Observations on the Interrelationship of Cholera phages and El Tor Vibrios[1]

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Since d'Herelle (1) observed cholera bacteriophages in 1920 a host of investigators have been active in this field. In recent years Dr. Mukerjee of India and his associates (2) have used four groups of cholera phages extensively and have obtained considerable data on the properties of these phages and their relationship towards cholera vibrios. These phages are in wide use for separating cholera vibrios into phage types and for the identification of the El Tor vibrios. El Tor vibrios are not susceptible to group II and group IV phages, but are lysed by group I and group III phages (3). The insusceptibility to group IV phages is presently the most generally accepted characteristic of the El Tor strains (4). This report will present our experience with the interaction between these phages and vibrios.

Materials and Methods

The four groups of cholera phages and the five type-strains of true cholera were kindly provided by Dr. Mukerjee. The El Tor strains from East and West Pakistan were isolated in our laboratory; some East Pakistan strains of El Tor were obtained from Dr. C. H. Yen. El Tor strains from other countries were the gifts of Drs. Feeley, Gomez, Mukerjee, Noyes, and Maitland.

The broth used for bacterial growth contained 1 percent trypticase (BBL) and 1 percent sodium chloride in distilled water. Bacto-agar (Difco) was added to a concentration of 1.5 percent to prepare basal agar plates and to a concentration of 0.7 percent to prepare soft agar. All media were autoclaved at 20 lbs. pressure for 20 minutes; the pH was about 7.1 and did not need adjustment.

Phage stocks with high titer (10^{11} p.f.u./ml.) were obtained as follows:

Mukerjee cholera vibrio strain 154 was grown in 50 ml. of trypticase broth in Erlenmeyer flasks (125 ml.) under aeration at 37° C. for 5 hours. The total bacterial count at the end of this incubation period was about 10^{11} per flask. Enough phage stock was added to total approximately 2×10^{11} p.f.u. of phage to insure infection of all bacteria; aeration was continued for an additional period of 3 hours at 37° C. Chloroform was then added and the flask stored overnight in the refrigerator. In the morning the lysate was centrifuged at low speed to sediment the bacterial bodies. The supernatant was used as the phage suspension. Dilutions were made in trypticase broth. Phage plaque-forming units were enumerated according to the agar layer method of Adams (5).

Antisera were prepared in rabbits. Two intraperitoneal injections with 5 ml. of the chloroform-treated and centrifuged phage lysate were given at 1-day intervals. After 1 week, an intravenous injection was given with 1 ml. of the same phage lysate. One week after the last injection the rabbits were bled. The sera obtained were adsorbed with the host bacteria to remove bacterial antibodies. Serum dilutions were made in normal saline.

Action of the four groups of Mukerjee phages was studied by the following methods: (1) log dilutions of various phage preparations were dropped on lawns of the bacteria; (2) log dilutions of the phage were mixed with young bacterial cultures in soft agar and overlaid on basal agar plates; and (3) phage and bacteria were mixed in trypticase broth and the viability of the phage as well as of the bacteria was determined.

Results and Discussion

Figure 1 shows the patterns of lysis obtained when serial log dilutions of the four Mukerjee phages were dropped on a lawn of strain Burma J, an El Tor vibrio. The number of p.f.u. deposited at each spot ranged from $0.5–1.2 \times 10^{9}$ at 10^{-0} to $0.5–1.2 \times 10^{6}$ at 10^{-3}. Only phage II showed no clearing or discrete plaque formation; phages I and IV showed clearings were up to $0.5–1.2 \times 10^{8}$ p.f.u. of phage were dropped. Phage III showed semi-confluent lysis due to numerous discrete plaques containing $0.5–1.2 \times 10$ p.f.u/drop and clearings at high-phage concentration. On lawns of some El Tor strains drops of phage III produced a pattern exactly like that produced by phages I and IV.

