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LYSOGENY IN *CORYNEBACTERIUM RENALE*

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INTRODUCTION

The microorganism *Corynebacterium renale* is known to be widely distributed in the urine and urinary tract of apparently healthy cattle (JONES & LITTLE, 1926; WEITZ, 1947; MORSE, 1950; CRUTCHLEY et al., 1961; HIRAMUNE et al., 1967). In a previous report YANAGAWA et al. (1967) classified *C. renale* into 3 types, on the basis of its serological and biochemical properties. Nutritional requirements (HIRAI & YANAGAWA, 1967) and piliation (YANAGAWA et al., 1968) differences of the 3 types of *C. renale* contributed additional information toward their classification.

The epidemiological picture of the parasitism of *C. renale* is quite incomplete. Although the serological typing of *C. renale* is now applicable (YANAGAWA et al., 1967), the application of bacteriophage-typing would be of value in identifying, in detail, and in tracing the microorganism *C. renale*. Eventually bacteriophage-typing will give us additional information on the ecology of *C. renale*. However, so far no reports have been published on bacteriophage-typing of *C. renale*. An attempt was made by the authors to obtain bacteriophages by ultraviolet irradiation of strains of *C. renale*. The results are described in this paper. The characteristics of the *C. renale* phage will be reported in a following paper.

MATERIALS AND METHODS

A total 83 strains of *C. renale*, 42 strains of type I, 31 strains of type II and 10 strains of type III, were used. These strains were collected from various parts of Hokkaido; 59 were from Hidaka, 15 from Sapporo, 5 from Shintoku, 2 from Hayakita, and 1 each from Iwamizawa and Asahikawa. The serological properties of many of these strains were previously described by YANAGAWA et al. (1967). Later, 4 strains received from J. E. PHILLIPS of the Royal (Dick) School of Veterinary Studies, Summerhall, Edinburgh, were also used. These 4 strains were classified in our laboratory and were found to be type I.

The induction of phage was done in the usual manner using ultraviolet light. Bacteria grown in a nutrient broth for 7~8 hrs were sedimented by centrifugation, and then resuspended in a saline solution in an amount equal to about half of the original culture, usually

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3 ml, which was then poured into a petri dish 90 mm in diameter. This bacterial suspension was then irradiated with a 15 w germicidal lamp at a distance of 60 cm for 1 min. Then the irradiated bacteria were mixed with an equal volume of 6% tryptose phosphate broth (Difco) or a nutrient broth twice as concentrated as usual, and incubated at 37°C overnight. Then, these mixtures were centrifuged at 4,000 rpm for 30 min. The resulting supernate was used as "induced material" and stored at -20°C. The term "induced material" is used instead of "induced lysate" in this report, because no visible reduction of the density of any strain was observed following ultraviolet irradiation. This induced material was obtained from all the strains studied in this work.

Each induced material was then examined on nutrient agar to determine whether it contained phage by a cross spot test. All strains were examined as indicator strains.

When lysis occurred, the induced phages were then propagated. Any strain which was lysed by the spot test could be used as a propagating strain. Incidentally, all the phages induced from the strains of Japanese origin lysed *C. renale* No. 71. So this strain was used as a universal propagating strain for these phages. For the phage of non-Japanese origin, another appropriate strain was used as a propagating strain. The propagation of phages was done as follows. The propagating strain, grown on agar plate medium for 7~8 hrs, was harvested, then washed with saline and mixed in 0.3% soft agar with the induced material, and poured onto an agar plate. After incubating overnight at 37°C, the phage propagated in the soft agar culture was, after mixing with small amount of nutrient broth, collected by centrifugation at 4,000 rpm for 30 min. The supernate, which contained propagated phage, was again mixed with indicator bacteria in a soft agar. After repeated propagation (5~6 times), increasing the amount of indicator bacteria, the phage titer almost reached a maximum of $10^8 \sim 10^{10}$ plaque forming units (PFU) per ml. The phages thus propagated were mixed with 3% chloroform and immediately shaken vigorously, then centrifuged at 3,000 rpm for 10 min. The resulting supernates were used as propagated phages, and stored at -20°C.

Induced phages were designated by the prefix "RP"; for instance, "RP 6" was the phage isolated from *C. renale* No. 6.

The phage titer was determined by the agar layer technique (ADAMS, 1959). Plaques were counted after a 48 hr incubation at 37°C.

