



Bacteriocins of *Vibrio cholerae* in relation to defective bacteriophage
by John Vander Schalie

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology

Montana State University

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Abstract:

Defective bacteriophage of *Vibrio cholerae* were studied, A mutant phage which retained its viability was found by electron microscopy to have an aberrant structure, Phage-associated particles released from a vibrio host upon mitomycin C induction were isolated and partially characterized. These particles had activity similar to bacteriocin of *V. cholerae* called "vibriocin," and exhibited serological homologies with several cholera phages. Upon mitomycin C induction, phage were isolated from two strains of non-agglutinable *V. cholerae*.

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December 4, 1970

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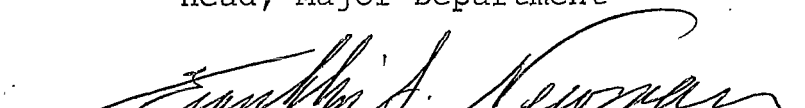
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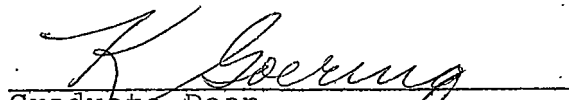
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ABSTRACT

Defective bacteriophage of Vibrio cholerae were studied. A mutant phage which retained its viability was found by electron microscopy to have an aberrant structure. Phage-associated particles released from a vibrio host upon mitomycin C induction were isolated and partially characterized. These particles had activity similar to bacteriocin of V. cholerae called "vibriocin," and exhibited serological homologies with several choleraphages. Upon mitomycin C induction, phage were isolated from two strains of non-agglutinable V. cholerae.

INTRODUCTION

Bacteriocins were discovered in 1925. Since then, many genera of bacteria have been found capable of producing bactericidal or bacteriostatic substances. Certain agents, originally called bacteriocin, have subsequently been shown not to satisfy the formal definition of "bacteriocin." Even now, there remains a heterogeneity in the types of substances included in this category. It has been suggested that one of the two general bacteriocin types is a form of lysogeny for a bacteriophage that is defective.

This investigation involved a study of defective phage of Vibrio cholerae and a substance released from vibrio hosts which satisfies the formal definition of bacteriocin. The purpose of this investigation was to search for existing correlations between the defective cholera phages and a bacteriocin of V. cholerae called "vibriocin." These studies also define the first reported isolation of bacteriophage from the non-agglutinable vibrios, and reveal the aberrant structure of a viable mutant choleraphage.

REVIEW OF THE LITERATURE

Historical Observations

The discovery of bacteriocins and the development of knowledge on the relationships of these substances to the bacteria which they affect has followed quite closely the study of bacterial viruses. The initial association between these two fields was due to the similar methods used in detecting phage and bacteriocins and to similarities in the gross effect on the bacterial cell. As the bacteriocins have become better defined, the rationale for studying them in association with bacteriophages seems well justified.

Gratia (1925) was the first to isolate and define the class of substances known as bacteriocins. He found that a substance produced by one strain of Escherichia coli would inhibit another strain. The inhibition which he observed was similar to that of a bacteriophage with one essential difference; it did not reproduce in the sensitive strain. The term "colicin" was coined by Gratia to designate this class of inhibitory substances. Jacob et al. (1953) defined the protein nature of colicins, showed that biosynthesis by a colicinogenic strain was lethal for that strain, revealed the involvement of receptors on the bacterial cell, and introduced the general term "bacteriocin" to the antibacterial substances produced by various species of bacteria.

Many genera of bacteria have now been shown to possess the genetic capacity to produce bacteriocins. It seems clear that bacteriocinogeny will become as general a phenomenon as lysogeny.

Bacteriocin Characteristics

Bacteriocins are a natural class of antibiotics or bactericidal substances which are as efficient as they are unique in their mode of action. The narrow specificity of their action and their protein nature distinguish them from most of the other "classical" antibiotics. They affect sensitive bacteria by adsorption to a specific receptor site (Maeda and Nomura, 1966), which may also serve as a receptor for phage (Fredericq, 1948, Fredericq and Gratia, 1949), and act while remaining at the cell surface (Nomura, 1963). Adsorption of a single molecule is sufficient to kill a sensitive bacterium (Jacob et al., 1952). Bacteriocins are active on other bacterial strains of the same or closely related species. The producing cell, called bacteriocinogenic, is resistant or immune to the action of the bacteriocin it produces, or homologous "external" colicins (Nomura, 1967). Bacteriocins, unlike phage, contain no nucleic acid (Kageyama and Egami, 1962) and do not multiply in the cells they kill. Extensive studies

done on colicins have revealed unique features showing the close relationship of bacteriocins to bacteriophages on one hand (Endo et al., 1965, and Sandoval et al., 1965), and to F factors on the other (Bhaskaran, 1964, and Hayes, 1968), and testify that the classification of bacteriocins as a unique class of antibacterial substances is appropriate.

