	—
35	83 A		400	—	—	174	—	—
36	77		544	—	—	984	—	—
37	83 A		360	—	—	292	—	—
38	75/77	PrT ^s	130	—	.	188	—	.
39	77		176	—	.	98	—	.
40	47/81		306	—	.	243	—	.
41	6/7/47		292	—	.	200	—	.
42	77		280	—	.	465	—	.
43	7/42 E/54/81/+		300	—	.	450	—	.
44	77		763	—	.	1600	—	.
45	6/7/47		592	—	.	3000	—	.
46	6/7/47/53/54/83 A/81		520	—	.	507	—	.
47	42 E/42 D	PrTr	210	—	—	160	—	—
48	N.T.		486	—	—	400	—	—
49	N.T.		405	—	—	2905	—	—
50	N.T.		96	—	—	188	—	—

—, no loss observed; . = not tested; phage patterns shown in italics were obtained at 1000 × routine test dilution (RTD); N.T. = not typable. The three strains reported as N.T. (nos. 48, 49, 50) are the same as those described by Jevons & Parker (1964) and are common epidemic strains. P = penicillin; T = tetracycline; P^s = penicillin-sensitive; Pr = penicillin-resistant; T^s = tetracycline-sensitive; Tr = tetracycline-resistant.

the soft agar layer method of Swanstrom & Adams (1951). After filtration through sintered glass filters (A.P.D. $1.5\ \mu$), the filtrates were tested for bacterial sterility. Sufficient phage was added to log-phase broth cultures of the recipient strain to give a multiplicity of infection of from 0.4 to 0.7. After allowing 30 min. for phage adsorption the mixtures were centrifuged and the bacteria resuspended in broth containing 0.02 M-sodium citrate to prevent further phage adsorption. Incubation was continued for a further 2-3 hr before screening for transductants by plating the organisms on plates containing either tetracycline 5.0 $\mu\text{g./ml.}$ or penicillin 0.1 $\mu\text{g./ml.}$

RESULTS

Table 1 lists the 50 strains of *Staphylococcus aureus* which were tested for loss of resistance to penicillin or tetracycline or both during growth at 43-44°. The phage type of the strains and their resistance to the two antibiotics are also shown. Thirteen of the 50 strains tested showed a loss of penicillin resistance when grown at 43-44°. With twelve of these strains no loss of penicillin resistance was detected when the strains were grown at 37°. The loss observed during growth at 43-44° can therefore be assumed to have resulted from growth at this higher temperature.

With one strain (no. 15), however, the loss of penicillin resistance that occurred during growth at 43-44° was no greater than that observed when the strain was grown at 37°. Loss of tetracycline resistance occurred with 5 out of 28 strains tested; but with two of these (nos. 14, 27) it was no greater than the rate of spontaneous loss at 37°. If these three strains be excluded, certain conclusions can be drawn: (1) Loss of penicillin resistance as a result of growth at 43-44° occurred with greater frequency than loss of tetracycline resistance. (2) Strains which lost tetracycline resistance always lost penicillin resistance as well, but these two resistances were lost independently, i.e. it was rare to find a clone which had lost both resistances, although this did occur occasionally. For example, with strain no. 2, loss of resistance to penicillin occurred in 2.5 % of clones, loss of resistance to tetracycline in 8 % of clones, loss of resistance to both antibiotics in 1.7 % of clones.

An examination of the phage-typing pattern of those strains in which loss of resistance was accelerated by heating revealed that they all belonged to the '52, 52A, 80, 81 complex'. This complex, which comprises strains lysed by various mixtures of the four typing-phages (52, 52A, 80, 81) is believed to contain organisms which arose as a result of lysogenizations of a single 'basic' strain by a number of different temperate phages. However, not all members of this complex showed a loss in antibiotic resistance when grown at 43-44°. Eighteen such strains were tested and six of these (nos. 19, 20, 22, 23, 24, 26) did not show any loss in penicillin resistance. None of these six was resistant to tetracycline. On the other hand, all of the twelve strains in the complex in which loss of penicillin resistance or tetracycline resistance, or both, occurred, were naturally resistant to both antibiotics. It seemed possible, therefore, that it was necessary for a strain to be doubly resistant and possibly, therefore, to carry two plasmids, for loss of one or both to occur during growth at 43-44°.

