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Role of Temperate Phage in Determining Lytic Phage Sensitivity and Serotype of *Vibrio cholerae*

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The effect of lysogenization with five temperate phages from various sources on serotype and lytic phage sensitivity was investigated in six cultures of *Vibrio cholerae* of both classical and El Tor biotypes. No changes in serotype or in classical phage sensitivity in the classical biotype were observed. Four of the temperate phages were homoimmune and induced resistance to one of the El Tor typing phages, E3, thereby causing a type change in El Tor strains. The sensitivity to the other phages was not changed. In 14 natural isolates too, E3 (group III) phage resistance correlated with the presence of temperate phage. Postadsorption exclusion was found to be the mechanism of resistance involved. The fifth phage, VcA-1, had a unique immunity profile. It could infect the El Tor biotype of *V. cholerae* but caused no change in the host properties investigated.

Asheshov et al. (1) first showed the difference in sensitivity of *Vibrio cholerae* strains to different bacteriophages. Later, Mukherjee et al. (11) developed a highly successful phage typing scheme for the classical biotype which, however, soon became redundant with the sudden widespread emergence of the new El Tor biotype. Several phage typing schemes were then developed for these new vibrios (6, 12, 14, 15), and, finally, the modified scheme of Basu and Mukherjee (3), using a different set of five lytic phages, was introduced and is still in widespread use. The El Tor vibrio scheme, although independently found to be valid epidemiologically (4), still remains problematical. In recent epidemics, all of the phage types originally described could no longer be found. Virtually the only types encountered are types 2 and 4, with a substantial number of untypable strains (8, 9). Type variability on subculture is also common. After more than a decade of effort, attempts to improve the typing scheme are still in progress (5).

In 1963, Takeya and Shimodori (19) reported that the majority of El Tor vibrios carry a temperate phage which they named kappa (K-phage) (21). Several other temperate phages have also been demonstrated in classical and El Tor biotypes (7, 15). High phage activity in platings of cholera stool is a common observation, and, indeed, Takeya et al. found that the presence of K-phage is more sensitive than culture test for diagnosis of *V. cholerae* (20). Yet the role of temperate phages in the biology of *V. cholerae* has not been thoroughly investigated. Recently, serotype conversion induced by a temperate phage has been reported (16). We therefore investigated the correlation between serology, phage type, and lysogeny in *V. cholerae*. We used natural isolates as well as lysogens constructed with five well-characterized temperate phages, including K-phage (20), α phage (15), VcA-1 phage (7), and VcA-2 phage (7).

MATERIALS AND METHODS

Cultures. The 24 strains used are listed in Table 1. They were maintained in sealed tubes of peptone-agar at ambient temperature and cultured in nutrient broth or agar (Oxoid) at 37°C for 18 h unless otherwise stated. Viable counts (colony-forming units [CFU]) were determined by standard methods. The cultures were examined for serotype, biotype, phage type, and sensitivity to and presence of temperate phages by the methods described below.

Bacteriophages. El Tor (E series) and classical (C series) lytic typing phages were obtained from the Cholera Research Centre, Calcutta, India. Their respective propagating strains were Mak757 and 154. Standard procedures for propagation, qualitative spot assay of routine test dilution for phage type, and quantitative assay of PFU by the soft agar dilution method were used (10).

Temperate phages were usually obtained (10^4 to 10^6 PFU/ml) without induction from chloroform-treated, centrifuged broth supernatants of 18-h host cultures. For test phage 22 from strain SLH22 (20), phage α from variety 2 (15), and phage 241 from strain PS241 (our collection), these supernatants were additionally treated with DNase (1 μg/ml; $MgCl_2$, 5×10^{-3} M) for 1 h at 37°C before filtering through G5 scintered glass