Bacteriocin Types

There are two distinct basic types of bacteriocins. The properties of the two types are summarized in Table 1 (from Bradley, 1967). Low molecular weight bacteriocins can be released with the absence of cellular lysis, whereas following the induction of high molecular weight bacteriocins, which resemble bacteriophage tails, release follows lysis of the host cell. The common feature of the two types is their protein nature and use of specific receptor sites on the host cell (Reeves, 1965). The two bacteriocin types are not necessarily associated with different bacterial species. Both types may be found in the same species. For example, colicin V of E. coli forms a large clear area on agar and is dialyzable and sensitive to trypsin. These properties make it a member of the first bacteriocin type. Colicin 15, also produced by an E. coli strain, has a molecular weight exceeding 2×10^5 , is sedimentable, and

TABLE 1. Properties of the two groups of bacteriocins*

Low molecular wt. bacteriocins	High molecular wt. bacteriocins
Not sedimentable	Sedimentable
Trypsin-sensitive	Trypsin-resistant
Thermostable	Thermolabile
Cannot be resolved in electron micro- scope	Visible in electron microscope as phage- like objects or components

* From Bradley, D. E., 1967. Ultrastructure of bacteriophages and bacteriocins. Bact. Rev. 31:230-314.

can be observed in the electron microscope as phage-like components (Bradley, 1967). Campbell (1969) states that the high molecular weight structures are "obviously a form of lysogeny for a phage that is defective" and that their inclusion with other types of bacteriogenesis is a "semantic accident."

There are instances where bactericidal substances released from a cell were termed bacteriocins, and later were shown to be erroneously classified as such. Megacin A, produced by B. megaterium, is an inducible bacteriolytic substance which has since been shown to be a simple hydrolytic enzyme, with phospholipase A activity (Nomura, 1967). Nomura states that it is likely that this enzyme may be the product of a highly defective lysogenic strain which, after induction, produces no particle with visible phage structure, but produces several proteins, one of which is phospholipase A which can be detected by its killing action.

Every group of the Enterobacteriaceae has been shown to produce bacteriocins except Proteus-Providencia (Hamon and Peron, 1963). Bacteriocins have been discovered in other bacterial families including a few Gram positive organisms such as Staphylococcus, Streptococcus, and Bacillus. A partial listing of bacteriocins and the producing genera is contained in Table 2 (from Reeves, 1965).

TABLE 2. Various families of bacteriocins*

Bacteriocin name	Producing genus
Colicins	<u>Escherichia</u> <u>Paracolobactrum</u> <u>Shigella</u> <u>Salmonella</u> <u>Aerobacter</u>
Alveicins	<u>Hafnia</u>
Caratovoricens	<u>Erwinia</u>
Arizonacins	<u>Paracolobactrum</u>
Cloacins	<u>Enterobacter</u>
Marcescins	<u>Serratia</u>
Pneumocins	<u>Klebsiella</u>
Aerocins	<u>Aerobacter</u>
Pyocins	<u>Pseudomonas</u>
Fluocins	<u>Pseudomonas</u>
Pesticins	<u>Pasteurella</u>
Megacins	<u>Bacillus</u>
Monocins	<u>Listeria</u>
Cerecins	<u>Bacillus</u>
Enterococcins	<u>Streptococcus</u>
Staphylococcins	<u>Staphylococcus</u>
Vibriocins	<u>Vibrio</u>

* Compiled from Reeves, P. 1965. The bacteriocins. Bact.
Rev. 29:24-45.

Some explanation of the nomenclature of bacteriocins should be included here. It is obvious, and stated by Bradley (1967), that the classification and hence the nomenclature of bacteriocins has room for improvement. The existing names are given to the various bacteriocin families based on the specific rather than the generic name of the host organism. This is due perhaps to the high degree of specificity of bacteriocins. Thus, colicins are bacteriocins of E. coli, monocins of Listeria monocytogenes, and so on. Fredericq (1948a) based a classification of colicins on the spectrum of resistance of various mutants of E. coli. From this code, nomenclature is used based on the number of the producer strain, followed by the original letter designation given by Fredericq (1948b). For example, colicin CA23-D is a type D colicin produced by E. coli strain CA23.

Mode of Action

Bacteriocins, as with bacteriophages, adsorb to specific host receptors located within the cell wall. The first stage of killing is the specific, irreversible (in most instances), adsorption of the bacteriocin. With most low molecular weight bacteriocins, trypsin "rescue" is possible for a short time following adsorption by the addition of trypsin to the medium (Bradley, 1967). Nomura and Nakamura

(1962) found that the complete inhibition of macromolecular synthesis by a small molecule type colicin was reversible by trypsin treatment. This suggests that some bacteriocins are bacteriostatic rather than bactericidal. It also suggests that bacteriocins exert their action while attached to the outside of the cell. This has been shown in several instances (Nomura, 1963). Trypsin added to the growth medium in which the host cells are suspended will also effectively prevent the adsorption of low molecular weight bacteriocins (Ozeki, 1968).

The biochemical effects produced by colicins of E. coli have been studied in the most detail. Table 3 summarizes these effects as listed by Nomura (1967).

A theoretical explanation for how a bacteriocin present on the outside of the cell can arrest the synthesis of macromolecular components was given by Nomura (1963). He postulated a functional alteration of some specific element of the cytoplasmic membrane caused by the attachment of the bacteriocin. The bacteriostatic or bactericidal effect is then exerted through hypothesized "amplification" mechanisms residing in the cell envelope. Luria (1970) suggests this amplification mechanism may be mediated by conformational changes of the cell membrane.

