TRANSDUCTION OF RESISTANCE TO SOME MACROLIDE ANTIBIOTICS IN *STAPHYLOCOCCUS AUREUS*¹

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ABSTRACT

PATTEE, P. A. (Iowa State University, Ames) AND J. N. BALDWIN. Transduction of resistance to some macrolide antibiotics in *Staphylococcus aureus*. J. Bacteriol. **84**:1049-1055. 1962.—By use of phage 80 of the International Typing Series, propagated on appropriate strains of *Staphylococcus aureus*, two related markers controlling resistance to certain macrolide antibiotics (erythromycin, oleandomycin, spiramycin, and carbomycin) were transduced among a variety of strains of *S. aureus*. Unlike the markers controlling penicillinase production and resistance to chlortetracycline and novobiocin, the determinants of resistance to the macrolide antibiotics were transduced at normal frequencies (at least 300 transductants per 10⁹ phage) only to certain of the recipient strains. One of the markers studied appears to control an inducible enzyme system which is specifically induced by sub-inhibitory concentrations of erythromycin and which controls resistance to erythromycin, oleandomycin, spiramycin, and carbomycin. The other marker examined confers resistance to erythromycin, oleandomycin, spiramycin, and carbomycin, and shows no evidence of being dependent upon an inducible mechanism.

Transduction in *Staphylococcus aureus* was first reported in 1958 by Ritz and Baldwin (1961), who demonstrated that several phages of the International Typing Series were capable of transducing the capacity to produce penicillinase

among members of this species. Morse (1959), Edgar and Stocker (1961), and Dowell and Rosenblum (1962) also reported staphylococcal transductions involving a variety of characteristics. Pattee and Baldwin (1961) reported that phages 29, 52A, 79, 80, and 53 of the International Typing Series were capable of transducing the capacity to produce penicillinase, as well as resistance to chlortetracycline and novobiocin, among a variety of strains of *S. aureus*. The present report is concerned with the transduction of resistance to several of the macrolide antibiotics among strains of *S. aureus*.

Among the pathogenic staphylococci, resistance to the macrolide antibiotics, which include erythromycin, oleandomycin, spiramycin, carbomycin, and leucomycin (Murai et al., 1959), appears to be controlled by two distinctly different general mechanisms. In vitro adaptation results in a step-wise development of resistance to the macrolide antibiotics as a group (Jones, Nichols, and Finland, 1956). Resistance in this instance probably is determined by a series of independent mutations and may be similar to erythromycin resistance in the pneumococcus (Ravin and Iyer, 1961). The other mechanism of resistance, which predominates among naturally resistant strains of *S. aureus*, was extensively studied by Garrod (1957). His results generally were in agreement with others (Jones et al., 1956; Waterworth, 1960; Rantz et al., 1957); i.e., in vitro adaptation to one of the macrolide antibiotics results in a considerable degree of cross resistance to other antibiotics of the macrolide group. During these studies, Garrod (1957) encountered certain strains of *S. aureus*, called dissociated strains, which were naturally resistant to erythromycin but apparently sensitive to the other macrolide antibiotics. Garrod (1957) was able to show that, while populations of dissociated strains consisted primarily of erythromycin-sensitive cells, such populations did not readily

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yield erythromycin-sensitive mutants. Further examination of dissociated strains revealed that growth in broth containing subinhibitory concentrations of erythromycin resulted in a population which consisted entirely of cells resistant not only to erythromycin, but also to oleandomycin, spiramycin, and carbomycin. Reversion of dissociated strains to the original phenotype occurred after several transfers in the absence of erythromycin. Waterworth (1960) reported that dissociated strains acquired temporary resistance to leucomycin after growth in the presence of erythromycin.

MATERIALS AND METHODS

The methods employed for determining the characteristics possessed by strains of *S. aureus*, the methods used for the maintenance of bacteria and bacteriophages, and the transduction techniques have been described in detail (Pattee and Baldwin, 1961). The strains of *S. aureus* employed were originally isolated from several different sources, and all were of human origin. The genetic markers possessed by these strains are included in the strain designations, and include the capacity to produce sufficient penicillinase to grow on Brain Heart Infusion (BHI; Difco) agar containing 100 units of penicillin per ml (*Pase* marker), and the ability to grow on BHI agar containing either 75 µg of chlortetracycline per ml (*Tet* marker) or 20 µg of novobiocin per ml (*Nov* marker). Strains exhibiting erythromycin resistance of the dissociated type possess the *Ery* marker. From strain U9(*Pase*, *Tet*, *Ery*, *Nov*), a mutant was isolated by the gradient-plate technique (Szybalski, 1952), which was resistant to oleandomycin. This mutant was resistant to 800 µg of oleandomycin per ml of BHI agar (*Ol* marker) and was designated U9(*Pase*, *Tet*, *Ol*, *Nov*). The designations and phage types of the strains employed are given in Table 1.

