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# NOTES

## Genotypic Stability of Methicillin Resistance in *Staphylococcus aureus* at Supraoptimal Temperature

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Methicillin resistance in a highly resistant mutant of *Staphylococcus aureus* could not be diminished by elevated temperature. After 100 subcultures at 43°C the resistance to methicillin was retained when determined at 37°C.

It was shown that the susceptibility of the methicillin-resistant (MR) strains of *Staphylococcus aureus* to methicillin was directly related to temperature and increased markedly with it (2, 5, 9). The phenotypic expression of the resistance, however, was suppressed by supraoptimal temperatures (2, 9). Subsequently, Al Salihy and James (1) reported that cells of the MR strain 13136 irreversibly lost their methicillin resistance after 35 subcultures at 43°C. This finding has suggested a reexamination of the question in as clear a system as possible.

Most of the naturally occurring MR strains of *S. aureus* have appeared to consist of mixed populations in which the majority of the cells are susceptible to methicillin and only a small minority are highly resistant (3-10, 12, 14, 15). Therefore, the results of experiments with such strains reflect the major susceptible fraction of the cell population rather than the really MR ones.

To avoid this ambiguity I have separated the methicillin-susceptible (MS) fraction of the population from the highly MR one of the same isolate. For this purpose the naturally occurring MR *S. aureus* strain 5814/1966 was used. This strain had been isolated from pus, produced coagulase, and consisted of a heteroresistant population (10).

The MS substrain was obtained as follows. A 24-h culture of the parent strain was diluted up to $10^{-7}$ with isotonic NaCl solution, pH 7.2, and 0.1-ml portions of the diluted culture were plated onto methicillin-free blood agar plates to obtain discrete colonies. Incubation lasted for 24 h at 37°C. Each colony was tested for methicillin susceptibility. Tube susceptibility tests were carried out using twofold dilutions of the drug from 16 to 0.5 μg/ml in Difco Casitone broth (16), pH 7.2, containing 1% glucose. Tubes were inoculated with 0.1 ml of a 1:100 dilution of a 24-h broth culture of the colony incubated in the same medium. This test was done in duplicate. Cultures inhibited by 8 μg of methicillin per ml or less after incubation at 37°C for 48 h were considered susceptible.

For selection of the MR mutant the Casitone broth containing 500 μg of methicillin per ml was seeded with an appropriate dilution of a 24-h culture of the parent strain so that the final inoculum amounted to $10^6$ organisms/ml. After a 48-h incubation at 37°C the culture was subcultured on a blood agar plate free of the antibiotic. The subculture was tested for methicillin resistance by the tube dilution technique mentioned above.

A population analysis was also carried out and for the MR 5814R mutant 70% of the population has been able to grow in the presence of 500 μg of methicillin per ml and 55% in the presence of 800 μg of methicillin per ml on nutrient agar at 37°C (11). This mutant has maintained these levels of methicillin resistance since 1968.

The effect of elevated temperature on methicillin resistance was studied on this MR mutant. For comparison, the MS substrain 5814S was used. This strain was fully inhibited by 4 μg of methicillin per ml at 37°C after 2 days of incubation as tested by the tube dilution technique. The whole population of the strain proved also to be susceptible when it was cultured at 30°C on nutrient agar plates containing increasing concentrations of methicillin. After seeding each plate with $1.8 \times 10^8$ organisms and after incubation for 2 days, many discrete colonies were visible at 3 μg of methicillin per ml but none at 6 and 12 μg/ml. The biological, biochemical probes usually tested in the diagnostic laboratory appeared to be identical to those of the 5814R, and both substrains

TABLE 1. *Effect of elevated temperature on the methicillin susceptibility of the MS substrain 5814S and that of the MR mutant 5814R derived from the naturally occurring heteroresistant strain Staphylococcus aureus 5814/1966*

| Temp of repeated growth (°C) | Test temp (°C) | 5814S (MIC, μg/ml) | | | 5814R (MIC, μg/ml) | | |
|---|---|---|---|---|---|---|---|
| | | 0.8 | 1.6 | 3.12 | <12.5 | 1,600 | 3,200 |
| 37 | 37 | | | +[a] | | | + |
| | 43 | | + | | + | | |
| 43 | 37 | | | + | | | + |
| (9 subcultures) | 43 | | + | | + | | |
| 43 | 37 | | | + | | + | |
| (27 subcultures) | 43 | | + | | + | | |
| 43 | 37 | | + | | | | + |
| (40 subcultures) | 43 | + | | | + | | |
| 43 | 37 | | + | | | | + |
| (50 subcultures) | 43 | + | | | + | | |
| 43 | 37 | | | + | | | + |
| (80 subcultures) | 43 | + | | | + | | |
| 43 | 37 | | + | | | | + |
| (100 subcultures) | 43 | + | | | + | | |

[a] + indicates inhibition.

were identical in phage type to one another and to the parent strain. All of them were lysed by phage 77 only at 100 times the routine test dilution of the phages.

The effect of elevated temperature on the methicillin susceptibility of the MS substrain 5814S and that of the MR mutant 5914R is shown in Table 1.

The cells of both the substrain 5814S and the mutant 5814R in parallel were subcultured repeatedly in Difco broth (16) in the absence of the antibiotic at 37 and 43°C. After a number of subcultures at a given temperature the susceptibility to methicillin was determined at each of the two temperatures by the method described. Cells of the MS substrain 5814S grown repeatedly at 43°C retained a minimal inhibitory concentration (MIC) of 3.12 μg of methicillin per ml at 37°C and an MIC of 1.6 μg/ml at 43°C through 27 subcultures. Further repeated growth at 43°C generally lowered the 37°C MIC value to 1.6 μg of methicillin per ml and the 43°C MIC value to 0.8 μg/ml.

The behavior of cells of the MR mutant 5814R grown repeatedly at 43°C differed considerably. When the MIC of methicillin for mutant 5814R was tested at 43°C for 48 h, the cells appeared to be susceptible to methicillin regardless of the previous subculturing temperatures. On the contrary, on determining the MIC at 37°C, the MR mutant proved to be as resistant to methicillin after 100 subcultures at 43°C as it had been before the beginning of the passage. Neither the antibiotic susceptibility pattern nor the phage type of the strains changed during the subculturing at 43°C.

In conclusion, a supraoptimal growth temperature does not abolish the genotype of methicillin resistance but rather suppresses the phenotypic expression of this genetic property. The phenomenon seems likely to be similar to the finding of Sabath et al. (13), who demonstrated that acidification of the test medium resulted in a significant decrease in the level of methicillin resistance in *S. aureus*. This was also only a suppression and not a loss of methicillin resistance.

The reason why Al Salihy and James (1) obtained irreversible loss of resistance might derive from two facts. On the one hand, the strain 13136 has been shown to have only a few highly MR cells in its population (5, 8). Secondly, the highly MR cells are thought to have a longer generation time than the MS cells (6–10, 14, 15). Therefore, if such a heteroresistant strain is subcultured at an elevated temperature, which is not favorable for growth of the MR cells, the major MS fraction of the population will overgrow the minor MR fraction, and consequently the proportion of MR cells will decrease in parallel with the extent of the subculturing. After a sufficient number of passages the MR portion of the population will be reduced to the extent that it may be easily lost upon further dilution of the culture.

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