Drug Resistance of Staphylococci

II. Joint Elimination and Joint Transduction of the Determinants of Penicillinase Production and Resistance to Macrolide Antibiotics

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Received for publication 16 November 1964

ABSTRACT

Mitsuhashi, Susumu (Gunma University, Maebashi, Japan), Hajime Hashimoto, Megumi Kono, and Masato Morimura. Drug resistance of staphylococci. II. Joint elimination and joint transduction of the determinants of penicillinase production and resistance to macrolide antibiotics (PCase+) and resistance to macrolide antibiotics (MAC+) of these strains were irreversibly eliminated by treatment with acridine or with ultraviolet light. Among the 18 strains tested, PCase+ and MAC+ were eliminated from all strains except one, which lost only PCase+ but not MAC+. The characters PCase+ and MAC+ were jointly transduced with the aid of phage lysates, obtained from the resistant donors by ultraviolet irradiation, into staphylococcal strains sensitive to PC and MAC. Segregation of PCase+ and MAC+ was rarely observed after transduction. From these results, it is suggested that the determinants of both PCase+ and MAC+ of staphylococci are located close together on a single genetic element, i.e., a plasmid (or episome), which exists extrachromosomally.

It was reported by Vourekia (1952) that virulence and resistance to penicillin (PC) and streptomycin (SM) of a strain of Staphylococcus aureus decreased after an exposure to injurious agents, such as chloramphenicol (CM), tetracycline (TC), nitrogen mustard, etc. Also, the new characters remained unchanged through more than 50 passages in normal laboratory media.

We reported previously that resistance to macrolide antibiotics (MAC), i.e., erythromycin (EM), oleandomycin (OM), and leucomycin (LM), of S. aureus was jointly eliminated upon treatment with acriflavine, and that these characters were transduced together even at a multiplicity of phage infection of 0.1 (Mitsuhashi et al., 1963). It was also found that the PC resistance (determinant of penicillinase production; PCase+) of a strain of S. aureus MS57 was eliminated irreversibly by treatment with acridine or with ultraviolet light (Hashimoto, Kono, and Mitsuhashi, 1964). The present paper deals with the joint elimination of PCase+ and MAC+ and with a transductional analysis of both determinants.

MATERIALS AND METHODS

Bacterial strains. Strains used were 18 coagulase-positive strains of Staphylococcus aureus, which were isolated from clinical sources. They were highly resistant to macrolide antibiotics (EM, OM, LM, SP), TC, SM, and sulfanilamide (SA), and were capable of producing penicillinase. Several strains of staphylococci which were sensitive to PC and MAC, selected at random from the stock cultures of the present authors, along with those which became sensitive to PC and MAC by treatment with acriflavine, were used as recipients in the transductional experiment.

Media. The nutrient broth, used routinely for the propagation of bacteria, consisted of 1% beef extract, 1% peptone, and 0.5% NaCl adjusted to pH 7.2. The nutrient broth plus 1.5% agar was used as the solid medium. Heart infusion (HI) agar (Difco) was used for the preparation of phage lysate from the drug-resistant donor strain and as the selective medium in the transduction experiment. HI agar plates were also used for the determination of phage type and of drug resistance. Mueller-Hinton (Difco) agar was used for the determination of SA resistance.

Determination of drug resistance, phage type, and penicillinase production. These procedures were carried out as described previously (Mitsuhashi et al., 1965).

Elimination of drug resistance by treatment with acriflavine. Acriflavine treatment was done as described by Hirota (1960). One loopful of a 100-fold diluted bacterial culture, grown in nutrient broth for 18 hr, was inoculated into 2 ml of nu-
trient broth containing a serial twofold dilution of acriflavine. After incubation for 24 hr at 37 C, the culture containing the maximal concentration of acriflavine (usually 12.5 μg/ml) which permitted the growth of bacteria was spread onto nutrient agar plates. Plates showing fewer than 200 colonies after incubation for 24 hr at 37 C were replicated to nutrient agar plates containing EM (12.5 μg/ml) or PC (3.1 units per ml). Colonies on the master plate which failed to show corresponding colonies on the nutrient agar plates containing EM or PC were picked and streaked on nutrient agar plates containing EM (12.5 μg/ml) or PC (3.1 units per ml) to confirm the elimination of drug resistance and to make a single-colony isolation. Thereafter, resistance to various drugs and phage types was determined.

