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STUDIES ON BACTERIOPHAGE TYPING OF STAPHYLOCOCCI ISOLATED FROM BOVINE MILK

II. SOME OBSERVATIONS ON THE LYSOGENIC STRAINS

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INTRODUCTION

In the previous report⁶⁾, it was concluded that the phages of the International Series were not entirely suitable for the typing of staphylococcal strains originated from bovine milk and that a new set of typing phages should be designed.

The present study deals with investigations of three problems. The first is to ascertain the frequency with which phage carrying or lysogenic strains will be detected from milk strains of staphylococci. The second is to examine the range of hosts of phages revealed from the carriers and the availability of these phages for the typing of milk strains. And the last is to determine whether the carriage of phage may be responsible for the milk strains being resistant to lytic action of the typing phages of the International Series.

MATERIALS AND METHODS

Strains employed One hundred and eighty-nine strains of coagulase-positive bovine milk staphylococci were employed in this study. Of these, 169 were selected at random from 272 strains isolated from bovine milk, on which bacteriophage typing had been carried out with the phages of the International Series⁶⁾. The remaining 20 were propagating strains of the International phages. Their details are as follows.

Type I: 29, 52 A, 52, 925
Type II: 3 C, 3 B, 3 A, 55, 71
Type III: 70, 42 E, 6, 7, 73, 47, 54, 75/76, 53, 77
Type IV: 42 D

Each of the former (169 strains) was examined for lysogenicity and was also used as an indicator strain of other cultures. The latter (propagating strains), however, were used only as indicators for the detection of potential lysogenic milk strains.

Induction of phages Phages were induced from lysogenic strains by irradiation with ultraviolet light (UV). Each strain of staphylococci was incubated in broth at 37°C for 6 hours

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and 5 ml of this culture was poured into a sterile petri dish in a layer of 2 to 3 mm thickness. This sample was irradiated at a distance of 80 cm for 80 seconds with a 10 watt "germicidal" lamp (wave length 2537 Å) as UV source. One milliliter of the irradiated broth culture was inoculated into 9 ml of nutrient broth and incubated at 37°C overnight. Subsequently, the broth culture was centrifuged for 40~60 minutes at 3,000 rpm, and a piece of crystal thymol was put into the supernatant for sterilization. The supernatant was examined for the presence of free phage particles within 5 days of its preparation. Strict precaution to intercept visible light was maintained through the irradiation and incubation, because the induction effect is reversible by irradiation with visible light^{5,10}.

Detection of lysogenic strains Lysogenic strains were detected by plaque formation on agar plates. The culture medium⁶ (10 g of beef extract, 10 g of polypeptone, 2 g of sodium chloride and 12 g of powdered agar dissolved in 1,000 ml of distilled water and pH adjusted to 7.4) was poured into a petri dish. The medium was dried for 90~120 minutes at 37°C and then 0.2 ml of a 6- to 18-hour broth culture of an indicator strain was seeded on its surface.

When the culture had been absorbed into the agar, 0.2 ml of the supernatant of the broth culture which had been irradiated with UV was spread over the plate with a glass spreader, leaving a small margin as a control area for ascertainment whether the indicator strain presented a spontaneous lysis. Then, the plate was inverted and incubated at 37°C overnight. The presence of phage was shown by the development of lysis, usually in the form of isolated plaques and less often as semi-confluent lysis. When the plaque formation was not apparent due to its scant development, an area which seemed to be a plaque was rubbed up with a loop and suspended in 0.5 ml of broth, and 0.2 ml of this suspension was spread again over the indicator strain which had been seeded on an agar plate.

RESULTS

1. Occurrence of Lysogenic Strain

The irradiated broth cultures of the 169 milk strains were examined for the carriage of phage by plating each with all the other untreated strains including the 20 propagating strains of the phages of the International Series. In this way, a total of 31,772 combinations $\{169 \times (169 - 1 + 20)\}$ were made. And it was proved that out of the 169 milk strains, 74 (43.8%) were phage carriers and that 121 were susceptible to any and all of them. More than a half (40 strains) of the carriers were lysogenic to none of the propagating strains.