TABLE 3. Summary of the apparent biochemical effects of several colicins
on sensitive cells*

Effect	Colicin E1	Colicin E3	Colicin K	Colicin I
Inhibition of:				
Respiration	-		-	-
ATP production	+	-	+	+
Active transport of potassium	+	-		
DNA synthesis	+	-	+	+
RNA synthesis	+	-	+	+
Protein synthesis	+	+	+	+
DNA degradation	-	-	+	-
Induction of phage λ	-	-	-	

10

* Condensed from Nomura, M. 1967. Ann. Rev. Microbiol. 21:257-284.

Genetic Determinants for Bacteriocinogeny

The possession of a genetic determinant termed a "colicinogenic factor" (col factor or C factor) is in general a stable genetic character giving a bacterial strain the ability to produce a particular bacteriocin. Where studied, the chemical nature of col factors has been DNA, and their size is comparable to the DNA of temperate phages or F factors (Silver and Ozeki, 1962).

Jacob and Wollman (1958) originally described col factors as one of three types of episomal elements, including temperate phage and fertility factors or F factors. Subsequent work has shown few examples of col factors attaching to the genome. In one instance, the factor also had fertility promoter activity as well (Fredericq, 1963). This was manifested by (1) synthesis of structures with receptors for male-specific phages (presumably pili), (2) promotion of chromosomal transfer, and (3) ability to interfere with the establishment of the F agent. During conjugation studies on V. cholerae, Bhaskaran (1964) also associated bacteriocin production with the fertility factor. Unlike temperate phage, col factors can replicate in phase with the bacterial genome while in the autonomous state without killing the cell. They rarely become integrated into the

host genome and therefore are probably best designated as plasmids (Clowes, 1963). In this way the col factors appear more similar to the F factors than to temperate phage.

Significance of Bacteriocins

Bacteriocinogeny is probably of considerable selective advantage for bacteria. The fact that the synthesis of bacteriocin may be lethal does not detract much from the advantage, because only a very small percentage of cells produce bacteriocin and die. By killing nearby competing bacteria, this small advantage to other members of the clone offsets the disadvantage to give the bacteriocinogenic property an overall selective advantage. Col factors also confer immunity to the same bacteriocin produced by other clones.

Bacteriocins have been advocated and utilized for bacterial typing and for use in epidemiological studies. Typing schemes have been successfully applied to Shigella sonnei (Abbott and Shannon, 1958), Shigella flexneri (Chakrabarty, 1964), Pseudomonas pyocyanea (Darrell and Wahba, 1964), and Pseudomonas aeruginosa (Holloway, 1960).

Bacteriocins may be a useful tool to explore the functions of the cytoplasmic membrane of bacteria. This

structure contains enzymes and other constituents involved in permeation, active transport, and biosynthesis of cell wall components. It is the site of terminal respiration, and may also play an important part in DNA replication and in the segregation of DNA copies at cell division (Ryter et al., 1968). A possibility suggested by Luria (1970) is that the cytoplasmic membrane may play a role in the positioning and functioning of its active components by transmitting conformational signals. He postulates that elucidation of the molecular mechanisms involved in the response of the bacterial cell to bacteriocins may suggest correlations in the functional organization of cellular membranes of higher organisms and that the study of bacteriocin action may contribute to the understanding of membrane functions in general.

Relation of Certain Bacteriocins to Defective Phage

High molecular weight bacteriocins have a structure resembling phage tails, and some have been observed in electron micrographs to have typical phage tail appendages (Parker, Richardson, and Romig, 1970). Their mode of action can be duplicated by T-even phages which are unable to inject their nucleic acid (French and Siminovitch, 1955). It has also been shown in many instances that these bacteriocins share common receptor sites with bacteriophage.

Possession of a col factor confers immunity to a specific bacteriocin and other closely related bacteriocins as well as determining the ability to produce a bacteriocin. Bacteriocinogenic strains adsorb the agent, but are insensitive to its action (Reeves, 1965, and Jayawardene and Farkas-Himsley, 1969a). This immunity resembles the immunity conferred by a prophage against a closely related, superinfecting phage.

Phage-tail type bacteriocins are known to be induced with identical agents shown to be effective inducers of temperate phage. This is in contrast to the low molecular weight bacteriocins which have their genetic determinant in the form of a plasmid, rarely in close association with the bacterial genome.

Bacteriocins of *Vibrio cholerae*

Genetic studies have suggested the occurrence of bacteriocinogeny among certain vibrio species (Bhaskaran, 1960). While testing for the presence of the fertility (P) factor, Bhaskaran noted an inhibition of indicator strains observed as a hazy clearing around the periphery of a loopful inoculation of a test strain on indicator cells. This activity, said to possibly be due to the production of bacteriocin, was found to be invariably associated with

donor ability (P+) in V. cholerae, but not in V. cholerae biotype el tor. The possibility that the P factor determines the production of a bactericidal agent was therefore suggested.