TABLE 1. Cultures used and their relevant characteristics

Culture[a]	Serotype[b]	Phage type[c]	Source (reference)[d]
El Tor biotype			
Mak 757	OG	1	El Tor phage propagating strain (3)
Variety 2	IN	4	Alpha producer (15)
SLH22[e]	OG	4	Kappa producer (19)
HP47 (1966)	IN	2	Ubon-cured strain (19)
VRC276 (1971)[e]	OG	1	CRCC
VRC242 (1971)[e]	IN	2	CRCC
VRC221 (1971)[e]	OG	4	CRCC
VRC241 (1971)[e]	IN	3	CRCC
VRC245 (1971)[e]	OG	4	CRCC
VRC317 (1971)[e]	OG	4	CRCC
VRC821 (1971)[e]	OG	4	CRCC
PS172 (1967)	OG	4	Laboratory collection
PS188 (1970)	OG	4	Laboratory collection
PS190 (1970)	OG	4	Laboratory collection
PS191 (1970)	OG	4	Laboratory collection
PS192 (1974)	OG	4	Laboratory collection
Phil 6973	IN	4	Laboratory collection
Classical biotype			
154	OG	1	CRCC, classical phage propagating strain (11)
H218	IN	1	(12)
PS241	IN	1	Laboratory collection, kappa producer
T2	OG	2	CRCC, type strain (11)
NIH41	OG	1	NIH serotype reference strain, Ogawa. Double lysogen for VcA-1 and VcA-2 (7)
NIH35a3	IN	1	NIH serotype reference strain, Inaba
CA385	RO	UT	Rough serotype strain (isolated by Sakazaki et al.)

[a] Isolation year is shown within parentheses.
[b] OG, Ogawa; IN, Inaba; RO, rough.
[c] See references 3 and 11. UT, Untypable.
[d] CRCC, Cholera Research Centre, Calcutta, India; NIH, National Institutes of Health, Bethesda, Md.
[e] Lyophilized shortly after isolation.

filters. Phages VcA-1 and VcA-2 (7) were purified from the filtered, untreated culture supernatant of the doubly lysogenic host NIH41 by repeated single-plaque isolation of the two morphological types on lawns of Mak757. Mak757 was then lysogenized with these phages and used as a source of pure VcA-1 and VcA-2 phages (7). Assay methods were similar to those used for lytic phages except that soft agar overlay cultures were used for both qualitative and quantitative procedures, using indicator strain Mak757.

Biotyping and serotyping. Resistance of cultures to lytic phage C4 was used as the criterion of El Tor biotype. Ogawa, Inaba, and rough O typing antisera were raised in rabbits against autoclaved cells of international serotype strains (Table 1), absorbed, and used in slide agglutination tests by standard methods.

Purification of phage. Phage was precipitated from 2-liter batches of broth culture with 10% polyethylene glycol (13). The precipitate was suspended in 0.2 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.002 M $MgSO_4$ and centrifuged for 10 min at 10,000 rpm in the cold. Phage was sedimented from the supernatant at 40,000 rpm for 90 min. The phage pellet was suspended in 10 ml of normal saline, filtered through a G5 scintered glass filter, distributed in small vials, and kept at −15°C. A 100-fold concentration was obtained resulting in counts of 10^{11} PFU/ml for lytic phage E3 and 10^8 PFU/ml for temperate phages.

Rabbit antiphage sera. Three intramuscular injections of purified phage in complete Freund adjuvant (Difco Laboratories) followed by four intravenous injections were given in graded doses (0.1 to 1.0 ml) over a period of 6 weeks. Antisera were absorbed of bacterial antibody with packed cells of Mak757, and complement was inactivated at 56°C for 0.5 h. The antiviral titers (50% endpoint) were in the region of 1/1,000 by the neutralization test. The sera were used routinely to neutralize phage at five times this concentration.

Preparation and testing of lysogens. Four El Tor (Mak757, VRC276, VRC242, and HP47) and two classical (H218 and T2) biotype strains were used as recipients. They were all sensitive to phage E3, and no temperate phages were detected in them. From stock phage suspensions, 0.1 ml of a 10^4-PFU/ml suspension was added to 0.1 ml of test culture (CFU, 10^3/ml), incubated for 10 min, and diluted to 2 ml in nutrient broth. The mixture was incubated for 2 h at 30°C and then at 37°C for 18 h and plated. Forty to fifty colonies were picked up and phage typed. The colonies showing change in type were examined for sensitivity to all of the lytic and temperate phages for the presence of infecting phage and for serotype. To confirm the

