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Production of Bacteriophage-Associated Materials by *Vibrio cholerae*: Possible Correlation with Pathogenicity

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Classical and El Tor strains of *Vibrio cholerae* were examined for production of bacteriophage or bacteriophage-related material by electron microscopy of mitomycin C-induced cultures. The strains were also tested for pathogenicity by using the ligated ileal loops of adult rabbits. Of the 27 strains tested, 25 showed a correlation between the production of bacteriophage-related material and pathogenicity. Both P^+ and P^- strains produced bacteriophage tails. The bacteriophage tails of strains 569B and ATCC 9168 and other strains were shown to occur in an extended form with tail appendages.

Bacteriocins are defined by Nomura as "bactericidal substances, apparently protein in nature, which are synthesized by certain strains of bacteria and are active against some other strains of the same or closely related species" (15). Many bacteriocins, though not all, are particulate in nature and resemble defective bacteriophages or bacteriophage parts (3). Farkas-Himsley and Seyfried (5) reported a bacteriocin from *Vibrio cholerae*, and later Jayawardene and Farkas-Himsley (10) showed that this bacteriocin resembles bacteriophage tails with contracted sheaths.

Since Freeman's work with *Corynebacterium diphtheriae* (6, 7), it has been recognized that temperate bacteriophages may affect the pathogenicity of the host bacteria. The correlation between lysogeny and toxigenicity is well established for *C. diphtheriae* (8, 16). In 1963, Takeya and Shimodori (17) reported a correlation between lysogeny and pathogenicity in El Tor strains of *V. cholerae*. They examined 89 El Tor strains for evidence of lysogeny by using the cross-lysis technique. Seventy-five strains isolated from cholera El Tor cases could be classified as "Celebes" type strains because they were lysogenic for a particular bacteriophage. The remaining 14 strains were nonpathogenic or produced mild diarrhea and did not carry this bacteriophage. These strains were designated "Classic-Ubon" strains. In 1965, Takeya et al. (19) were able to develop a sensitive method of diagnosing carriers of "Celebes" type El Tor vibrios by detection of the bacteriophages in stool specimens. Several workers have reported that classical strains of *V. cholerae* are not usually lysogenic (13, 14). Since these strains are pathogenic, Takeya's observations did not seem applicable to classical strains of *V. cholerae*.

In this paper, we present the results of experiments which suggest that a correlation exists between pathogenicity and bacteriophage influence in both classical and El Tor strains of *V. cholerae*. By bacteriophage influence we mean that the bacterial strain is capable of producing material of bacteriophage nature. This material may be bacteriophages, defective bacteriophages, or bacteriophage parts. We class the vibriocin particles of Farkas-Himsley as bacteriophage parts.

MATERIALS AND METHODS

Bacterial strains. The strains used and the donors are listed in Table 1. The strains were maintained frozen at −60 C in nutrient broth with 15% glycerol.

Media. Nutrient broth and nutrient agar for strain maintenance were prepared by making up Nutrient Broth (Difco) as directed, adjusting the *p*H to 7.5, and adding 1.5% agar if desired. Peptone water for pathogenicity studies consisted of 2% peptone (Difco) and 0.5% NaCl, adjusted to *p*H 8.0. The induction medium was HB, consisting of 1% Casamino Acids (Difco), 1% beef extract (Difco), and 0.5% NaCl, adjusted to *p*H 7.5. Another medium used for induction was TRB, consisting of 1% Casamino Acids (Difco), 0.25% beef extract (Difco), 0.005 M tris(hydroxymethyl)aminomethane (Tris) maleate, 0.25% NaCl,