The agar layer method was found to disclose phage effects sometimes not evident by the drop method. With the agar layer method all El Tor strains available to us

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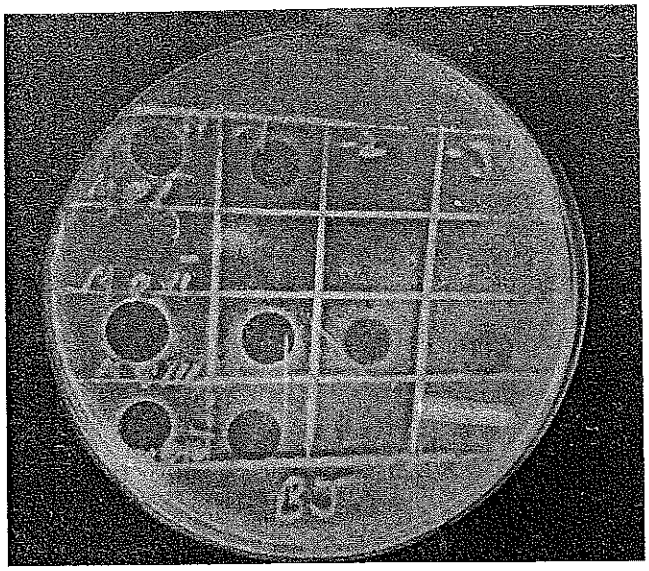

FIGURE 1.—Pattern of lysis of El Tor vibrio with four Mukerjee phages.

were tested for their behavior towards the Mukerjee phages and the following picture emerged:

(i) *Thinning of the Lawn.*—With most El Tor strains phages I, III, and IV caused thinning of the lawn at high-phage concentration but not discrete plaque formation at any concentration of the phage. Phage II showed thinning of the lawn only with rare strains of El Tor.

Thinning of the bacterial lawn at only high-phage concentrations, with no discrete plaque formation at lower phage concentrations, indicates that a large number of bacteria die but that the phage does not multiply in that particular strain of bacteria. This will explain the clearing at high-phage concentrations which is observed by the drop method.

The interaction of phages and El Tor vibrios was tested by mixing the four Mukerjee phages with El Tor strains Burma J, Indonesia 40, Karachi 28373, and Karachi 30369 in trypticase broth. Viable bacteria and phage were determined after 3 hours at 37° C. The results obtained fell into two types represented by Burma J and Karachi 30369, as shown in table 1: (a) Phage I is lost in the presence of El Tor vibrios but this phage does not reduce the viability of the bacteria to any significant extent; (b) viability of phage II is not affected by the El Tor vibrio nor does it affect the viability of the vibrio; (c) phage III multiplies on Burma J and its titer increases with time, but it does not multiply on Karachi 30369 and its titer decreases slightly. When this phage multiplies the bacterial count falls considerably, and no fall in vibrio count is noted if the phage titer does not increase; (d) both phage IV and El Tor vibrio counts fall markedly when these are mixed.

TABLE 1.—Mutual effect on viability of Mukerjee bacteriophages and El Tor vibrios in trypticase broth mixtures

Initial mixtures		Titer of vibrio and bacteriophage			
Vibrio strain	Bacteriophage group	Vibrio		Bacteriophage	
		0 hour	3 hour	0 hour	3 hour
Burma J	I	7×10^8	7×10^8	5×10^9	6×10^4
Do	II	7×10^8	9×10^8	4×10^9	1×10^9
Do	III	7×10^8	3×10^5	3×10^9	2×10^{10}
Do	IV	7×10^8	8×10^4	5×10^9	5×10^5
Karachi 30369	I	6×10^8	1×10^9	5×10^9	6×10^6
Do	II	6×10^8	1×10^9	4×10^9	2×10^9
Do	III	6×10^8	7×10^8	3×10^9	1×10^9
Do	IV	6×10^8	3×10^5	5×10^9	4×10^4

(ii) *Minute Plaque Formation by Phage III.*—A total of 156 strains of El Tor from various places were tested by the agar layer method for sensitivity towards Mukerjee phage III. Results are given in table 2. Phage III produced minute plaques on 72 strains (46 percent). Some strains showing this phenomenon were found among cultures from every country, indicating that both the resistant as well as the minute plaque-forming types of El Tors are widely distributed. The ability of phage III to form minute plaques on El Tor strains seems to be a fixed property of the vibrio strain, with no change in susceptibility to the phage on subculture. The morphology of the minute plaques is shown in figure 2.