Phage antiserum was prepared by injecting concentrated phage RP 6 into a rabbit intravenously. The concentrated phage used for the injection was prepared, from a large amount, about 3,000 ml, of the phage propagated as described above by centrifugation, at 30,000 rpm for 60 min. Injection of the concentrated phage into the rabbit was done 8 times at 4 day intervals. After this immunization procedure was completed the titer of the serum was found to be sufficient. The rabbit was bled, and the anti-RP 6 serum thus obtained was stored at -20°C. This serum was assayed for anti-phage activity by the method described by ADAMS (1959). The neutralization constant (K value) of the immune serum for phage RP 6 was 243.8.

RESULTS

1 Isolation of bacteriophages

After irradiation, no visible decrease of bacterial density was found in any strains. The

induced materials were tested against all the other strains by the cross spot test. The results were shown in table 1. Twenty-nine of the 46 (63%) strains of type I produced phages, which included 1 from Scotland. No phages could be detected in 31 type II strains or in 10 type III strains. Electron microscopic examination was done, after centrifugation at 30,000 rpm for 60 min, on some of the induced materials. Phages could be detected morphologically in the materials from the strains of type I but not of types II and III.

TABLE 1 *Number of C. renale strains producing phages after ultraviolet irradiation*

TYPE OF <i>C. renale</i>	NUMBER OF STRAINS		
	Irradiated	Produced bacteriophage	%
I	46	29	63
II	31	0	0
III	10	0	0

2 Host range

Of the 29 induced phages, 28 lysed *C. renale* No. 71, many of them also lysed *C. renale* Nos. 8, 74, 11 and 31. The phage titer of these induced materials, estimated with the indicator strain No. 71 ranged from $10^2 \sim 10^8$ PFU/ml. After propagation of these induced phages in *C. renale* No. 71, as described in Materials and methods, the titer reached $10^8 \sim 10^{10}$ PFU/ml. They all lysed *C. renale* Nos. 71, 11, 8, 31, 29 and 49. The only exception was phage RP 50 which lysed *C. renale* Nos. 71 and 11. This phage did not propagate enough, and after the propagation procedure reached 10^5 PFU/ml with difficulty, which indicated the above host range.

Another phage, induced from *C. renale* FS 113-63 isolated in Scotland, lysed only strain R-4, the Scottish isolate. This phage was also difficult to propagate. The host range of this phage was the same following propagation.

In table 2, the host range of these phages is shown. The host range is clearly different between the phages isolated from the strains of Japanese origin and that of Scottish origin. In addition, the phages derived from the strains isolated in Hidaka, Hokkaido, had the same

TABLE 2 *Host range of the 3 phage groups*

PHAGES	ORIGIN OF THE STRAIN	HOST RANGE
RP 6 and other 26 phages	Hidaka, Hokkaido	*
RP 50	Sapporo, Hokkaido	71/11
RP FS 113-63	Scotland	R-4

* Name of strain

host range, and were slightly different from the phage RP 50, which was derived from a strain isolated in Sapporo, Hokkaido.

Although many strains isolated in Hidaka, Hokkaido, produced the phages of the same host range, there were other strains which did not produce phages. The strains which did not produce phage were examined to determine whether or not they were rough in colonial morphology. Under the microscope, however, colonies of these strains were found to be smooth.

3 The relationship of phage-type to the epidemiological features of *C. renale* infection

Twenty-seven phages were produced from the strains isolated on a farm in Hidaka, Hokkaido. Since these phages showed the same host range they belonged to the same phage-type. Of these 27 strains, 7 were isolated from cattle with pyelonephritis, while 20 were isolated from the urine and urinary tracts of apparently healthy cattle. Therefore, it is obvious that strains of the same phage-type were distributed among the diseased and apparently healthy cattle in the same farm. This indicates that the establishment of this disease was primarily influenced by certain predisposing factors of the host animal, such as pregnancy, etc., and not by the phage-types of *C. renale*.

4 Confirmation of true lysogeny

Whether the lysogeny in *C. renale* is true or not was determined by incubating *C. renale* No. 6, which could produce phage RP 6, in nutrient broth containing a 10% anti-phage RP 6 serum. Another lysogenic strain, *C. renale* No. 27, which could produce a similar phage

TABLE 3 *Retention of lysogenic properties after incubating 2 lysogenic bacteria in anti-phage RP-6 serum*

LYSOGENIC BACTERIA	INCUBATION WITH ANTI-PHAGE RP-6 SERUM * ¹	COLONIES	
		examined	cured * ²
<i>C. renale</i> No. 6	Yes	76	0
	No	52	1
<i>C. renale</i> No. 27	Yes	59	0
	No	81	0

*¹ Incubation was done in a nutrient broth containing 10% anti-phage serum at 37°C for 2 days.