TABLE 2. Phage type changes induced in six strains of *V. cholerae* by K-phages

Culture	El Tor phage sensitivity[a]					Phage type	Proportion of colonies (%)
	E1	E2	E3	E4	E5		
El Tor biotype							
Mak757 wild type	+	+	+	+	+	1	
Mak757 (241)	+	+	−	+	+	3	20
Mak757 (22)	+	+	−	+	+	3	30
Mak757 (VcA-2)	+	+	−	+	+	3	50
Mak757 (α)	+	+	−	+	+	3	70
Mak757 (VcA-1)	+	+	+	+	+	1	50
HP47 wild type	+	+	+	−	+	2	
HP47 (241)	+	+	−	−	+	4	98
HP47 (22)	+	+	−	−	+	4	90
HP47 (VcA-2)	+	+	−	−	+	4	80
HP47 (α)	+	+	−	−	+	4	80
HP47 (VcA-1)	+	+	+	−	+	2	30
VRC276 wild type	+	+	+	+	+	1	
VRC276 (241)	+	+	−	+	+	3	100
VRC276 (22)	+	+	−	+	+	3	90
VRC276 (VcA-2)	+	+	−	+	+	3	70
VRC276 (α)	+	+	−	+	+	3	80
VRC276 (VcA-1)	+	+	+	+	+	1	50
VRC242 wild type	+	+	+	−	+	2	
VRC242 (241)	+	+	−	−	+	4	82
VRC242 (22)	+	+	−	−	+	4	80
Classical biotype							
T2 wild type	−	−	+	−	−	2	
T2 (241)	−	−	−	−	−	2	60
T2 (22)	−	−	−	−	−	2	58
H218 wild type	−	−	+	−	−	1	
H218 (241)	−	−	−	−	−	1	100
H218 (22)	−	−	−	−	−	1	100

[a] +, Sensitive; −, resistant.

absence of lysogeny in colonies not showing type change, at least five were tested for lack of immunity to infecting temperate phage in each experiment.

Controls were run similarly by treatment with phage inactivated at 70°C for 0.5 h. Results were further confirmed by plating and testing lysogens from the center of isolated turbid plaques on soft agar cultures.

Stability of lysogens. Phage-carrying lines were periodically subcultured and checked for the stability of the lysogeny established. The minimum inhibitory concentration of rifampin in broth culture was determined for wild-type cultures and found to be 0.2 to 0.5 μg/ml. Lysogens were serially passaged in subminimal concentrations (0.1 to 0.2 μg/ml) of rifampin (17), the last broth containing in addition temperate phage antiserum. After plating out, 40 to 50 colonies were tested for the presence of phage.

Superinfection exclusion. Superinfection exclusion of lytic phage E3 by a few of the lysogens was studied. Broth cultures (4 h) (CFU, 10^8/ml) were incubated with E3 phage (PFU, 10^7/ml) for 10 min at 30°C, cooled in ice to stop development, and centrifuged in the cold. The supernatant was collected, treated with chloroform, and then assayed for unabsorbed phage. The cell sediment was suspended in normal saline, and free phage was inactivated with anti-E3 phage serum at 37°C for 5 min and then cooled in ice. The cells were centrifuged, washed with normal saline, and suspended in nutrient broth to the original volume in the cold. A sample (0.5 ml) was plated directly and after further dilution to determine the number of productively infected cells.

RESULTS

Phage sensitivity and lysogeny in El Tor wild-type cultures. All wild-type El Tor strains (Table 1) of phage types 3 and 4 were resistant to phage E3 and were found to carry temperate phage. The strains sensitive to phage E3 belonged to phage types 1 and 2 and did not produce temperate phage. Similarly, the three type 4 El Tor strains used as temperate phage producers were resistant to phage E3 (cultures of variety 2, SLH22, and VRC241).

TABLE 3. Immunity patterns of induced lysogens with the series of test phages

Culture	Phage sensitivity profile[a]						
	Temperate					Lytic	
	α	241	22	VcA-2	VcA-1	Clear plaque mutant of 22	E3
Mak757 wild type	+	+	+	+	+	+	+
Mak757 (α)	−	−	−	−	+	−	−
Mak757 (241)	−	−	−	−	+	−	−
Mak757 (22)	−	−	−	−	+	−	−
Mak757 (VcA-2)	−	−	−	−	+	−	−
Mak757 (VcA-1)	+	+	+	+	−	+	+
HP47 wild type	+	+	+	+	+	+	+
HP47 (α)	−	−	−	−	+	−	−
HP47 (241)	−	−	−	−	+	−	−
HP47 (22)	−	−	−	−	+	−	−
HP47 (VcA-2)	−	−	−	−	+	−	−
HP47 (VcA-1)	+	+	+	+	−	+	+

[a] See Table 2, footnote *a*.