The difficulties in demonstrating the bacteriocins of V. cholerae have prompted controversy as to the ability of vibrios to produce vibriocin. Some predicted detectable bacteriocins of V. cholerae may not exist at all (Barua, 1963, and Nicolle et al., 1962), and others stated that the production may be a special phenomenon of transitory inhibition and detectable only by unusual methods (Wahba, 1965). These views resulted mainly from the lack of evidence of bacteriocin effects while typing large numbers of vibrio strains by bacteriophage sensitivity. Wahba (1965) suggested the lack of observing the phenomenon may be due to the inhibition of vibriocin activity by strong proteolytic enzymes produced by the V. cholerae hosts.

Early successful investigations in the production of bacteriocin by V. cholerae were done by Farkas-Himsley and Seyfried (1962a & b). Under conditions closely related to those for induction of bacteriophage in lysogenic bacteria, a test strain underwent lysis, liberating a substance lethal to part of the homologous population. The name

"vibriocin" was proposed for this substance by Farkas-Himsley and Seyfried (1962a). The specific environmental conditions necessary for its production included reduced redox potential, brought about by thioglycollate, ultra-violet irradiation, or chilling. The production of this inhibitor was found to occur only in a complex medium, and was shown to be a lethal biosynthesis, not merely a secretion as with many low molecular weight colicins. Streptomycin resistant (SMr) mutants, derived from producer strains were found to be "predominantly sensitive" to the inhibitor and curved as indicator cells. The method used to detect vibriocin was to spot the lysates of streptomycin sensitive (SMs) cells on a lawn of SMr cells. Chemical and physical properties of the vibriocin originally isolated included inactivation by trypsin, insensitivity to a wide range of pH (1.3 to 12.5), insensitivity to 56 C for 3 hr and boiling for 1 min, but sensitivity to boiling for 10 min or more. The size, determined by filtration, was estimated to be approximately 100 m μ .

The relationship of SMs producer cells and SMr indicator cells prompted Farkas-Himsley and Seyfried (1962b), in a second publication, to implicate bacteriocins as a factor contributing to the "Welsh Phenomenon" where the

growth of a mixed population of SMs and SMr bacteria in broth results in a steady decrease of the SMr mutants to the level normally present in a sensitive culture.

Subsequent publications by Jayawardene and Farkas-Himsley (1968, 1969a,b) involved purification methods and electron microscopy, a mitomycin C induction method, and studies on the interaction of the bacteriocin with sensitive bacteria. All studies were done using bacteriocin produced by one strain. Electron micrographs revealed rod-shaped particles, 1,100 A in length, consisting of a core, covered with a sheath 240 A in width. The sheath was seen both in the extended and contracted state (as describing tail sheath structures of bacteriophage). The purified bacteriocin elicited the formation of a vibriocin-neutralizing antibody and was devoid of antigenic material from the vibriocinogenic host strain. A common receptor site was shared with cholera phage IV, to which the bacteriocin was not related immunologically or morphologically (phage IV has a long curved tail structure). Vibriocin was adsorbed both by the indicator and producer strains, with the former being sensitive and the latter immune to the lethality of the bacteriocin.

Studies on the interaction of vibriocin with sensitive bacteria revealed that it inhibited motility and reduced

the colony forming ability of sensitive V. cholerae cells without causing their lysis. Anaerobically growing cells manifested a tolerance to vibriocin, implicating the necessity for a cell with an active oxidative phosphorylation system. Adsorption of a single unit of vibriocin was sufficient to kill a bacterium, however 35 units could be adsorbed from an environment of excess vibriocin.

Wahba (1965) developed a technique for detecting vibriocin using stab inoculated cultures with colonies killed by exposure to chloroform and overlaid with a sensitive indicator strain. A period of cold shock for 4 hr, developed by Farkas-Himsley and Seyfried (1962a) was used prior to incubation. This method, involving cell to cell contact, detected inhibition produced by 13 of 16 randomly selected vibrio strains against four indicator strains. Wahba's purpose was to define similarities between V. cholerae and V. cholerae el tor, a biotype formerly considered of little clinical significance, which more recently has shown the ability to cause a disease identical to that produced by "classical" V. cholerae. The results indicated a close relationship between the two biotypes.

In an attempt to distinguish V. cholerae from V. cholerae el tor and to establish a typing scheme, Nasu and

Aoki (1966) used the detection method of Wahba (1965) with a complete "checker-board" experiment testing 51 classical and 77 el tor cholera strains for their production and sensitivity to vibriocins. Their results showed about half of the V. cholerae strains tested were sensitive to vibriocin produced by el tor vibrios. Those sensitive to vibriocin produced by either classical or el tor vibrios were predominately V. cholerae el tor. However, in a second experiment, using 101 strains as producers and 15 selected strains as indicators, the results were not reproducible.