When phage III was titrated on three El Tor strains known to be susceptible to it (Burma J., Indonesia 40,

TABLE 2.—Minute plaque formation by Mukerjee group III bacteriophage on El Tor vibrios from different countries

Origin of strain	Total number tested	Number showing plaques
East Pakistan	14	7
West Pakistan	84	24
Burma	10	10
Hong Kong	7	3
Indonesia	7	6
Malayasia	17	10
Philippines	9	6
Thailand	8	6
Total	156	72

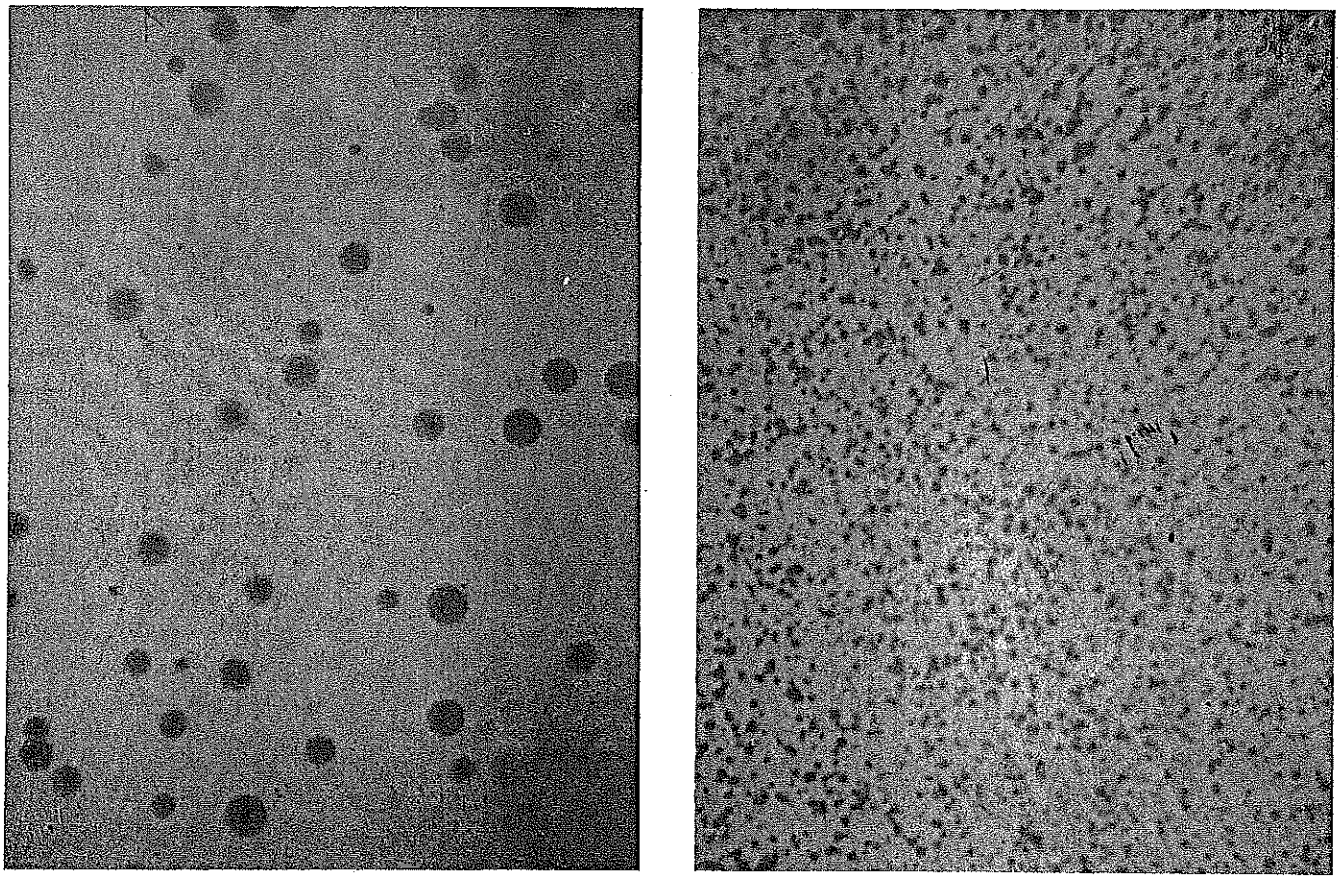

FIGURE 2.—Plaque morphology of Mukerjee phage III(a) on host strain 154(b) on El Tor strain. (x2)

and Muar 1360) the number of plaques appearing on the El Tor lawns was about 50 percent of that noted on the host strain 154. Mukerjee Phage III evidently is not altered by growth on the El Tor strains; the phage recovered from the supernate after growth on Burma J forms minute plaques on Burma J, but produces normal-size phage II-like plaques on strain 154.