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TABLE 1. *Strains used*

Strain	Type of vibrio	Serotype[a]	Donor[b]
569B	Classical	I	SB, JF
ATCC 9168	Classical		HF-H
VC 154	Classical	O	SB
VC 12	Classical	O	SB
NIH 41	Classical	O	SB
1450B	Classical	I	SR
B1307	Classical	O	SR
VC 13	Classical	I	SR
NIH 35A3	Classical	I	SB
V-58 sm^rp^+	Classical	O	KB
V-58 sm^rp^-	Classical	O	KB
V-63 sm^rp^-	Classical	I	KB
ATCC 14035	Classical	O	SB
GS/1/65	Classical	I	SB
Phil 6973	El Tor	I	SB
Phil Q 15	El Tor	O	SB
Phil 1418	El Tor	O	SB
V-86	El Tor	I	SB
cq 1651	El Tor	I	SB
HK-1	El Tor	O	SB
GS/9/65	El Tor		SB
Ubon 13	El Tor		SB
EW 6	El Tor		SB, JF
ME 7	El Tor	O	JF
65-42915	El Tor	I	SB
65-43090	El Tor	I	SB
10-30-CB	El Tor	H	SB

[a] I indicates Inaba, O indicates Ogawa, and H indicates Hikojima.

[b] SB indicates S. Basu, JF indicates J. Feeley, HF-H indicates H. Farkas-Himsley, SR indicates S. Richardson, and KB indicates K. Bhaskaran.

0.25% KCl, 0.02% Na_2HPO_4, 0.05% $MgSO_4$, 0.005% $FeCl_3$, and 0.005% $MnCl_2 \cdot 4H_2O$, adjusted to *p*H 8.0 with NaOH and readjusted to *p*H 7.5 with 1 M maleic acid. BNB, a rich nutrient broth described by Bhaskaran (1), was used to grow his auxotrophic mutants (V58 and V63) for pathogenicity tests and consisted of 0.5% peptone (Difco), 0.1% beef extract (Difco), 0.1% yeast extract (Difco), 0.5% NaCl, with the *p*H adjusted to 8.0.

Mitomycin C induction. Our induction technique was based on that of Jayawardene and Farkas-Himsley (11). A 20-ml amount HB or TRB in a 125-ml Erlenmeyer flask was inoculated from an overnight slant culture. The vibrios were grown at 37 C with shaking to a density of about 10^8 to 3×10^8 cells/ml. The flasks were rapidly chilled in an ice bath and held in the cold for 1 to 3 hr. We ordinarily tested eight strains at a time, by using the chilling period to synchronize the cultures. After the cells had reached the appropriate density and all flasks had been chilled for at least 1 hr, the flasks were replaced in a water bath at 37 C. After shaking for 5 min, 0.3 μg of mitomycin C per ml was added. After 15 min, the cells were removed by centrifugation in the cold and resuspended in 20 ml of fresh, prewarmed medium. After 2 to 3 hr of incubation, the cells were removed by centrifugation at $3,600 \times g$ for 15 min and at $6,000 \times g$ for 20 min. The supernatant fluid was centrifuged at $33,000 \times g$ for 1 hr. The sediment was taken up in 1 ml of growth medium and preserved with a drop of chloroform. In some cases, including all negative strains, the incubation period after induction was increased to 7 hr.

Electron microscopy. All sediments were examined by negative staining by using a Hitachi HU11A electron microscope. The staining procedure was to allow a drop of the suspended final sediment to remain on a grid for 1 to 2 min. This was washed three times with water and twice with 1% uranyl acetate. The grids were 400 mesh copper, with carbon-stabilized parlodion films. The grids were glow discharge-treated just before use. In some instances, the sediments were taken up in 0.1 M ammonium acetate. These preparations were mixed with stain and dried down onto the grid without washing.

The original microscope magnification was 37,500. Several areas of each grid were screened for bacterio-