The bacteriophages of the International Typing Series used in this study were originally obtained from John E. Blair and were maintained in the Department of Microbiology, Ohio State University. Phages 29, 52A, 79, 80, and 53 were employed for transduction after propagation on appropriate donor strains. The designation of the transducing phages includes both the international number designation of the phage and the designation of the donor strain. Accordingly,

TABLE 1. *Designations and phage types of strains of Staphylococcus aureus*

Strain designation	Phage type
U9 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	80/81
U9 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i> , <i>Nov</i>)	80/81
U9 (<i>Pase</i> , <i>Tet</i> , <i>Ol</i> , <i>Nov</i>)	80/81
U35 (<i>Pase</i> , <i>Tet</i>)	80/81
U40 (<i>Pase</i> , <i>Tet</i>)	80/81
U61 (<i>Pase</i> , <i>Tet</i>)	80/81
U66 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	80/81
U91 (<i>Pase</i> , <i>Tet</i>)	80/81
U114 (<i>Pase</i> , <i>Tet</i>)	80/81
461 (<i>Pase</i> , <i>Tet</i>)	80/81
588 (<i>Pase</i> , <i>Tet</i>)	80/81
655 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	29/52A/79/7/83/47/ 53/54/73/77
4A (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	79/83/53/54/77
Ps 42B	42B
Ps 29	29
Ps 52	52
C-72	29/52A/79/80
Ch-50	80
W-26	29/80
N-135	29
608	52A/80/81
1	52A/80/81
769	81
D-1	52A/80
152	52A/79/80
112	29/52A/79/83/42E/ 80/81
248	52A/80

phage 80 propagated on donor strain U9(*Pase*, *Tet*, *Ery*) was designated 80/U9(*Pase*, *Tet*, *Ery*).

Briefly, the transduction procedure involved combining 1.0 ml of a cell-free phage lysate (10^{10} plaque-forming units per ml) with between 2 and 6×10^{10} cells of the recipient strain suspended in 1.0 ml of P and D broth (nutrient broth containing 0.25% K_2HPO_4 and 0.2% dextrose). The mixture was then shaken for 1 hr at 37 C, after which the cells were washed once with 1 ml of P and D broth, resuspended in 1.0 ml of P and D broth, and 0.05-ml quantities spread over the surface of each plate of the selective medium with the aid of a sterile bent glass rod. Control suspensions consisted of an identical suspension of recipient cells shaken with 1.0 ml of sterile P and D broth.

RESULTS

Characteristics of strains possessing the Ery and Ol markers. When a heavy (10^9 cells) inoculum of strain U9(Pase, Tet, Ery), prepared from an overnight BHI agar slant culture, was inoculated onto BHI agar containing 800 μ g of erythromycin per ml, apparent uninhibited growth was obtained. Oleandomycin, spiramycin, and carbomycin (10 μ g per ml of BHI agar) were inhibitory to identical inocula, except for a few resistant mutants present in the cell population. In contrast, strain U9(Pase, Tet, Ol, Nov) was capable of uninhibited growth on BHI agar containing either 800 μ g per ml of oleandomycin or erythromycin, or 400 μ g per ml of spiramycin or carbomycin. Strains U9(Pase, Tet, Ery) and U9(Pase, Tet, Ol, Nov) were then examined for resistance, by performing plate counts on BHI agar and BHI agar containing 25 μ g per ml of erythromycin, oleandomycin, or spiramycin. The results of this experiment (Table 2) indicate that strain U9(Pase, Tet, Ery) consisted primarily of erythromycin-sensitive cells. In contrast, strain U9(Pase, Tet, Ol, Nov) consisted entirely of cells resistant to all of the macrolide antibiotics examined. It was also observed that strain U9(Pase, Tet, Ery) possessed a significant mutation frequency, conferring resistance to oleandomycin and spiramycin. When such mutants were further examined, approximately 30% were found to possess the *Ol* marker.