These minute plaques are obviously due to multiplication of the phage. Figure 3 shows the results of a one-step growth experiment of phage III with Burma J and strain 154. In this experiment 4.5 ml. of young cultures of Burma J or strain 154 (at 37°) were mixed with 0.5 ml. of a suitable dilution of the phage at (37°). Twenty minutes were allowed for the phage to attack and then the mixture was diluted 100 times and 1,000 times in trypticase broth (at 37°). Aliquots of 0.1 ml. from the undiluted preparation and the two dilutions were plated at 15-minute intervals on *V. cholerae* strain 154 only. The plates were incubated overnight and the number of plaques counted in the morning. It can be seen from this figure that (i) 50 percent of the phage count was lost when added to the El Tor strain, as compared with the count obtained after the same preparation was mixed with strain 154; (ii) burst time in both strains of the bacteria is the same; and (iii) the burst size on Burma J is slightly less but is within the same order of magnitude as that for strain 154.

(iii) *Turbid Plaque-forming Phages.*—Of the 156 El Tor strains tested with the four Mukerjee phages by the agar layer method, 17 strains from West Pakistan and two from Burma gave rise to turbid plaque-forming phages. Plaque morphology of these phages is shown in figure 4. One turbid plaque appeared for each 10^5 to 10^6 p.f.u. of the Mukerjee phage added. An interference phenomenon was found between the phage added and the number of turbid plaques appearing: 5 to 10 plaques were seen when 10^9 phages were added, but 100 to 500 plaques appeared when only 10^6 phages were added. This interference was not seen with pure phage prepared by infecting the host bacterium in broth with phage from a turbid plaque. The host used for these phages was the El Tor strain under test when the turbid plaques appeared.

None of these turbid plaque-forming phages grew on the universal host, cholera strain 154. The plaque morphology of the various turbid plaque forming phages obtained from a number of El Tor strains after treatment with any of the Mukerjee phages was very similar.

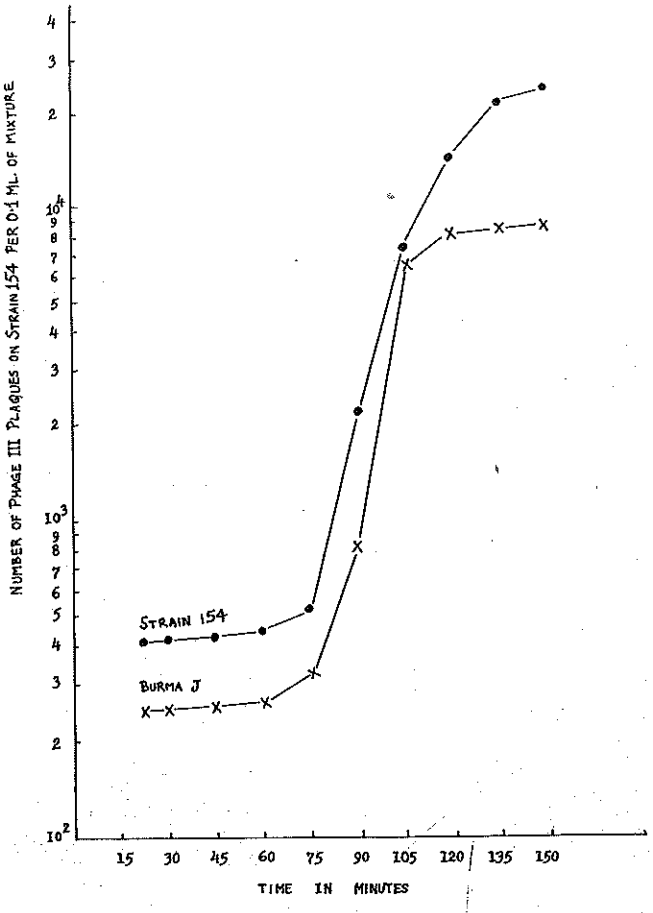

FIGURE 3.—One-step growth experiment of phage III with Burma J and strain 154.

The turbid plaque is characterised by growth of bacteria in the center surrounded by a clear zone of lysed cells. Bacteria from the center of the plaque were streaked on gelatin agar plates and many colonies were found after overnight incubation. Twenty such colonies were individually cultured in trypticase broth and found to be fully susceptible to the turbid plaque-forming phages.

In order to exclude the possibility that the stock phage was contaminated with the turbid plaque-forming phage, single plaque isolations of phages, III and IV, carefully passed three times through strain 154 grown on trypticase broth and gelatin agar, were tested with the agar layer method. Turbid plaque-forming phages were observed consistently on strain Karachi 29119, on which such plaques were originally seen.

A single turbid plaque together with the surrounding bacteria, when added to 10 ml. of trypticase broth and incubated overnight, yielded about 10^6 to 10^7 p.f.u./ml. of the phage.