Elimination of drug resistance by ultraviolet light. Bacterial suspensions were prepared in nutrient broth, grown in stationary culture for 18 hr, and harvested by centrifugation. Bacterial cells suspended in saline were irradiated with ultraviolet light according to the method described previously (Mitsushashi et al., 1965). Irradiated suspension (0.1 ml) was streaked onto nutrient agar plates. Thereafter, the procedure for the isolation of a colony, whose drug resistance was eliminated, was the same as that described for treatment with acriflavine.

Phage lysates. Lysates were prepared from the donor strains of drug-resistant staphylococci by ultraviolet irradiation, according to the method described previously (Mitsushashi et al., 1965). Plaque formation of each phage lysate was checked on a nutrient agar plate seeded with each of the propagating strains of the International Typing Series. The determination of the plaque-forming titer of each phage lysate was carried out according to the agar-layer technique as described by Swanstrom and Adams (1951). As an indicator, the strain which showed the highest titer of plaque formation was used. The phage lysates obtained from S. aureus MS642, MS643, and MS646, used as donor strains, were grown in nutrient broth, 0.5 ml of the recipient culture (3 × 10^9 to 5 × 10^9 cells per milliliter) was mixed with an equal volume of phage lysate of the donor strain. Multiplicity of infection was 0.1 to 1.0. After 60 min of incubation at 37 C, the mixture was centrifuged to sediment the cells, which were then resuspended in 0.1 ml of nutrient broth and spread onto HI agar plates containing EM (12.5 μg/ml) or PC (3.1 units per ml). The colonies which developed on the selective plates after incubation for 48 hr were touched with a straight needle and subjected to two successive single-colony isolations, and their drug resistance and phage type were determined. As controls for the transduction experiment, sterility tests of the phage lysate and mutation tests of the recipient organisms were carried out by plating on a medium without the transducing phage.

RESULTS

Elimination of the characters MAC* and PCase*. By the use of ultraviolet irradiation, 12 mutants sensitive to MAC and PC were obtained from 9,893 colonies of S. aureus MS620 which were resistant to TC, SM, SA, PC, and MAC. They were all sensitive to PC, as demonstrated by the loss of their ability to produce penicillinase, and they were sensitive to macrolide antibiotics (EM, OM, LM, SP; Table 1).

Eighteen strains of S. aureus were treated with acriflavine according to the method described in Materials and Methods. As shown in Table 2, the characters of MAC* and PCase* of 16 strains were eliminated altogether: they lost the ability to produce penicillinase and became sensitive to all four macrolide antibiotics (EM, OM, LM, SP). The percentage of elimination was 3.5 to 0.1. No segregation of PCase* and MAC* was found among 16 of the strains examined. However, a mutant which was sensitive to PC but not to MAC was obtained from a strain of S. aureus S18 after its treatment with acriflavine. None of these mutant colonies regained resistance to PC or MAC, even after successive cultivations in a medium containing different concentrations of PC, EM, or both, indicating that PCase* and MAC* were irreversibly eliminated. Resistance to TC, SM, and SA was not affected when these strains were exposed to ultraviolet light or to acriflavine.

Transduction of resistance to PC and MAC. The strains of S. aureus, MS642, MS643, and MS646, resistant to PC and MAC, were used as donors in transduction. The phage lysates were obtained by ultraviolet irradiation of the donor strains described above. Among the 34 strains of staphy-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies scored</th>
<th>Elimination of resistance to</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PC</td>
<td>MAC</td>
</tr>
<tr>
<td>UV</td>
<td>9,893</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Without UV irradiation</td>
<td>5,065</td>
<td>0</td>
<td>0</td>
</tr>
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* Abbreviations: MAC, macrolide antibiotics (erythromycin, leucomycin, oleandomycin); PC, penicillin; UV, ultraviolet. Numbers in parentheses indicate the percentage of elimination.
lococci, 25 were competent recipients of PC or MAC resistance. Most of the competent recipients were found to be the phage type of group 1 or of 80/81. Representative results are shown in Table 3. The phage-typing and drug-resistance patterns of the transductants, which were selected on PC or EM plates, were determined after two successive single-colony isolations. Among the 79 strains of transductants, 74 strains (90%) were found to be resistant to PC and MAC (EM, OM, LM, SP), 4 strains were resistant to MAC and sensitive to PC, and 1 strain was resistant to PC and sensitive to MAC (Table 4). All transductants resistant to PC were found to be capable of producing penicillinase. Segregation of MAC and PC resistance was found, but not segregation of resistance to various macrolide antibiotics, i.e., EM, OM, LM, SP. The degree of drug resistance noted among the transductants was almost as high as that of the donor strains. The joint transduction of the characters MAC and PCase, even at the low multiplicity of infection of 0.1, indicates that the genetic loci controlling both characters are located closely on a single genetic element.