Takeya and Shimodori (1969) introduced a new method for the detection of "a lethal factor in vibrios." Involved was the plating of 50-100 vibriocinogenic vibrios with indicator cells in an overlay medium. They failed to demonstrate vibriocin by other methods, but with this technique inhibition was noted as a circular growth-inhibition zone with a microcolony of vibriocin producing vibrios at the center. P+ and P- strains also proved to produce lacunae by this method. All attempts by various methods including mitomycin C induction, to separate vibriocins from vibriocinogenic cells were unsuccessful. Treatment with proteolytic enzymes such as trypsin had no effect on either the viability of vibrios or vibriocin

activity. By electron microscopy, particles resembling phage or phage tails could not be detected, with or without mitomycin C induction of vibriocinogenic (by this method) cells. It was hypothesized that the vibriocin was inseparable from the bacteria. Vibriocin resistant colonies were isolated and it was found that they had acquired not only vibriocinogeny but also resistance to phage IV. Phage IV is known to be lytic for all Asiatic or classical cholera vibrios but not for V. cholerae el tor.

Datta and Prescott (1969a) found three vibriocin producers (one of which was very weak) in 45 V. cholerae and el tor vibrios by an agar well technique described in the subsequent materials and methods section. Their purpose was to examine the possibility of using the vibriocins as a taxonomic tool in differentiating V. cholerae from V. cholerae el tor. Primary purification studies pointed to the presence of a heat labile and high molecular weight bacteriocin by Bradley's (1967) classification. In a general screening, the vibriocins showed equal inhibition of six V. cholerae and nine el tor strains; therefore patterns useful for taxonomic information were not apparent.

In a second study, Datta and Prescott (1969b) examined the effect of two vibriocins on members of the Enterobacteriaceae. A wide range of antibacterial activity was

found throughout the Enterobacteriaceae. Although inhibition was minimal, most of the indicator strains, regardless of their genus, were affected by one of the vibriocin producers. Only five of 23 strains examined showed no zones of inhibition on such indicators as E. coli, Enterobacter aerogenes, Klebsiella pneumonia, Enterobacter cloacae, Proteus vulgaris and Shigella. Zones of inhibition measured 1-2 mm. A negative control organism of V. cholerae proven female and non-vibriocinogenic was checked against all indicator strains with no effect against any of them.

In a large bacteriocin typing experiment, Chakrabarty et al. (1970) found 87% of 425 strains of V. cholerae to produce vibriocin providing three essential factors were included in the production method. These factors were (1) a citrate-phosphate buffer concentration of 0.5 to 0.7%; (2) pH 7.5 to 7.6; and (3) cold shock treatment. The bacteriocins isolated could be grouped into 11 vibriocin types on the basis of their reactions on eight indicator strains including E. coli Row, S. sonnei, S. flexneri and V. cholerae. Also, thermal treatment of indicator organisms at 45 C for 12 min increased the percentage of typable strains.

Few researchers have published electron micrographs of vibriocins. Jayawardene and Farkas-Himsley (1968, 1969a)

showed the particulate nature and some characteristics of the vibriocin they isolated. Lang et al. (1968) found particles "resembling pyocins" associated with one bacteriocinogenic strain of V. cholerae and did not find the particles with six non-bacteriocinogenic strains. Sheaths of some particles were shorter and appeared fragmented while still surrounding a central core. The suggestion is made that the particles may be identical with tail portions of defective V. cholerae phages.

Parker, Richardson, and Romig (1970) examined mitomycin C induced lysates of 14 classical and 13 el tor strains of V. cholerae by electron microscopy for the production of bacteriophage-related materials. The strains were also tested for pathogenicity using the ligated ileal loops of adult rabbits. A correlation between the bacteriophage related material and pathogenicity was observed in 25 of 27 strains tested. Typical bacteriophage tails were found to be produced by both P+ and P- strains. Thus, according to Parker et al. (1970), the fertility character does not appear to be related to the presence or absence of bacteriophage tails. In several strains, including the one used by Jayawardene and Farkas-Himsley for vibriocin production, bacteriophage tails were shown to occur in an extended form

with tail appendages. The vibriocin particles of Farkas-Himsley are classified as bacteriophage parts by these authors.

MATERIALS AND METHODS

Organisms

The strains of Vibrio cholerae used in this study and their sources are listed in Table 4. Strains of V. cholerae phage are listed in Table 5.

Media

Cultures of V. cholerae were maintained by monthly transfer of slants of tryptose agar, Difco, and stored at 4 C. Heart infusion broth (HIB), Difco, was used as the growth medium, the diluent for titrations, as the suspending medium in thermal stability experiments, and for the preservation of high titered phage stocks. Tryptose agar plates were used as the base medium for overlaying indicator strains, and screening techniques for detection of bacteriocin. Overlay medium consisted of heart infusion broth, Difco, with 0.75% Difco Bacto-Agar. Minimal broth consisted of the medium of Finkelstein and Langford (1955) as given in Table 6.