To test this possibility strain no. 2 (phage pattern 52/52A/80), which was resistant to both penicillin and tetracycline (P^rT^r) and consistently showed a loss of both resistances during growth at 43-44°, was examined further. From this strain two

P^rT^r (penicillin-sensitive, tetracycline-resistant) clones and two P^rT^s (penicillin-resistant, tetracycline sensitive) clones were isolated after growth at 43–44°. These 4 clones, together with the original P^rT^r parent, were tested for loss of resistance as result of growth at 43–44°. The results are summarized in Table 2. The P^rT^r isolates lost their penicillin resistance, and the P^rT^s isolates their tetracycline resistance, at approximately the same rate as the P^rT^r parent lost each resistance separately. It seems therefore that the presence of more than one plasmid is not necessary for this loss to occur.

Table 2. *Loss in antibiotic resistance during growth at 43–44° in a strain of Staphylococcus aureus resistant to penicillin and to tetracycline (P^rT^r) and isolates from this strain resistant to penicillin only (P^rT^s) and to tetracycline only (P^sT^r)*

Strain	Colonies screened (no.)	% losing resistance during growth at 43–44°	
		Penicillin	Tetracycline
Strain 2 P ^r T ^r	934	1.1	2.2
Strain 2 P ^r T ^s —1	794	1.6	.
Strain 2 P ^r T ^s —2	1207	1.0	.
Strain 2 P ^s T ^r —1	601	.	2.1
Strain 2 P ^s T ^r —2	1360	.	2.4

. = not done.

Table 3. *Loss in antibiotic resistance during growth at 43–44° in strains of Staphylococcus aureus transduced to resistance with phage 80/2 and phage 80/80*

Recipient strain	Transducing phage	Resistance transduced	Clone no.	Colonies screened (no.)	% losing transduced resistance during growth at 43–44°	
					Penicillin	Tetracycline
Strain 2 P ^r T ^s	80/2	Penicillin	1	348	6.0	.
	80/2	Penicillin	2	316	2.5	.
Strain 2 P ^s T ^s	80/80	Penicillin	1	1410	< 0.07	.
	80/80	Penicillin	2	335	< 0.3	.
Strain 2 P ^s T ^s	80/2	Tetracycline	1	541	.	3.3
	80/2	Tetracycline	2	323	.	4.3
PS 80 P ^r T ^s	80/2	Tetracycline	1	1260	.	1.1
	80/2	Tetracycline	2	987	.	2.0

. = not done.

Transduction experiments

Transduction of antibiotic resistance was done with typing phage 80 grown on two different donor strains: (1) strain 2, which is P^rT^r, and in which both resistances are susceptible to loss during growth at high temperatures; (2) ps 80, the propagating strain for phage 80 (strain 20 in Table 1) which is penicillin-resistant and tetracycline sensitive, and in which the penicillin resistance was not lost during growth at 43–44°. These two phage preparations will be referred to as phage 80/2 and phage 80/80.

The recipient strains used were (1) a P^rT^s clone isolated from strain 2 after heat treatment. This strain was transduced to penicillin resistance with both phage

80/2 and phage 80/80, and also to tetracycline resistance with phage 80/2. (2) ps 80, the propagating strain for phage 80 (strain 20 in Table 1). This strain was transduced to tetracycline resistance with phage 80/2.

Two of each class of transductants were tested for loss of antibiotic resistance during growth of 43–44°. The results of these experiments (Table 3) show that the heat sensitivity of a particular antibiotic resistance was transduced with the resistance itself. Thus, strain 2 P^rT^r transduced to penicillin resistance by phage 80 grown in strain 2 P^rT^r, where the penicillin resistance is sensitive to heat, showed a similar loss in penicillin resistance when grown at 43–44°. The same strain, when transduced to penicillin resistance by phage 80 grown in strain ps 80, where the penicillin resistance was not lost during growth at 43–44°, showed no loss in resistance when grown at this elevated temperature. Similarly, both strain 2 P^rT^r and ps 80 P^rT^r, when transduced to tetracycline resistance by phage 80/2, showed a loss in tetracycline resistance when grown at 43–44°. The heat stability of the plasmid, therefore, is characteristic of the plasmid itself and seems to be independent of the cell which carries it.

