Inhibition Phenomenon of Staphylococci by Bacteriophages

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In the study of the relationship between bacteriophage and strains of staphylococci showing inhibition, slight differences were observed in the ability to adsorb phage between staphylococci of full phage sensitivity and those showing inhibition by phage. Only a few plaques were produced by inhibitory phages adsorbed on strains showing inhibition, whereas almost all of the phages adsorbed on corresponding phage-propagating strains produced plaques. Some strains showing inhibition were converted to full sensitivity to certain phages by heat shock or trypanflavine treatment. Treated strains adsorbed inhibitory phages to almost the same degree as nontreated strains, but most of the phages adsorbed on treated strains produced plaques. Killing was not always observed in cells adsorbing inhibitory phages. These results suggest that inhibition is not due to low adsorption rates, but rather to plaque formation by a small number of the sensitive fraction of the population and overgrowth by non-lysed cells.

Williams and Rippon (12) observed that some undiluted filtrates of high-titer bacteriophage inhibit the growth of certain strains of staphylococci on nutrient agar plates with the appearance of a thinning growth in the area on which the phage had been deposited. They concluded that the agent responsible for the inhibition of bacterial growth could only act in the presence of phages, since the preparation from which the phage had been removed could no longer produce the inhibitory effect. Rountree (7) and Rountree and Asheshov (8) noticed a relationship between the occurrence of the inhibitory phenomenon and the lysogenic state of type 80/81 strains and stated that strains showing inhibition could be converted to full sensitivity as a result of prophage substitution.

Beard and Rountree (2) concluded in their study that the inhibition is due to the phage-cell interaction in which the majority of infected cocci are killed and only a minority of cocci produce new phages. However, Wentworth and Romig (10) suggested that the infection of inhibitory phages does not always lead to the death of cells, and the inhibition may be associated with either a normal burst of phage from a small fraction of infectious centers or the release of a reduced burst from a higher proportion of infectious centers. On the other hand, West, Kelly, and Shields (11) reported inhibition of normally sensitive staphylococci by altering the adsorption of phages to

their host staphylococci; this was done by altering the salt concentration of media. The present investigation revealed that strains showing inhibition contain small numbers of infectious bacterial centers which result in plaque formation under favorable conditions, and the number of centers is markedly increased by heat shock or trypanflavine treatment of cells.

MATERIALS AND METHODS

Phages and staphylococci. A total of 21 phages included in the basic routine typing set were supplied with their propagating strains by M. T. Parker, Staphylococcus Reference Laboratory, Colindale, London, and propagated in this laboratory by the method of Blair and Williams (3). These are phages 29, 52, 52A, 79, 80, 3A, 3B, 3C, 55, 71, 6, 7, 42E, 47, 53, 54, 75, 77, 42D, 80, and 187. Phage typing was carried out by the method recommended by Blair and Williams (3), with concentrations 1,000 times the routine test dilution and with lower concentrations when necessary. The inhibition was differentiated from the overgrowth of resistant cells by the disappearance of phage action when typed with diluted phages (3). Test strains were selected from our collection of coagulase-positive staphylococci of pathological and nonpathological origins.

Adsorption of phages. The method described by West, Kelly, and Shields (11) was applied. This method, originally used by Delbrück and Luria (6), compares the change in the number of plaque-forming units (PFU) in the original phage-cell mixture with that in the centrifuged supernatant fluid after the

adsorption period. Briefly, 0.3 ml of the phages in Trypticase Soy Broth (TSB, BBL) containing approximately 1,400 PFU per ml was mixed with an equal volume of 6-hr TSB culture of the test strain. After standing for 20 min at room temperature to allow the adsorption of phages to cells, 0.1 ml of the sample was placed in a tube containing 3 ml of melted and cooled TSB containing 0.6% agar. The mixture was overlaid on a TSB (with 1.5% agar) plate, and plaques were counted after 20 hr at 37 C. After removing the initial sample, the phage-cell mixture was centrifuged at 4 C for 5 min at $1,350 \times g$. The supernatant fluid (0.1 ml) was mixed with an equal volume of a 6-hr TSB culture of propagating strains, and the PFU were counted by the overlay technique of Adams (1). Calcium chloride was incorporated to a concentration of 400 $\mu\text{g/ml}$ in all phage assay media. This experiment was repeated at least three times, and the plaque counts of five replicates were averaged.