Electron Microscopic Study of Defectiveness

Bacteriophages were propagated on a sensitive host strain of V. cholerae, HVC 49 (Host V. cholerae strain #49). The temperate phage, PVC 120 SP (Phage of V. cholerae #120, Small Plaque type) was propagated by the plate method of Adams (1959), whereas a virulent mutant of this phage,

TABLE 4. Strains of Vibrio cholerae

Culture No.		Donor or Source	Original Designation
HVc	36	National Collection of Type Cultures (England)	Egypt 117 Inaba
HVc	39	Dr. William Burrows (Chicago)	C-311
HVc	41	"	C-281
HVc	49	"	C-291
HVc	52	"	C-23
HVc	53	"	C-11
HVc	55	"	C-272
HVc	60	"	C-144
HVc	63	"	C-173
HVc	67	"	C-62
HVc	70	"	C-323
HVc	72	"	C-81
HVc	76	"	C-191
HVc	77	"	C-175
HVc	80	"	C-101
HVc	81	"	C-154
HVc	82	"	C-252
HVc	85	"	C-282
HVc	87	"	C-115
HVc	91	"	C-91
HVc	93	"	C-181
HVc	94	"	C-331
HVc	98	"	C-332
HVc	99	"	C-284
HVc	122	Institute of Pasteur (Paris)	C.75 Inaba Ishu
HVc	130	"	V.52 Ogawa K2996

TABLE 4 (Continued)

Culture No.	Donor or Source	Original Designation
HVc 140	Dr. G. Singh (India)	V. <u>cholerae</u> , Inaba 569B
HVc 152	Dr. D. C. Lahiri (India)	<u>V.</u> <u>cholerae</u> , Inaba P6
HVc 164	Haffkine Institute (India)	<u>V.</u> <u>cholerae</u> , Ogawa 41
HVc 166	"	<u>V.</u> <u>cholerae</u> , Inaba 3371
HVc 170	"	<u>V.</u> <u>cholerae</u> , Inaba 9
HVc 174	"	<u>V.</u> <u>cholerae</u> , Inaba
HVc 184	Dr. Y. Watanabe (Japan)	<u>V.</u> <u>cholerae</u> , Ogawa 8
HVc 189	"	<u>V.</u> <u>cholerae</u> , Ogawa 154
HVc 198	"	<u>V.</u> <u>cholerae</u> , Ogawa 1644
HVc 199	"	<u>V.</u> <u>cholerae</u> , Ogawa 1045
HVc 201	"	<u>V.</u> <u>cholerae</u> , Inaba 123
HVc 225	"	<u>V.</u> <u>cholerae</u> , Inaba P-175
HVc 233	"	<u>V.</u> <u>cholerae</u> , Inaba 194
HVc 236	"	<u>V.</u> <u>cholerae</u> , Inaba 2
HVc 237	"	<u>V.</u> <u>cholerae</u> , Inaba 203
HVc 239	"	<u>V.</u> <u>cholerae</u> , Inaba 6a-845
HVc 241	"	<u>V.</u> <u>cholerae</u> , Inaba 95
HVc 250	"	<u>V.</u> <u>el tor</u> , 17
HVc 299	American Type Culture Collection (U.S.A.)	<u>V.</u> <u>comma</u> , 14100
HVc 300	"	<u>V.</u> <u>el tor</u> , 14033
HVc 301	"	<u>V.</u> <u>el tor</u> , 14734
HVc 302	"	<u>V.</u> <u>comma</u> , 14101
HVc 303	"	<u>V.</u> <u>comma</u> , 14102
HVc 305	"	<u>V.</u> <u>comma</u> , 14104

TABLE 4 (Continued)

Culture No.	Donor or Source	Original Designation
HVc 307	Dr. Y. Watanabe (U. of Texas Medical Branch)	V. <u>el tor</u> , 86
HVc 309	"	V. <u>comma</u> , 368512
HVc 310	"	V. <u>comma</u> , 368996
HVc 311	"	V. <u>comma</u> , C-16660
HVc 312	"	V. <u>comma</u> , C-13340
HVc 313	"	V. <u>comma</u> , C-14705
HVc 314	"	V. <u>comma</u> , 791
HVc 315	"	V. <u>comma</u> , C-17336
HVc 316	"	V. <u>comma</u> , B-68516
HVc 317	"	V. <u>comma</u> , C-13676
HVc 318	"	V. <u>comma</u> , C-2010
HVc 319	"	V. <u>comma</u> , C-2015
HVc 320	"	V. <u>comma</u> , C-13667
HVc 321	"	V. <u>cholerae</u> , 569B
HVc 322	Dr. S. Mukerjee (Calcutta, India)	NAG, 34/68
HVc 323	"	NAG, 7/68
HVc 324	"	V. <u>el tor</u> , EW6
HVc 328	"	V. <u>cholerae</u> , Bangal 24/67
HVc 329	"	V. <u>cholerae</u> , Hygn 5/67
HVc 330	"	V. <u>el tor</u> , Mad 757
HVc 330 (301/41r)	Mutant of HVc 330, transduced to streptomycin resistance	
HVc 330 (A01)	Mutant of HVc 330, acridine orange treated (hemolytic)	
HVc 330 (A02)	Mutant of HVc 330, acridine orange treated (hemolytic)	
HVc 330 (A03)	Mutant of HVc 330, acridine orange treated (hemolytic)	