El Tor vibrios are known to harbor temperate phages (6), which can be liberated by treatment with heat or ultraviolet light. We were interested in learning whether or not the strains that liberate the turbid plaque-forming phages due to Mukerjee phages would liberate temperate phages under the conditions in which temperate phages are released. Nine El Tor strains known from previous work to release temperate phages by one treatment or another were simultaneously treated with the Mukerjee phages according to the agar layer method, with heat for 1 hour at 56° or with ultraviolet light for 4 minutes. The El Tor strains treated with Mukerjee phages or ultraviolet light were grown for a suitable length of time and then tested for lysogenic phages on Takeya strain and Malacca 3734. Appearance of plaques on the indicator strains was considered as evidence of release of a temperate phage under the influence of the treatment given. The results of this experiment are given in table 3. Evidently those El Tor strains that release a phage under the influence of heat or ultraviolet light are not those from which turbid plaque-forming phages appear after treatment with Mukerjee phages, and vice versa. The other important point about all these nine phages is that their plaques looked the same: that is, the phages coming out from the same strain due to treatment with heat or with ultraviolet light may be considered as one.

FIGURE 4.—Plaque morphology of turbid plaque-forming phages

TABLE 3.—Appearance of turbid plaque-forming bacteriophage from El Tor vibrio strains after various treatment

El Tor strain	Treatment giving rise to turbid plaque-forming phages		
	Infection with phages I to IV	Heat	Ultraviolet light
Bandung 8	—	+	+
Indonesia 9	—	+	+
Indonesia 40	—	+	+
Karachi 28879	—	+	+
Karachi 28885	+	—	—
Karachi 29114	—	+	+
Karachi 29119	+	—	—
Karachi 30369	+	—	—
Lahore 54931	+	—	—

+, phage isolated; —, no phage isolated.

The host range of the 9 turbid plaque-forming phages shown in table 3 (4 obtained by Mukerjee-phage treatment and 5 obtained by heat or ultraviolet treatment) was determined by the agar layer method on 37 strains of true cholera and El Tor vibrios. Plaque-formation was used to indicate the strain serving as host. Results are presented in table 4. All nine phages attack the same organisms.

Since some of the turbid plaque-forming phages were obtained from vibrios treated with the Mukerjee phages, it was possible that these phages were actually Mukerjee phages slightly modified by their growth in the El Tor strain. An antigenic relationship between the Mukerjee phages and the corresponding turbid plaque-forming phages could be studied by preparing an antiserum against one and testing its neutralizing power on the other phages. Table 5 gives some preliminary results with antisera prepared against Mukerjee phages III and IV and their neutralizing effect on themselves and on the turbid plaque-forming phages obtained under their own effect. Decrease in the titer of Mukerjee phages III and IV by about 4 logs in 1 hour in the presence of the specific antiserum indicates that these sera have specific neutralizing antibodies. Turbid plaque-forming phages obtained after treatment with Mukerjee phages III and IV were not neutralized by either of the antisera, indicating that the neutralizing antibodies of these phages do not have much in common.

TABLE 4.—Host range of turbid plaque-forming phages obtained from El Tor vibrios by various treatments

Host organism	Number of strains tested	Number of strains found sensitive
V. cholerae (Takeya strain)	1	[1] 1
V. cholerae (Mukerjee type strains)	5	0
V. cholerae (East Pakistan strains)	10	0
El Tor (Karachi strains)	7	[1] 5
El Tor (Burma strains)	3	0
El Tor (Indonesia strains)	3	0
El Tor (Malayasia strains)	5	[1] 5
El Tor (Philippines strains)	3	0

[1] These strains were sensitive to all the phages tested.

TABLE 5.—Effect of rabbit sera against Mukerjee phages III and IV on homologous phages and on turbid plaque-forming phages appearing after their use

Antiserum	Serum dilution	Plaque-forming units [1] after neutralization with serum			
		Mukerjee phage III	Mukerjee phage IV	Turbid plaque III [2]	Turbid plaque IV [2]
None	.....	10^9	10^9	10^6	10^6
Mukerjee phage III	1 : 25	10^4	10^9	10^6	10^6
	1 : 100	10^4	10^9	10^6	10^6
Mukerjee phage IV	1 : 25	10^9	10^4	10^6	10^6
	1 : 100	10^9	10^4	10^6	10^6

[1] Mukerjee phages III and IV were titrated on strain 154 and turbid plaque phages III and IV were titrated on Karachi 29119.