Almost all of the transductants were sensitive to the transducing phage and showed the same phage type as the parent strains of recipient staphylococci. Among the 79 strains of transductants tested, however, eight strains (0.1%)...
TABLE 4. Joint transduction of the resistance to macrolide antibiotics and to penicillin with phage lysates obtained from Staphylococcus aureus MS642, MS643, and MS646

<table>
<thead>
<tr>
<th>Donor</th>
<th>Drug-resistance patterns*</th>
<th>Change of phage typing patterns†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(MAC, PC)</td>
<td></td>
</tr>
<tr>
<td>MS642</td>
<td>24/27 2/27 1/27 2/27</td>
<td></td>
</tr>
<tr>
<td>MS643</td>
<td>20/22 2/22 0/22 3/22</td>
<td></td>
</tr>
<tr>
<td>MS646</td>
<td>30/30 0/30 0/30 3/30</td>
<td></td>
</tr>
</tbody>
</table>

* Number of transductants resistant to macrolide antibiotics (MAC) and penicillin (PC) per number of transductants tested.
† Number of transductants in which phage typing patterns were altered per number tested.

showed a change in their phage type after transduction. Results of the changes occurring in phage-typing pattern after transduction will be described elsewhere.

Discussion

In a previous paper (Mitsuhashi et al., 1963), it was reported that the resistance of S. aureus to macrolide antibiotics was jointly eliminated by treatment with acriflavine, suggesting that MAC resistance is controlled by a single genetic element which exists extrachromosomally. On the other hand, the resistance of S. aureus MS66 and MS282 to PC was not affected by treatment with acriflavine. Later, it was found that PC resistance of S. aureus MS57, a strain of our stock culture, was eliminated when treated with acriflavine along with the elimination of MAC resistance (Hashimoto et al., 1964).

Novick (1963) proposed the possibility that PC resistance in S. aureus is associated with a plasmid and is thus inherited extrachromosomally. As evidence against this possibility, however, he reported that no increase in the number of penicillin-sensitive mutants resulted from the propagation of staphylococci in the presence of acridine. The unsuccessful result obtained in the elimination of PC resistance in our previous experiment (Mitsuhashi et al., 1963) and in that of Novick (1963) may be accounted for by the low frequencies of elimination in the strains of staphylococci used in these experiments.

Harmon and Baldwin (1964) reported that it was possible to isolate penicillin-sensitive strains at a frequency that was 60 times greater than the spontaneous mutation rate by treating resistant strains with acridine orange. Thus, they concluded that the resistance to penicillin, i.e., the determinant which controls the capacity to produce penicillinase, appears to be a cytoplasmic element.

Many reports have been presented concerning the artificial elimination of various characters in microorganisms. It was found that various types of yeasts, which possess both respiratory and fermentative mechanisms, lose the former mechanism permanently, and that this loss is inheritable through both vegetative and sexual reproductions (Ephrussi, 1953). Hirota (1958, 1960) reported on the permanent loss of the sex factor of Escherichia coli resulting from treatment with acridine. It was found that some strains of Euglena gracilis can permanently lose their ability to form chloroplasts when exposed to temperatures of 34 to 35°C under growing conditions (Brawerman and Chargaff, 1960). It was reported by the present authors that the transmissible drug-resistance factor R was eliminated by treating the organisms with acridine (Mitsuhashi et al., 1961). Gundersen et al. (1962) and Gundersen (1963) reported that the new type of streptomycin resistance, "mutator resistance," can be lost, either spontaneously or by treatment with ultraviolet light and acriflavine. Furthermore, the elimination of characters MAC+ and PCas+ from S. aureus, as described in the present article, also substantiates the fact that the changes of characters in microorganisms caused by environmental effects are inherited permanently.

Acknowledgments

We are grateful to T. Ichikawa, H. Umezawa, and A. C. Sanders, for their helpful discussions and support, and to M. Tsunoda for her technical assistance.

This investigation was supported by grant DA-CRD-AG-G19 from the U.S. Army, Research and Development.

Literature Cited


Harmon, S. A., and J. N. Baldwin. 1964. Nature of the determinant controlling penicillinase...