TABLE 5. Strains of Vibrio cholerae phage

Phage	Source
PVc 117	Temperate phage isolated from HVc 117 (Newman and Eisenstark, 1964)
PVc 120SP	Temperate phage isolated from HVc 120 (Newman and Eisenstark, 1964)
PVc 120LP	Virulent mutant isolated from temperate phage PVc 120SP (Newman and Eisenstark, 1964)
PVc 300	Temperate phage isolated from HVc 300, el tor vibrio (Newman, unpublished data)
PVc 301	Temperate phage isolated from HVc 301 (Egan and Newman, unpublished data)
PVc 307	Temperate phage isolated from HVc 307 (Newman, unpublished data)
PVc 239	Temperate phage isolated from HVc 239
PVc 322	Temperate phage isolated from HVc 322
PVc 323	Temperate phage isolated from HVc 323
Mukerjee IV	Virulent typing phage isolated by Dr. S. Mukerjee (India) and purchased from the American Type Culture Collection

TABLE 6. Minimal medium*

Glucose	1.0 gm
KH_2PO_4	2.0 gm
K_2HPO_4	7.0 gm
Sodium Citrate . . .	0.5 gm
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 gm
$(\text{NH}_4)_2\text{SO}_4$	1.0 gm

Double distilled water to 1 liter, pH adjusted to 7.4.
Filter sterilized glucose added following autoclave sterilization at 121 C for 30 minutes.

* Finkelstein, R., and C. E. Langford. 1955. Nutrient requirements of Vibrio cholerae. Bact. Proc. 1955:49.

PVc 120 LP (Large Plaque type), was propagated in broth culture. These methods yielded titers of 2.5×10^8 and 1×10^{10} plaque forming units (P.F.U.) per ml respectively. The virus preparations were purified by centrifugation at $12,000 \times g$ for 10 min in a Sorvall SS-3 centrifuge, followed by filtration through a #03 Selas filter. The phage were pelleted using a Spinco Ultracentrifuge (Model L) at $65,000 \times g$ for 1 hr. These pellets were resuspended in a neutral solution of 1% ammonium acetate (Vieu, Nicolle, and Gallut, 1965).

Defective phage specimens were prepared by mitomycin C induction of strains by the method of Jayawardene and Farkas-Himsley (1969) as described in the section on mitomycin C induction.

Samples to be examined were mixed 1:1 (V/V) with 2% aqueous phosphotungstic acid (PTA) which had been adjusted to pH 7.0. Microdrops were then placed on grids and allowed to dry. After drying the preparations were examined by electron microscopy using a Zeiss EM9A, having an accelerating potential of 60 Kv., using a direct magnification of 41,000X.

Induction and Screening Methods

Colony Overlay Technique. The method used was based on the method of Wahba (1965). Organisms from a log phase

culture were plated on tryptose agar plates at a dilution which would give 10-100 colonies per plate. Following growth for 32-36 hr, at which time the colonies were about 2-3 mm in diameter, the plates were inverted over 2 ml of chloroform for 15 min, then left open at room temperature for 1 hr to allow the chloroform to evaporate. The overlay agar was seeded with 0.5 ml of an overnight broth culture of the indicator strain, and poured over the plate. After incubation for 18 hr, the plates were examined for zones of inhibition in the confluent growth of the indicator bacteria.

A modification of this technique based on a method of Chakrabarty et al. (1970) was also used. Equal amounts of sodium citrate and dibasic potassium phosphate (K_2HPO_4), at a concentration of 0.5% were added to the basal agar and the overlay in the above technique.

Mitomycin C Induction. Using the method of Jayawardene and Farkas-Himsley (1969a), 50 ml volumes of heart infusion broth were inoculated with log phase cultures and grown at 37 C with shaking to a density of approximately 1×10^9 cells per ml. Mitomycin C was added to each culture at a concentration of 0.3 μ g per ml. After incubation with shaking for 20 min, the cells were removed by centrifugation

at 12,000 X g for 10 min. The mitomycin C containing supernate was discarded, and the cells were resuspended and incubated in 25 ml prewarmed heart infusion broth (HIB) for 3 hr at 37 C with shaking. The lysate was then centrifuged at 12,000 X g for 10 min to remove bacteria and bacterial debris. The supernates were spotted on plates with a seeded overlay. In one experiment, 13 mitomycin C induced strains were tested on 83 indicator strains. In another experiment, 16 induced strains were tested on 30 indicator strains selected from the 83 used previously.

Strains producing inhibitory substances by mitomycin C induction in complete medium were also tested by this technique substituting minimal broth for the HIB.

Streak Plate Method. Following the method of Chakrabarty et al. (1970), strains of V. cholerae were grown in streaks 1 cm in width for 54 hr at 37 C on tryptose agar. The plates were then refrigerated for 18 hr at 4 C, the growth was scraped off and the plates sterilized with chloroform vapor for 15 min followed by aeration for 1 hr. Indicator strains were applied in parallel lines at right angles to the position of original inoculation, so as to give a heavy confluent growth. The plates were then incubated for 18 hr. Indicator strains were applied in

two streaks each, one streak being a heat treated preparation (45 C for 12 min). Also, the effect of citrate-phosphate buffer at a concentration of 0.5% was tested.

Overlay Seeded with Producer and Indicator Strains.

Approximately 50-100 vibrios to be tested and about 5×10^7 indicator vibrios were seeded into an overlay and plated over a basal layer of agar (Takeya and Shimodori, 1969). After incubation at 37 C for 6 hr, then at room temperature overnight, the plates were observed for plaque-like clearings or lacunae.