Therefore, they would have been missed if the milk strains had not been employed as indicators for the detection of the potential lysogenic strains.

The details of the phage carrying strains are listed in table 1. The lysogenicity was proved in 37 (44.0%) of 84 phage typable strains and in 37 (43.5%) of 85 untypable ones. The table, at the same time, indicates that the lysogenic strains were detected frequently in types I, II and III but rarely in type IV; 4 of 9 strains in type I, 2 of 3 strains in type II and 25 of 42 strains in type III were proven to be phage carriers while in type IV, however, only 3 lysogenic strains were detected from 22 strains tested. Such a low incidence of lysogenic strains might be accounted for by the unsuitability of the available indicators. Further work will be necessary to elucidate this point.

TABLE 1. Occurrence of Lysogenic Strains

PHAGE TYPES	NO. OF STRAINS	LYSOGENIC STRAINS	
		No.	Percent
I	9	4	} 37/84 = 44.0
II	3	2	
III	42	25	
IV	22	3	
Mixed type	8	3	
Untypable	85	37	37/85 = 43.5
Total	169	74	74/169 = 43.8

2. Grouping of Phages Carried by the Lysogenic Strains

An attempt was made to classify the bovine phages which were carried by the 74 lysogenic strains into the 4 groups (I, II, III and IV) which were established in the phages of the International Series, according to their host ranges. For convenience of description, each of the lysogenic strain was assumed to be carrying only one phage. Forty-three strains of definite phage types, including the 20 propagating strains of the International phages, were selected as indicators to determine host ranges of the phages. The details are listed in table 2. The phages attacking the indicator strains of type I were categorized as phages of group I, similarly

TABLE 2. Indicator Strains for Grouping on the Phages

PHAGE TYPES	NO. OF STRAINS
I	9
II	7
III	16
IV	11
Total	43

TABLE 3. Grouping of Phages Carried by the Lysogenic Strains

GROUPS	NO. OF STRAINS
I	1
II	3
III	12
IV	16
II+III	3
III+IV	20
Unclassifiable	19
Total	74

those active on the strains of type II, III or IV were regarded as phages of group II, III or IV respectively. And the phages which showed lytic action on strains belonging to different types were placed in the mixed group. The results revealed that a great part of the phages obtained from the lysogenic milk strains of staphylococci were active on indicators of types III and IV (Table 3). Namely, out of the 74 phages, 12 were regarded as group III, 16 were group IV and 20 were groups III and IV. Of the 36 phages in the 2 latter groups, 28 attacked milk strains of type IV, but not the propagating strain (type IV: 42D). Furthermore,

it is noteworthy that 10 of the 19 unclassifiable phages which attacked strains of untypable or mixed type only were active on strains which are quite resistant to the phages of the International Series.

3. The Relationship between Lysogenic Strains and their Indicators

HATANO and IWASAKI, in their study on lysogenicity of staphylococci originated from human beings, reported that the phages carried by lysogenic strains of type II were active on indicators of this type only, but such a correlation was not observed in strains of types I and III. SMITH⁸⁾ stated that the lysogenic strains of phage type 42D did not show lytic action on strains belonging to types other than this. He concluded provisionally that two strains, if lytic action was noted between them, were closely related to each other.

TABLE 4. *Relationship between Lysogenic Strains and their Indicators*

		NO. OF STRAINS	PHAGE TYPES OF INDICATOR STRAINS						
			I	II	III	IV	II+III	III+IV	Untypable
Phage Types of Lysogenic Strains	I	4			3				1
	II	2	1						1
	III	25			2	8		11	4
	IV	3						2	1
	I+III	2			2				
	III+IV	1	1						
	Untypable	37		3	5	7	3	7	12

In the present investigation, the relationship between the 74 phage carriers and strains susceptible to them is presented in table 4. The correlation which was noted in the previous studies can not be found from the table. In other words, correspondence in phage types between lysogenic strains and their indicators was not observed except for 2 cases in type III. It will be explained from this lack of correspondence that a phage carried by a lysogenic milk strain of staphylococci usually different from the phage which attacks the strain.