Treatment of cells. Ma and Mandle (Bacteriol. Proc., p. 111, 1961) reported the conversion of some nontypable strains of staphylococci into typable form by heat shock. This technique was applied in this study. Cells grown in TSB at 45 C for 4 hr were heated at 55 C for 5 min and then assayed as above. The treatment with trypaflavine was also applied, as Chun et al. (4) and Weiss and Nitzkin (9) reported that acridine dyes show some favorable effects on the phage typing of staphylococci. Cells subcultured three times in TSB containing 5 $\mu\text{F/ml}$ (approximately one-half to one-third the minimal inhibitory concentration) of trypaflavine (Merck & Co. Inc.) were grown in trypaflavine-TSB for 4 hr at 37 C.

RESULTS

Phage patterns before and after treatments. The original reaction to phages and the change in patterns of test strains after the treatment are shown in Table 1. The inhibition reaction is usually disregarded in phage typing, and strains showing only inhibition to some testing phages

are considered as the nontypable form (3). However, the nontypable form in this report refers only to the strains which were neither lysed nor inhibited by any typing phages. Test strains were typed and included the 52/52A/80/81 complex, group III, the mixed group, and the nontypable form. After heat shock and trypaflavine treatment, reactions of most strains to phages were changed in various degrees. Some strains originally inhibited by certain phages were lysed by those phages after treatment, and three nontypable strains became lysed or inhibited by certain phages.

Absorption of phages by test strains. Adsorption of phage 80 by test strains showing inhibition to this phage was studied and the results are summarized in Table 2. The test strains adsorbed phages less efficiently (58 to 69%) than PS 80 (92%). The mixture of phages and cells showing inhibition produced only a few plaques in soft-agar overlays after the adsorption period, in contrast to the mixture of phage 80 and its propagating strain in which almost all of the adsorbed phages produced plaques. Table 3 shows similar experiments with other inhibitory phage-host systems. Again, propagating strains adsorbed 86 to 91% of the corresponding phages, and cells showing inhibition adsorbed 47 to 84% of the phages. Likewise, only a few plaques were observed in the mixture of phages and cells showing inhibition, whereas large numbers of plaques were seen in the mixture of phages and their propagating strains. None of the culture supernatants of propagating strains contained phages active on test strains, indicating that plaques produced in the inhibitory phage-host systems do not result from lysogenic contaminating phages derived from the propagating strains. Nontypable

TABLE 1. *Phage patterns of test strains before and after treatments*

Strain	Phage pattern		
	Original	Heat shock	Trypaflavine
N-119.....	29/52A/80°/6/54/77°/81	29/52A/79°/80/81	29/52A/79°/53/6/81
S-123.....	52A°/80° ^a	52A°/80	52A°/80
181.....	80°/81	80/81	80/81
S-482.....	80°/81°	80/53°/81	52A°/80/53°/81
S-33.....	80°/6°/81°	6°/7°/81°	55°/6°/7°/81°
N-83.....	6°/53/77/81°	80°/53/81	80°/53/81
194.....	53/54°/77	53/54/77	53/54/77
204.....	53°/54°/75/77	53°/54°/75/77	53°/54°/75/77
S-112.....	NT ^b	52/80	52/80
S-284.....	NT	52A°/81	29°/52A/81
S-132.....	NT	54	6/54

^a Inhibition.

^b Nontypable.