TABLE 2. *Correlation of induction and pathogenicity*

Strains	Induction[a]	Pathogenicity[b]
Classical		
569B	Tails	Positive
ATCC 9168	Tails	Positive
VC 154	Tails	Positive
VC 12	Tails	Positive
NIH 41	Bacteriophages	Positive
1450B	Tails	Positive
B1307	Tails	Positive
VC 13	Tails	Positive
NIH 35A3	Tails	Positive
V-58 sm^rp^+	Tails	Positive
V-58 sm^rp^-	Tails	Positive
V-63 sm^rp^-	Tails	Positive
ATCC 14035	Tails	Positive
GS/1/65	Negative	Positive
El Tor strains		
Phil 6973	Bacteriophages	Positive
Phil Q 15	Bacteriophages	Positive
Phil 1418	Bacteriophages	Positive
V-86	Bacteriophages	Positive
cq 1651	Bacteriophages	Positive
HK-1	Bacteriophages	Positive
GS/9/65	Bacteriophages	Positive
Ubon 13	Bacteriophages	Positive
EW 6	Negative	Negative
ME 7	Negative	Negative
65-42915	Negative	Negative
65-43090	Negative	Negative
10-30-CB	Negative	Positive

[a] Refers to electron microscopic examination of induced cultures.

[b] Refers to ability to cause fluid accumulation in ligated rabbit ileum.

phage or tails. Bacteriophages were easily identifiable, but practice was necessary to identify tails. Criteria for identification of tails included the following: squared off ends of contracted sheaths, regular substructure of sheaths, projecting cores, stain filled hollows of empty sheaths, and tail appendages of extended sheaths. A minimum of four separate grids from two different induction experiments were examined from strains counted as negative. Grids from two inductions were examined for all strains. Photographs were taken if necessary to confirm typical particles.

Pathogenicity. Pathogenicity was tested for by the rabbit ileal loop method of De and Chatterje (4). A 1-ml amount of a 6-hr broth culture in peptone water was injected into a ligated loop of an adult rabbit. Loops of the ileum were approximately 10 cm in length and were separated by 6-cm uninoculated loops. The rabbits were sacrificed after 16 hr and the loops were examined. All loops were cultured for vibrios. Strains which gave negative reactions were retested after animal passage. Each strain was tested a minimum of two times in different animals. For the auxotrophic mutants, 1 ml of a 12-hr broth culture in BNB was used as the inoculum. Streptomycin-resistant strains had 100 μg of streptomycin per ml added to the growth medium.

RESULTS

The results of induction and pathogenicity experiments are summarized in Table 2. We tested a total of 27 strains of cholera vibrios. All 14 classical strains caused fluid accumulation in the ligated rabbit ileum. When induced with mitomycin C, 12 of these strains produced material which could be identified as bacteriophage tails, one strain produced typical bacteriophages, and one strain was negative for visible bacteriophage material. Bhaskaran (2) found that *V. cholerae* strains which possess a fertility factor (designated the P factor) function as gene donors and produce a bacteriocin, whereas P^- strains (without the P factor) serve as recipients and are sensitive to the bacteriocin. In our study, both P^+ and P^- strains were found to produce typical bacteriophage tails. Thus, the P character does not appear to be related to the presence or absence of bacteriophage tails. Likewise, the auxotrophic mutations in these strains (requirements for arginine, histidine, and isoleucine-valine in V58 and for purine and leucine in V63) had no detectable effect on their pathogenicity nor on any other of their characteristics which we tested.