[2] Turbid plaque phage III was obtained by treating Karachi 29119 with Mukerjee phage III, and turbid plaque phage IV was obtained by treating Karachi 29119 with Mukerjee phage IV.

The data obtained so far indicate that all these turbid plaque-forming phages resulting from any of the treatments have similar host ranges and plaque morphologies. They are not neutralized with antisera prepared against Mukerjee cholera phages. Those phages that are obtained by the action of ultraviolet light or of heat are truly temperate; those that are obtained after treatment with the Mukerjee phages may be defective temperate or lytic phages. Since temperate phages do not grow on the parent strain, and since the same phage seems to be liberated by treatment with any one of the four phages, it is indicated that the phage present inside the bacteria depends for its lytic power on some kind of nonspecific recombination with a Mukerjee phage genome. Heat and ultra-

violet light *per se* are incapable of fulfilling this requirement, and therefore no phage is detected in indicator strains after treatment with these agents. Although it is slight, the possibility that some of these are really mutants of Mukerjee phages can not be completely ruled out.

The work reported here suggests that the response of El Tor vibrio to bacteriophages depends on quantitative relationship. It raises more questions than it answers—which is in accordance with the announced purpose of this symposium.

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Lysogeny in El Tor Vibrio

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In our preliminary report in 1963 (*1*), a "prophage typing" of El Tor vibrio into Celebes and Classic-Ubon types was attempted. According to the prophage typing, El Tor vibrios isolated in the Ubon outbreak (1961) were considered as Classic-Ubon type (*1*), and nonhemolytic El Tor vibrios isolated in the west New Guinea outbreak (1962) were considered as Celebes type (*2*), even though the latter outbreak had been considered as being etiologically different from all other outbreaks in the west Pacific by DeMoor (*3*).

In this paper further investigations of lysogeny in El Tor vibrio will be reported, using newly isolated strains. In addition, biological properties of El Tor temperate phages and an application of the temperate phage detection method for early diagnosis of El Tor vibrio carriers will be reported.

1. Distribution of lysogenic strains among El Tor vibrios:

For examination of lysogeny each strain of El Tor vibrio was cultured for 24 hours in nutrient broth and the culture was centrifuged at 4,000 r.p.m. for 40 minutes. The supernatant was spotted on a plate which had been inoculated with the indicator organisms suspended in semisolid agar medium. The plate was incubated at 37° C. and observed the next morning. Since the lysis reaction by El Tor temperate phages is comparatively unstable, the experiment was repeated several times in case negative results were obtained. On the other hand, when lysis of the indicator bacteria at the spotted area was not clear, the plaque assay method was used to confirm positive results.

As shown in table 1, 135 strains of El Tor vibrio (83 hemolytic and 52 nonhemolytic) isolated in the epidemic of the west Pacific from 1960 to 1963, and 3 strains isolated in the 1959 outbreak in Makassar, Celebes, were examined for evidence of lysogeny. *Vibrio comma* strain H218 was used as the indicator organism, because it has proved to be one of the most susceptible strains to vibrio phages. Of 138 tested strains 136 were found to liberate temperate phages. The two strains which did not liberate temperate phage lytic to the indicator strain were VE 12, and VE 13. Since temperate phages from 133 strains showed a common very narrow host range, they were tentatively designated as "kappa type phages." Three strains isolated in Sarawak in 1961 liberated temperate phages lytic not only to the H218 strain, but also to many other vibrio strains. They were tentatively designated as "SE phages."

On the other hand, all El Tor vibrios isolated from mild cases or from water, including five classic Arabia strains, two Calcutta strains (1958), four Bangkok strains (1959, 1960), and three Ubon strains (1960), did not liberate temperate phage active against the H218 strain.

The results obtained here seem to support our attempt of "prophage typing" with a few exceptions, but further investigation using many other strains of El Tor vibrio will be necessary to determine whether or not our prophage typing can be used routinely.

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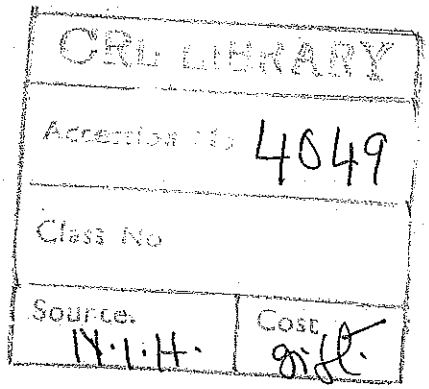

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