Phage sensitivity and serology of induced lysogens. True lysogeny was deemed to have been established in the phage-treated cultures by demonstrating (i) immunity to and presence of infecting phage after repeated subculture and (ii) inabilty to cure lysogeny by repeated single-colony isolation and also growth in rifampin and antiphage serum-containing broth (to rule out the possibility of pseudolysogeny) (17). Thus, the proportion of lysogenized colonies ranged from 20 to 100% among the six recipients of both biotypes tested, the lowest being in strain Mak757 (Table 2).

Phages α, 241, 22, and VcA-2 all caused 100% conversion to resistance to typing phage E3 in both biotypes (Table 2). These four phages also had similar immunity patterns (Table 3) and are therefore referred to as K-phages for ease of description. Temperate phage VcA-1 behaved differently. It did not induce a change in lytic phage sensitivity (Table 2) and had a unique immunity profile (Table 3).

The K-phages gave clear plaque mutants at a frequency of 10^{-6}. These mutants did not plate on K-lysogens (Table 3) and had the same plaque morphologies and host ranges as E3. No serological changes were detected in the lysogens.

Phage exclusion by lysogens. Adsorption of phage E3 in controls was efficient, being nearly 90% within 10 min. All of the lysogens tested (Table 4) were found to absorb phage E3, but unproductively.

DISCUSSION

Change in phage type by temperate phage is well known in several species (2, 18). The relative absence of temperate phage in only type 1 strains of the El Tor vibrio was noticed early on (3). Despite sustained interest in cholera phages, type conversion by temperate phages has not so far been reported except for an incomplete change in serotype from Ogawa to Hikojima (16). We could not demonstrate serological conversion by any of the phages investigated here. There was, however, a clear correlation between lysogeny and phage type.

We chose to compare temperate phages described by earlier workers with a local isolate. These phages have been taken from cultures of diverse origin including the epidemiologically earlier classical biotype over a long period of time. They are therefore considered to be representative of the stable temperate phage population of *V. cholerae*. Four of the five phages showed homology in their immunity profiles and type-converting activities. Typical of them was K-phage 22 (20). Changes in phage type caused

TABLE 4. Superinfection exclusion of E3 phage by K-lysogens

Culture	E3 phage count (PFU/ml) × 10^{-6}	
	Supernatant	Productively infected cell
HP47 wild type	2.6	27.0
HP47 (α)	3.1	0
HP47 (22)	2.3	0
HP47 (241)	3.2	0
HP47 (VcA-2)	1.7	0
Mak757 wild type	2.8	28.0
Mak757 (α)	3.0	0
Mak757 (22)	5.8	0
Mak757 (241)	1.3	0
Mak757 (VcA-2)	2.7	0

by K-phages involved only induction of resistance to lytic phage E3. Over the past few years, the great majority of typable isolates have belonged to El Tor types 2 and 4 (8, 9), which differ from each other only in sensitivity to this phage. One would therefore expect to find this difference to reflect lysogeny by K-phages. In our series of isolates (Table 1) taken from several epidemics between 1967 and 1974, this was found to be the case.

We were able to show that E3 resistance was due to postadsorption exclusion in K-lysogens. These lysogens were able to adsorb phage E3, but unproductively (Table 4). Clear plaque mutants of K-phage occurred at high frequency and resembled phage E3 in morphology and immunity profile. It is likely, therefore, that this phage is a coimmune type of a lytic mutant of K-phage and is repressed by K-repressor in these lysogens.

VcA-1 phage was found to be unrelated to the K-phages in its immunity profile. It also differed from them in lacking phage type-converting activity. This phage was reportedly ubiquitous in classical *V. cholerae* (7). There are no reports of its occurrence in the El Tor biotype. We have demonstrated that the El Tor vibrio can act as a host to this phage, and it is therefore still likely to occur. A derivative of this phage may prove to be a useful addition to the typing series.

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