Table 4. *Effect of growth at 43–44° on loss of resistance to streptomycin, erythromycin, novobiocin, oleandomycin, neomycin and bacitracin by strains of Staphylococcus aureus*

Strain	Phage pattern	Resistance pattern	Growth temperature	No. colonies screened	% losing resistance to					
					S*	E	Nv	Ol	Neo	Bac
1	80	P ^r T ^r E ^r	37°	495	.	—	.	.	.	.
			43–44°	156	.	—	.	.	.	.
15	29	P ^r T ^r S ^r E ^r Ol ^r	37°	360	—	—	.	—	.	.
			43–44°	420	—	—	.	—	.	.
16	52	P ^r T ^r S ^r E ^r Nv ^r	37°	340	—	—	—	.	.	.
			43–44°	696	—	—	—	.	.	.
30	83 A	P ^r T ^r E ^r Nv ^r	37°	723	.	—	—	.	.	.
			43–44°	290	.	—	—	.	.	.
33	83 A	P ^r T ^r S ^r E ^r	37°	197	.	—	.	.	.	.
			43–44°	498	.	—	.	.	.	.
50	n.t.	P ^r T ^r S ^r E ^r	37°	390	—	—	.	.	.	.
			43–44°	410	—	—	.	.	.	.
51	83 A	P ^r T ^r S ^r E ^r Nv ^r	37°	505	.	—	—	.	.	.
			43–44°	660	.	—	—	.	.	.
52	n.t.	P ^r T ^r S ^r E ^r Neo ^r Bac ^r	37°	383	—	.	.	.	—	—
			43–44°	715	—	.	.	.	—	—
53	n.t.	P ^r T ^r S ^r E ^r Neo ^r	37°	623	—	—	.	.	—	.
			43–44°	831	—	—	.	.	—	.

* S, streptomycin; E, erythromycin; Nv, novobiocin; Ol, oleandomycin; Neo, neomycin; Bac, bacitracin; . = not done; — = no loss observed; n.t. = not typable.

Effect of growth at 43–44° on loss of resistance to antibiotics other than penicillin and tetracycline

Nine strains of *Staphylococcus aureus* which were resistant to other antibiotics in addition to penicillin and tetracycline were examined to see whether growth at elevated temperatures accelerated the loss of resistance to these other antibiotics. The results are summarized in Table 4. Five strains were tested for loss of strepto-

mycin resistance; eight strains for loss of erythromycin resistance; three strains for loss of novobiocin resistance; one strain for loss of oleandomycin resistance; two strains for loss of neomycin resistance and one strain for loss of bacitracin resistance. In no case was there a detectable loss of resistance to these antibiotics, either during growth at 37° or at 43–44°.

DISCUSSION

The results reported here confirm those of other workers, particularly those of May *et al.* (1964) and support the hypothesis that resistance to penicillin and tetracycline is carried cytoplasmically, at least in some strains of *Staphylococcus aureus*. However, of the 50 strains examined only 12 strains showed a loss of penicillin resistance as the result of growth at 43–44°, and of these 12 only three showed a loss of tetracycline resistance. The two resistances were lost independently but penicillin resistance was lost only in strains that were also tetracycline resistant and *vice versa*.

All strains in which a loss of resistance occurred shared very similar phage-typing patterns (80, 52/52A/80, 80/81, 52/52A/80/81) characteristic of strains in the '52, 52A, 80, 81 complex'. All the evidence suggests that these strains are closely related and that the difference in typing patterns is the result of lysogenization of a single 'basic' strain by a series of temperate phages (Asheshov & Winkler, 1966). *Staphylococcus* strain E 169 of May *et al.* (1964) had the phage pattern 52/52A/80/81 and is, therefore, also a member of this complex of strains. In view of this it seemed possible that the loss of two plasmids in these closely related strains was a characteristic of the bacteria carrying them and independent of the plasmids themselves. The results of the transduction experiment would seem to rule this out, however, since they showed that the heat sensitivity of the plasmids was transferred together with the antibiotic resistance, which suggests that the heat sensitivity is a characteristic of the plasmid itself.

Failure to show a loss of resistance to penicillin or tetracycline in the other 38 strains may mean that the method used was not sufficiently sensitive (e.g. not enough colonies were screened) to detect a lower rate of loss. It seems possible that the rate of loss observed as the result of growth at 43–44° simply reflects an increase in the normal rate of spontaneous loss which occurs on storage. Two strains in which a high rate of loss of penicillin resistance occurred as the result of growth at elevated temperature (strains 1 and 2 of Table 1) also showed a very high rate of loss of penicillin resistance on storage. In the case of strain 1, 9% of colonies were penicillin sensitive after 4–5 months storage at room temperature: during the same period 40% of the bacteria in strain 2 showed a similar loss.

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