*² "cured" means loss of lysogenic property, that is, the loss of the ability to produce phage and the loss of prophage-immunity.

was also examined. After 2 days incubation, colonies of these strains were found to be still lysogenic because they retained: (1) the ability to produce phage and, (2) prophage-immunity (tab. 3). These results indicate that the lysogeny of *C. renale* is true and not pseudolysogeny.

DISCUSSION

In this paper, the authors reported the first isolation of phage from strains of *C. renale*. Despite the fact that *C. renale* is widely distributed among cattle, no report had been published on the phage of *C. renale*.

It is interesting to note that only strains of *C. renale* type I produced phages. The bacterial strains susceptible to these induced phages were also type I. Therefore, lysogeny was found only in the strains of type I and not in types II and III. This fact adds additional information to the differences noted between the 3 types of *C. renale*.

Phages were obtained from 29 of the 46 strains of type I. The ratio, 63%, indicates that many strains of *C. renale* type I are lysogenic. This ratio would increase when more indicator strains are used. No particular relationship of these lysogenic strains with other properties, such as serology and piliation, has been found yet.

Isolated phages lysed only those strains belonging to type I and, accordingly, they could be said to be type I-specific. The host range of these phages was rather limited, and they did not lyse all strains of type I. For the purpose of identifying *C. renale* type I, the phages obtained in this experiment could be used, but only partially.

Two or three different phage types were demonstrated in this report. The host range of phage RP 50 might be the same as that of the other Japanese phages. It was especially interesting that the phage of Scottish origin had a host range quite different from the phages of Japanese origin. The existence of different phage-types of *C. renale* is thus obvious. It will be possible in the near future to classify strains of type I of *C. renale* into more phage-types. In this experiment, we used strains of *C. renale* originating only from Japan and Scotland. A complete phage-typing of all type I strains of *C. renale* could be attained by collecting more lysogenic strains from various parts of the world.

Both phage RP 50, originating from *C. renale* No. 50, which was isolated in Sapporo, Hokkaido, and another phage originating from a strain isolated in Scotland were difficult to propagate. The difficulty in propagation could be characteristic of some of the *C. renale* phages.

A routine test dilution, which was not assayed at this time, will be needed when phage-typing is routinely applied to *C. renale* in the future.

Strains of *C. renale* of the same phage-type were found in the same area, both in the cattle affected with pyelonephritis, and in apparently healthy cattle. This indicates that *C. renale* of the same phage-type was widely distributed in this area, and there may be some possibility detecting the source of infection of

C. renale by applying phage-typing.

It was indicated that the urine and urinary tracts of many cattle raised in this area were parasitized by the same phage-type of *C. renale*. Whether or not pyelonephritis was established in a particular animal may be largely dependent upon certain predisposing factors of the host animal, such as feeding, pregnancy, parturition, etc., and not on the phage-type of *C. renale*. Although this was roughly postulated before (MERCHANT & PACKER, 1961; CRUTCHLEY et al., 1961), it is substantiated in this report, on the basis of our more recent and accurate knowledge of the types of *C. renale*, such as serological type and phage-type.

While conducting this work we noticed that prophage was eliminated spontaneously from a part of bacterial population. Although this finding is being investigated and will be reported in another paper it prompted us to determine whether the lysogeny in *C. renale* is true lysogeny or pseudolysogeny. The result of the experiment along this line using anti-phage serum indicated that it was true lysogeny.

SUMMARY

In order to isolate temperate phages, ultraviolet irradiation was applied to the total 87 strains of *C. renale*. These strains included those of 3 types of *C. renale*, and were isolated mainly in Hokkaido except for 4, of which were received from Scotland. Phages were isolated from 29 of the 46 (63%) strains of type I. No phages were obtained from 31 strains of type II or 10 strains of type III.

Of the 29 phages isolated from type I strains, 28 were from the strains isolated in Hokkaido and 1 was from a strain isolated in Scotland. The former phage group, all had a similar host range and lysed strains of *C. renale* isolated in Hokkaido while the latter only lysed another strain isolated in Scotland. No common host range was found between the two phage groups. This suggests that phage-typing is applicable for *C. renale* and that different phage-types exist in Japan and Scotland.

It was found on a farm, in Hokkaido, that the urine and urinary tracts of many apparently healthy cattle were parasitized by *C. renale* of the same phage-type. Pyelonephritis occurred only in a small percentage of these cattle. Thus, based on the knowledge obtained from the phage-typing of *C. renale*, we concluded that the factors influencing the establishment of the disease, were largely predisposing factors of the host animal and were not the type of *C. renale*.

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