TABLE 2. *Adsorption of phage 80 on staphylococci showing inhibition to this phage*

Strain	PFU ^a		Per cent adsorbed ^b
	Phage-cell mixture before centrifugation	Supernatant after 20 min of adsorption	
PS 80 ^c	573	54	92.3
S-123	3	296	57.7
181	2	296	57.7
S-482	4	212	69.7
N-119	7	221	68.4
S-33	3	216	69.1
S-112	0	258	63.1
S-132	0	624	10.9

^a Plaque-forming units per 0.1 ml.^b Phages not in the supernatant after adsorption period were considered to be adsorbed. Number of phages before adsorption was approximately 700 PFU per 0.1 ml.^c Phage propagating strain.TABLE 3. *Adsorption of phages on staphylococci showing inhibition to respective phage*

Phage	Strains for adsorption	PFU ^a		Per cent adsorbed
		Phage-cell mixture before centrifugation	Supernatant after 20 min of adsorption	
52A	PS 52A	592	72	89.7
	S-123	2	293	58.1
	S-112	0	282	59.7
	S-132	0	584	16.6
81	PS 81	593	61	91.0
	S-482	4	248	64.6
	S-33	7	111	84.1
	N-83	3	235	66.4
	S-284	0	205	70.7
6	PS 6	538	98	86.0
	S-33	4	199	71.6
	H-83	7	231	67.0
	S-132	0	316	54.9
53	PS 53	576	83	88.1
	204	3	368	47.4
54	PS 54	581	98	86.0
	194	3	281	59.9
	S-132	0	329	53.0

^a Plaque-forming units.

strains adsorbed phages to the same degree as did the strains showing inhibition, but after the adsorption period the phage-cell mixture produced no plaques. Strain S-132 did not become

sensitive to phages 80 and 52A after treatments and had a reduced ability to adsorb these phages. It is evident from these results that there is a marked difference in plaque formation in soft agar after the adsorption period between strains having full sensitivity and those showing inhibition, with a relatively minor difference in the degree of adsorption.

Adsorption of phages by strains converted to typable forms. It was shown above (Table 1) that the treatment of strains with heat shock or trypanflavine not only converts nontypable forms into typable ones but also converts some inhibitory reactions into full sensitivity to phages. Strains which became typable after treatments were studied for the adsorption of phages (Table 4). About 58 to 77% of phages added to treated cells were adsorbed, a rate similar to that of untreated cells (Tables 2 and 3). However, almost all of the adsorbed phages on treated cells produced plaques, quite unlike the results with untreated cells. These findings indicate that the treatment of cells with heat or trypanflavine en-

TABLE 4. *Adsorption of phages on staphylococci treated with heat shock or trypanflavine*

Phage	Strain for ^a adsorption	PFU ^b			Per cent adsorbed
		Treat-ment	Phage-cell mixture before centrifugation	Supernatant after 20 min of adsorption	
80	PS 80		584	65	90.7
	181	H	384	283	58.1
		T	481	162	76.8
	N-119	H	448	216	69.1
	S-123	T	401	187	73.3
	S-112	H	402	240	65.7
81		T	392	205	70.7
	PS 81		589	51	92.7
	S-482	H	399	248	64.6
		T	378	261	62.7
	N-83	H	412	255	63.6
		T	382	282	59.7
54	S-284	H	384	263	62.4
		T	271	275	60.7
54	PS 54		556	98	86.0
	S-132	H	294	291	58.4
		T	351	285	59.3
	194	T	391	201	71.3

^a Strains for adsorption became typable to indicated phages after heat shock (H) or trypanflavine treatment (T).^b Plaque-forming units.

ables the adsorbed phages to produce plaques without increasing adsorption rates.

Growth of test strains in the presence of phages.

To observe how strains showing inhibition grow in the presence of phages, cells were grown in TSB at 37 C for 18 hr and then diluted with TSB to contain the desired numbers of colony-forming units (CFU). CFU were counted by a routine method after thoroughly shaking the culture to obtain the maximum dispersion of clumped cells. Cells were then infected with phages at a multiplicity of infection (MOI) of 1/CFU and placed in an incubator (37 C) for 3 to 5 hr. CFU were counted at indicated intervals (Fig. 1). The number of CFU of strain S-482 infected with inhibitory phage 80 increased as the incubation time progressed, and there was no significant difference in the number of CFU between phage-infected and noninfected cultures, whereas a marked decrease in CFU was observed in PS infected with phage 80. Similar experiments were carried out with strains 194, 181, S-123, and N-119 infected with inhibitory phages at an MOI of 1 or 2, and similar results were obtained (figures not shown).