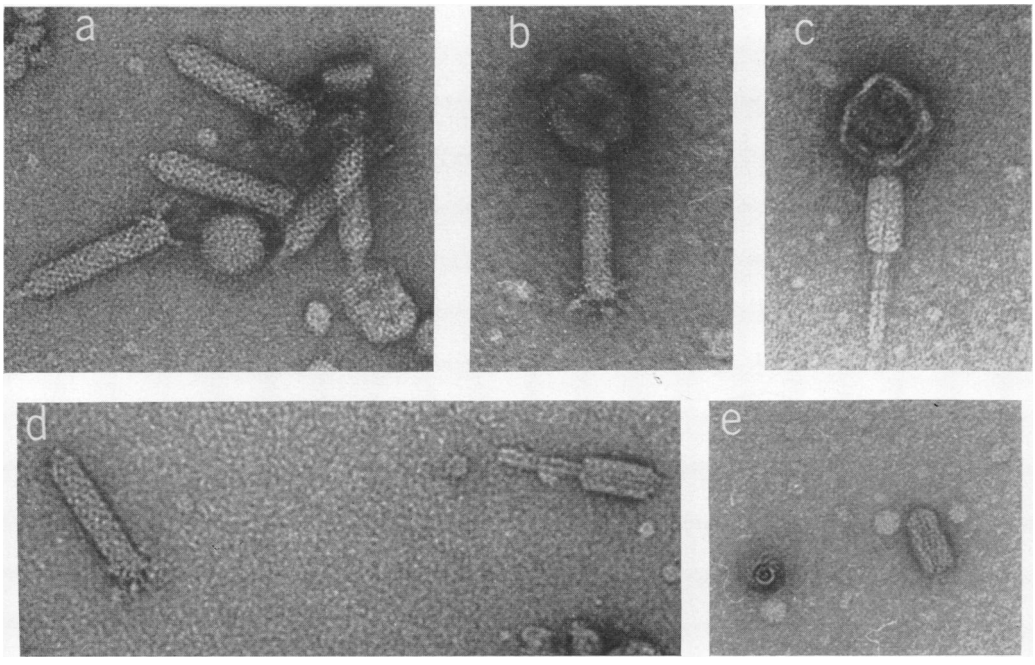

FIG. 1. *Electron micrographs of mitomycin C-induced Vibrio cholerae strains stained with uranyl acetate.* $\times$ *200,000.* (*a*) *Extended tails of V. cholerae strain 569B. Note the tail appendages and the neck.* (*b*) *Bacteriophage particle from V. cholerae strain NIH41.* (*c*) *Rare particle from V. cholerae strain ATCC 9168, which appears to have an empty head attached to the contracted tail.* (*d*) *Contracted and extended tails of V. cholerae 569B in the same field.* (*e*) *Contracted sheaths of V. cholerae strain 569B. The core has broken off the sheath on the right. The sheath on the left is end on, showing the helical arrangement of the subunits.*

Of the 13 El Tor strains tested, 9 gave a positive reaction in the ileal loop. Eight of these strains produced typical bacteriophages, whereas one was negative for bacteriophage material. The four strains which were consistently negative in the rabbit gut were all negative for bacteriophages or tails.

We also found that when the bacteria were induced in TRB we could demonstrate tails with extended sheaths (Fig. 1). Higerd et al. (9) recently reported a similar phenomenon with pyocin. Our measurements of the particles are in agreement with those of Jayawardene and Farkas-Himsley (11). We found the extended sheaths of strain 569B to be 104 by 21 nm. There is a distinctive neck at one end of the tail and fibers or appendages at the other end. The contracted sheaths are about 24 nm in width and 45 nm in length. The protruding core is about 9 nm in width and 104 nm length. The bacteriophage tails of strain NIH 41 are about this size as well as the vibriocin particles from strain ATCC 9168.

DISCUSSION

We were surprised to find that bacteriophage material was so consistently associated with *V. cholerae*, particularly the classical strains. Lang et al. (12) found bacteriophage tails in one of six noninduced strains. Takeya and Shimodori (18) failed to detect bacteriophage-associated material in some bacteriocinogenic strains. However, we found bacteriophage tails from strain VC154, which they were unable to induce in mixed culture. Several reports (5, 11) show the importance of cell density and medium composition for successful induction. Temperature may also be important. Although there may be noninducible bacteriocinogenic strains, it is clear that specific conditions are required for successful induction.

The consistent finding of these particles in classical cholera strains raises questions about the nature of the relationship between lysogeny and pathogenicity in vibrios. Our results with El Tor strains confirm earlier observations that infection with temperate bacteriophage is correlated with capacity to produce severe disease (17). Our results with classical strains suggest that a similar correlation may occur with defective lysogeny and pathogenicity. The nature of the relationship which leads to this correlation is not clear. At present, there is no evidence that any causal relationship exists between lysogeny and toxigenicity in either classical or El Tor strains. We intend to investigate the nature of the relationship between bacteriophage influence and the factors which are responsible for pathogenicity.

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