DISCUSSION

It was demonstrated by CALLOW, BURNET and LUSH, and by ROUNTREE that lysogenic staphylococci are released free phage into the surrounding medium. Recently, HATANO and IWASAKI examined for bacteriophages in the supernatants which were obtained by centrifugation of broth culture of staphylococci originated from humans and they proved lysogenicity in 35 of 99 strains tested. In this way, however, some phage carriers may fail of detection if they grow slowly or feebly and release few phage particles into the surrounding medium. In the present study, therefore, irradiation with ultraviolet light was made on staphylococci isolated from bovine milk in order to discover as many phage carriers as possible. Thus the carriage of phage was proven in 74 or 43.8% of 169 cultures tested.

This incidence of lysogenic strains is similar to that in staphylococci isolated from humans^{3,4}.

The phages carried by those lysogenic strains, however, seem to be significantly different from those of human origin. HATANO and IWASAKI observed that all of the lysogenic strains which originated from humans, except one which was coagulase-negative, exhibited lytic reactions on some of the propagating and test strains of the International phages; at the same time, they discovered many phages belonging to group III, but none of group IV from the phage carriers. The results obtained in the present work, on the contrary, reveal that more than half of the lysogenic strains which originated from bovine milk were not active on the 20 propagating strains of the International phages; in addition, the phages attacking type IV indicators were found most frequently while the phages which were active on type III indicators were common (Table 3).

In the previous study⁶ in which bacteriophage typing on milk strains of staphylococci was taken up, the author reported the existence of many type IV strains as well as type III strains; he pointed out the unsuitability of the International phages for typing on the milk strains of staphylococci, indicating the 4 following defects: (1) more than a half of the strains tested were untypable, (2) results obtained were often inconsistent, (3) no more than one phage was included in group IV, (4) cross reactions by phages belonging to different groups were frequently observed. In the present study, however, it was clearly demonstrated that most of the phage-carrying milk strains were lysogenic to strains belonging to either type III or IV while some others attacked strains which were quite resistant to the phages of the International Series. From these facts, it is expected that out of the above mentioned 4 defects, at least 2 defects, (1) and (3), will be considerably overcome by the use of phages which were released from the milk strains.

One of the purposes with which this work was undertaken was to search for the relationship between lysogenicity and phage susceptibility in staphylococci. SMITH^{8,9} investigated phage carriage of staphylococcal strains of 42D type and concluded that lysogenicity was a factor of importance in the matter of acquired phage resistance. On the other hand, ROUNTREE published the opposite view that the interference effects were very limited among staphylococci and the possession of lysogenicity does not make the staphylococci resistant to the lytic action of bacteriophages. If SMITH's opinion is correct, lysogenicity must be proven to occur more frequently in phage untypable strains than in typable ones. In the present study, however, phage carriers were detected from both groups of milk strains in almost equal frequency (Table 1). Moreover, the discrepancy in phage types between the lysogenic strains and their indicators affords basis for the

suggestion that a phage carried by a lysogenic strain is usually different from a phage which shows lytic action on the strain. These results do not seem to support the causal relation between lysogenicity and non-susceptibility to the typing phages in staphylococci.

SUMMARY

Lysogenicity was examined of 169 strains of coagulase-positive staphylococci which had been isolated from bovine milk samples. The results are summarized as follows.

1. Out of the 169 strains, 74 (43.8%) were proven to be phage carriers and 121 were susceptible to any of the phages. The lysogenic strains were detected with almost equal frequency from both of two groups of which the one consisted of phage-typable strains and the other of untypable ones.

2. The greater part of the carriers released phages which attack strains of types III and IV; about a half of the remaining part carried phages which were active on strains quite resistant to the phages of the International Series.

3. It was clearly recognized that more than a half of the phage-carrying milk strains, especially those attacking type IV cultures, were lysogenic toward none of the propagating strains of the International phages.

4. No significant correlation was observed in phage types between lysogenic strains and their indicators.

5. It was concluded that lysogenicity is not an important factor in an acquired phage resistance.

6. It was suggested in this paper that the phages carried by the lysogenic milk strains might be available for the typing of staphylococcal strains isolated from milk samples.

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