The growth of strains which were converted to full sensitivity from inhibition by treatments with heat shock or trypanflavine was studied with and without infection of phages. Typical results were obtained with strain S-482 (Fig. 2). Treated and nontreated cultures of S-482 diluted with TSB

were infected with phage 80 at an MOI of 1, and the growth of cells was compared. The growth of phage-infected cells which became typable by treatment was very slow with a slight initial decrease in the number of CFU, whereas uninfect-treated cells grew well.

DISCUSSION

The results of this study indicate that strains showing inhibition adsorb phages with lower efficiencies than sensitive strains, and this has been described by Beard and Rountree (2) and Wentworth and Romig (10). Although West, Kelly, and Shields (11) stated that the change in the absorption rate may cause the inhibition of sensitive bacteria, the difference in the adsorption rate of phages does not seem to play a major role in the inhibition, since strains converted to full sensitivity from inhibition by treatments with heat shock or trypanflavine show the same efficiency of adsorption as the nontreated strains. The fact that few plaques are formed by phages adsorbed on cells showing inhibition but that most of the phages on cells which become typable by treatments produce plaques seems to be important for the explanation of inhibition.

It is clear from the report of Beard and Rountree (2) and Rountree (7) and from our results of single-burst experiments (*unpublished data*) that inhibited strains produce phages. In inhibitory phage-host systems, plaques can be produced under favorable conditions. On typing plates on which inoculated cells are great in number,

FIG. 1. Growth of strains S-482 and PS 80 in the presence and absence of phage 80. Solid and dotted lines indicate growth in the absence and presence (MOI of 1 per CFU) of phage, and closed and open circles indicate S-482 and PS 80, respectively.

FIG. 2. Growth of strain S-482 treated with heat shock or trypanflavine in the presence and absence of phage 80. Solid and dotted lines indicate growth in the absence and presence (MOI of 1 per CFU) of phage, and closed and open circles indicate strains treated with heat shock and trypanflavine, respectively.

plaques produced may be overgrown by large numbers of nonlytic cells during or after the formation of plaques, resulting in an appearance of reduced growth. When the number of infectious centers leading to the formation of plaques in strains showing inhibition is increased to a certain degree, the strain may become typable; the treatments with heat shock or trypaflavine can serve as a means of increasing the number of infectious centers. Low levels of plaque formation despite considerable phage adsorption by strains showing inhibition suggest that inhibition is an indication of the degree of sensitivity to phage of strains. From this viewpoint, the grouping of strains showing inhibition to one or more of group III phages as "atypical group III" (5) is a pertinent method of typing. Results showing no marked difference between the growth of strains infected with inhibitory phages at low MOI and the growth of those not infected and the marked decrease in the growth rate of strains in the presence of phages after conversion to typability suggest that the adsorption of inhibitory phages by cells does not necessarily result in the death of cells. This result supports the observation of Wentworth and Romig (10) and is contrary to the report of Beard and Rountree (2) that the inhibition was caused by the death of a majority of cells on plates having a phage to CFU ratio of 1:1.

The plaque-forming ability of most of the inhibitory phages adsorbed on cells treated with heat or trypaflavine is interesting. Although Ma and Mandle (Bacteriol. Proc., p. 111, 1961) did not show the mechanism of the conversion of a nontypable form into a typable one by heat shock, it can be supposed that the treatment with heat shock or trypaflavine may suppress a genetic

mechanism which normally prevents the propagation of phages, as suggested by Weiss and Nitzkin (9), or may eliminate or substitute prophage immunity to adsorbed phages as in the case of lysogenicity (7, 8).

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