To verify the fact that erythromycin resistance possessed by strain U9(Pase, Tet, Ery) was of the dissociated type, an overnight BHI agar slant culture of this strain was suspended uniformly in 10 ml of P and D broth. The cell suspension was then shaken at 37 C, and 1 μ g of erythromycin added per ml. Immediately before and for several hours after the addition of erythromycin, samples were removed and plate counts made by using BHI agar and BHI agar containing 50 μ g of erythromycin per ml. The results (Table 3) clearly indicate that a brief exposure to low concentrations of erythromycin caused a rapid

expression of resistance to erythromycin in a strain possessing the *Ery* marker. When the colonies obtained on BHI agar containing erythromycin were replicated with velveten to BHI agar containing 100 μ g of oleandomycin, spiramycin, or carbomycin per ml, they all were found to be resistant to these antibiotics. When similar colonies were replicated serially three times on BHI agar at 24-hr intervals, and then replicated to BHI agar containing 100 μ g of oleandomycin, spiramycin, or carbomycin per ml, the majority of the colonies failed to develop. Those few colonies which retained resistance to these antibiotics were resistant mutants, a result which was expected in view of the high mutation frequency previously observed with strain U9(Pase, Tet, Ery).

Transduction of oleandomycin resistance. Transduction of resistance to oleandomycin was performed by using as a selective medium BHI agar containing 25 μ g of oleandomycin per ml. Control suspensions of erythromycin-sensitive recipient strains yielded approximately 20 resistant mutants per 10^9 cells when BHI agar containing either 3 or 5 μ g of oleandomycin per ml was used. When from 10 to 800 μ g of oleandomycin per ml were employed, the transduction frequencies for the *Ol* marker were not changed significantly, and control suspensions were completely inhibited. The frequencies of transduction of the *Ol* marker obtained by use of 15 recipient strains transduced with phage 80/U9(Pase, Tet, Ol, Nov) are shown in Table 4. All control suspensions were completely inhibited, with the exception of strain U9(Pase, Tet, Ery) which yielded 10 mutants per 10^9 cells. When erythromycin, spiramycin, or carbomycin (25 μ g per ml) was employed in place of oleandomycin in the selective medium, no significant changes in the transduction frequencies were observed, although overgrowth of strain U9(Pase, Tet, Ery) was obtained with both control and transduced suspensions on BHI agar containing erythromycin. Replication experiments revealed

TABLE 2. Plate counts of strains U9(Pase, Tet, Ery) and U9(Pase, Tet, Ol, Nov) as determined on BHI agar and BHI agar containing macrolide antibiotics

Strain	Viable cells per ml on BHI agar containing (25 μ g/ml):			
	—	Erythromycin	Oleandomycin	Spiramycin
U9(Pase, Tet, Ery)	1.7×10^{10}	6.0×10^7	1.0×10^2	2.0×10^2
U9(Pase, Tet, Ol, Nov)	2.3×10^{10}	1.8×10^{10}	2.0×10^{10}	1.9×10^{10}

that all of the transductants were uniformly resistant to at least 100 μg of any of the four macrolide antibiotics per ml of BHI agar. The transductants were stable after many subcultures, and no evidence of linkage between the *Ol* marker and the *Pase*, *Tet*, and *Nov* markers was obtained.

Transduction of erythromycin resistance. The concentration of erythromycin required to inhibit the growth of spontaneous mutants present in control suspensions of erythromycin-sensitive recipient strains was determined, after which the isolation of *Ery* transductants was attempted by using an appropriate concentration

TABLE 5. *Effect of erythromycin concentration in the selective medium on the frequency of transduction of erythromycin resistance using phage 80/U9 (Pase, Tet, Ery) and recipient strain 112*

Erythromycin (μg per ml of BHI agar)	No. of colonies recovered per 10^9 cells	
	Control suspension	Transduced suspension
0.5	200	1,000
1.0	40	600
2.0	20	50
4.0	10	20
6.0	4	4
8.0	0	0
10.0	0	0

TABLE 3. *Effect of erythromycin on the incidence of erythromycin-resistant cells in a population of strain U9 (Pase, Tet, Ery)*

Time of exposure to 1.0 μg of erythro- mycin per ml	Viable cells per ml as determined on BHI agar containing:	
	—	Erythromycin (50 $\mu\text{g}/\text{ml}$)
<i>hr</i>		
0	7.9×10^9	8.6×10^7
2	8.1×10^9	8.8×10^9
4	1.2×10^{10}	1.1×10^{10}
5½	1.0×10^{10}	1.2×10^{10}

TABLE 4. *Frequency of transduction of oleandomycin resistance by phage 80/U9 (Pase, Tet, Ol, Nov)*

Recipient strain	Frequency of transduction*
U9 (Pase, Tet, Ery)	4,100
112	2,000
152	3,000
248	800
D-1	3,000
Ps 42B	0
Ps 29	0
Ps 52	32
C-72	12
Ch-50	0
W-26	6
N-135	4
608	6
1	2
769	14

* Transductants recovered per 10^9 phage particles employed in the transduction suspension.

of erythromycin. Typical results of such experiments (Table 5) indicated that there existed a higher incidence of erythromycin-resistant cells in the transduced population than in the control. However, those concentrations of erythromycin allowing the direct isolation of erythromycin-resistant transductants also permitted the growth of numerous mutants in the control suspensions. A modified technique for the selection of *Ery* transductants, termed the agar-transfer technique, was therefore devised. After the cell suspensions were washed with P and D broth, 0.05-ml quantities of the transduced and control suspensions were inoculated onto BHI agar (2% agar) plates and incubated at 37 C for 1 hr. The agar was then removed intact from the dish with a sterile 6-in. spatula and placed, inoculated surface uppermost, onto the surface of a BHI agar plate containing 25 μg of erythromycin per ml. Incubation was continued for 24 hr at 37 C. Transductants were readily obtained by this technique, while spontaneous mutants present in control suspensions were inhibited. The initial 1-hr incubation period prior to transfer and the time required after transfer for the erythromycin to diffuse into the upper agar layer resulted in a light film of background growth which, however, did not interfere with the scoring and isolation of *Ery* transductants. When the concentration of erythromycin in the base layer was reduced to 12 μg per ml, numerous mutants and a heavy background growth were obtained. Increasing the concentration of erythromycin in the base layer to 50 or 100 μg per ml caused a significant depression in the observed transduction fre-

TABLE 6. Frequency of transduction of erythromycin resistance using phage 80/U9 (*Pase*, *Tet*, *Ery*) and the agar-transfer technique

Recipient strain	Frequency of transduction*
U35 (<i>Pase</i> , <i>Tet</i>)	700
U40 (<i>Pase</i> , <i>Tet</i>)	520
U61 (<i>Pase</i> , <i>Tet</i>)	620
U91 (<i>Pase</i> , <i>Tet</i>)	820
U114 (<i>Pase</i> , <i>Tet</i>)	320
461 (<i>Pase</i> , <i>Tet</i>)	720
588 (<i>Pase</i> , <i>Tet</i>)	360
112	1,200
152	1,000
248	1,000
D-1	800
Ps 42B	0
Ps 29	0
Ps 52	50
C-72	4
Ch-50	6
W-26	10
N-135	6
608	6
1	6
769	20

* Transductants recovered per 10⁹ phage particles employed in the transduction suspension.

quency. The results of transducing 21 recipient strains with phage 80/U9(*Pase*, *Tet*, *Ery*), by means of the agar-transfer technique, are shown in Table 6. All control suspensions were completely inhibited, the transductants were stable after many subcultures, and no linkages were detected between the *Ery* marker and the *Pase*, *Tet*, and *Nov* markers.

Frequencies of transduction. In contrast to the transduction frequencies previously reported for the *Pase*, *Tet*, and *Nov* markers (Pattee and Baldwin, 1961), the *Ery* and *Ol* markers were transduced by phage 80 at normal frequencies (at least 300 transductants per 10⁹ phage) only to recipient strains 112, 152, 248, and D-1, and seven 80/81 strains of independent origin. The remainder of the recipient strains, including strain C-72, were transduced at frequencies significantly lower than normal, but only with respect to the *Ery* and *Ol* markers. To determine whether the competence of a strain to serve as a recipient of the *Ery* marker was a function of the

TABLE 7. Frequency of transduction of the *Ery* and *Tet* markers by use of recipient strains C-72 and 152 and various transducing phages

Transducing phage	Frequency of transduction*			
	C-72		152	
	<i>Tet</i>	<i>Ery</i>	<i>Tet</i>	<i>Ery</i>
29/655 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	6,000	0	8,000	1,000
52A/655 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	4,000	0	4,000	600
79/655 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	40	0	60	400
53/655 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	560	0	190	500
80/U66 (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	5,000	2	1,000	600
79/4A (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	400	0	140	200
53/4A (<i>Pase</i> , <i>Tet</i> , <i>Ery</i>)	500	28	330	600

* Transductants recovered per 10⁹ phage particles employed in the transduction suspension.

transducing phage or the donor strain, three additional chlortetracycline-resistant dissociated strains obtained from natural sources were examined by use of a variety of transducing phages. The frequencies of transduction of the *Tet* and *Ery* markers were determined with recipient strains 152 and C-72 transduced with each phage. The results (Table 7) indicated that neither the donor strain nor the transducing phage significantly influenced the relative transduction frequencies obtained. In all cases, the transduction frequencies were significantly higher for the *Ery* marker when recipient strain 152 was employed.

DISCUSSION

The results of this study generally are in agreement with earlier studies concerning the transduction of the capacity to produce penicillinase and resistance to novobiocin and chlortetracycline in *S. aureus* (Pattee and Baldwin, 1961). None of the aforementioned characteristics was detectably linked to either the *Ery* or *Ol* markers, lysogenization of the transductants by the transducing phage was not observed, and the frequencies of transduction for all markers were similar in certain instances.

A logical interpretation of the characteristics displayed by strains of the dissociated type is that resistance to erythromycin involves an inducible enzyme system. Garrod (1957) reported that as

little as 0.0015 μg of erythromycin per ml "bred" a population which was uniformly resistant to at least 64 μg of erythromycin per ml. Although the results of the present study indicate that low concentrations of erythromycin were capable of inducing resistance, no attempt was made to determine the minimal active concentration of this antibiotic. Thus far, only erythromycin has proven to be capable of inducing resistance. In agreement with Garrod (1957) is the finding that growth of an induced population in the absence of erythromycin results in a population which no longer is resistant to oleandomycin, spiramycin, and carbomycin, and which contains only a few cells capable of growth in the presence of inhibitory concentrations of erythromycin.

When cells of a dissociated strain are induced, resistance is acquired to erythromycin, oleandomycin, spiramycin, and carbomycin. Therefore, in the induced state, dissociated strains are indistinguishable from strains possessing the *Ol* marker. This observation, and the fact that strain U9(Pase, Tet, *Ol*, Nov) was obtained as a mutant of strain U9(Pase, Tet, Ery, Nov), make it reasonable to assume that the *Ol* marker represents a mutant of the *Ery* locus in which resistance no longer is due to an induced enzyme system but rather is due to the same enzyme system functioning constitutively.

In view of the nature of dissociated resistance to erythromycin, the necessity of employing the agar-transfer technique to isolate the *Ery* transductants is quite apparent. After incorporation of the *Ery* marker by the recipient bacterium, induction is necessary before complete phenotypic expression of the marker is obtained. An identical situation concerning the phenotypic expression of resistance exists among naturally occurring dissociated strains. The induction process is conveniently accomplished by means of the agar-transfer technique. When the appropriate concentration of erythromycin is employed in the base layer, the *Ery* transductants first are exposed to subinhibitory concentrations. During subsequent incubation, the concentration of erythromycin gradually increases to a level which is inhibitory to the growth of all cells except the induced transductants. When excessively high concentrations of erythromycin are used, then inhibition occurs prior to complete induction of all cells genetically capable of dissociated resistance.

The *Ery* and *Ol* markers were transduced at normal frequencies to strains 112, 152, 248, and D-1, and to seven epidemic strains, whereas the majority of the recipient strains, including strain C-72, were transduced at low frequencies by both markers. When a variety of phages were propagated on several naturally occurring chlortetracycline-resistant dissociated strains, and employed to transduce strains 152 and C-72, in every case the *Ery* marker was transduced at normal frequencies only to strain 152. The *Tet* marker was transduced to both recipient strains at frequencies which were uniform within the capacities of the phages employed. The transducing phages used vary in their transductional competence. Phage 79 was the least effective and phage 80 the most effective transducing phage when the *Pase*, *Tet*, and *Nov* markers were examined by using many of the same recipient strains used in the present study (Pattee and Baldwin, 1961). These results suggest that a degree of genetic incompatibility associated with the region of the chromosome occupied by the *Ery* and *Ol* markers exists among certain strains of *S. aureus*. The observation that the *Ery* marker was transduced to seven 80/81 strains at normal frequencies simply adds to the available evidence that all 80/81 strains are closely related (Shaffer, Baldwin, and Wheeler, 1958).

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