Use of Streptomycin Sensitive and Resistant Strains.

Following the method of Ozeki (1968), six strains of Vibrio cholerae, each having a corresponding SMr sister strain were grown in heart infusion broth. By dilution, approximately 100 SMs cells were added to an overlay which had been seeded with about 1×10^8 SMr cells. The overlay contained 100 µg per ml SM. Plates were incubated 24 hr at 37 C and examined for zones of inhibition.

Phage Lysis of UV Induced Strains. In an attempt to liberate vibriocin from the cells, bacteria sensitive to a virulent tail-less phage, PVC 120 LP, were induced with UV irradiation. Growth from 10 ml broth was centrifuged and resuspended in 5 ml sterile saline in a petri plate.

Irradiation with a 15 watt G.E. G15T8 source at a distance of 45 cm was for 10 sec with constant stirring. These cells were then added to 50 ml prewarmed broth, and incubated for 120 min in the dark. The virulent phage were then added, and following 4 hr further incubation, the culture was centrifuged for 10 min at 12,000 X g, and the supernate was tested for bacteriocin activity by spotting on indicator strains resistant to PVC 120 LP.

Agar Well Technique. Employing an agar well technique introduced by Datta and Prescott (1969a), 20 ml of tryptose broth with 1% agar were dispensed into petri dishes. Circular reservoirs were made in the solidified agar with a 6 mm diameter cork borer. The agar circles were removed, and the wells filled with melted heart infusion broth containing 0.75% agar, seeded with the organism to be tested. The plates were incubated for 48 hr at 37 C, and left for 6 hr at 4 C. The organisms were then killed by exposure to chloroform vapor for 1 hr. Following exposure of the plates to the air for 1 hr to allow for the evaporation of the chloroform, an indicator strain was added to overlay agar and the mixture was poured over the reservoir containing basal layer. The plates were held for 18 hr at 4 C, incubated for 24 hr at 37 C, and finally examined for

the presence of zones of inhibition of the indicator strain around the reservoirs containing the test strain.

Eh Reduction Method. The method of Farkas-Himsley and Seyfried (1962b) was used. Vibrios to be tested were grown in heart infusion broth at 37 C with constant shaking to give about 1×10^9 cells per ml. Sodium thioglycollate was added to a final concentration of 0.15% resulting in a redox potential of about -230 mv. After 2.5 hr, the culture was centrifuged for 10 min at $12,000 \times g$, and the supernates were spotted on various SMr strains. The plates were incubated 24 hr at 37 C and examined for zones of inhibition.

Ammonium Sulfate in the Growth Medium. Ammonium sulfate, shown to inhibit the production of proteases (Liu and Hsieh, 1969), was added to the broth growth medium at a concentration of 4%. Mitomycin C induction followed when the cell density reached approximately 1×10^9 cells per ml. Lysates were spotted on indicator strains overlayed on tryptose agar plates. The plates were observed for any sign of bacteriocin activity after incubation at 37°C for 18 hr.

Bacteriocin Concentration Methods

Concentration by Ultracentrifugation. Certain mitomycin C induced lysates were ultracentrifuged in quantities

of 13.5 ml in Beckman/Spinco Lusteroid Cellulose Tubes (5/8" X 3"). Centrifugation was at 95,000 X g for 1 hr using a Spinco Model L Ultracentrifuge. The pellet was taken up either in 1 ml saline or heart infusion broth. The activity of the supernate and pellet was tested.

Concentration by Pervaporation. For concentration of larger volumes of mitomycin C induced lysates, dialysis tubing (Union Carbide Corp.) was filled with the preparation to be concentrated. The tubes, tied at each end, were hung in an incubator at 37 C in the path of air being circulated by a fan. Pervaporated specimens, ten-fold concentrated, were used in host range studies, heat stability tests, and in the serological relatedness test.

Characterization of Isolated Vibriocin

Thermal Stability. Tubes containing 0.5 ml samples of ten-fold pervaporation concentrated bacteriocin of HVc 239 were put into a 65 C water bath. Temperature equilibrium was reached in 45 sec. At 1 min intervals through the first 10 min, then at 2 min intervals through 30 min, tubes were removed from the high temperature bath and placed into an ice water bath. Samples were plated on a lawn of sensitive indicator cells and their activity compared with that of a control.

Sensitivity to Freezing. Tubes containing 1 ml samples of concentrated BVc 239 were subject to repeated freeze-thaw treatments. Samples frozen and thawed ten times at -70 C and 37 C were plated on sensitive indicator cells.

Trypsin Sensitivity. Trypsin at a concentration of 200 µg per ml was incorporated into the soft agar overlay seeded with indicator bacteria. Lysates which exhibited vibriocin activity were spotted on the overlay. In a second test, trypsin was added directly to the vibriocin preparation prior to spotting on the indicator cells suspended in a trypsinized medium.

Serological Relatedness to Choleraphage. The method used for preparation of antisera against the isolated bacteriocin and phage was a modification of that recommended by Adams (1959). Emulsions of a BVc 239 preparation in Freund's incomplete adjuvant or phage preparations were injected subcutaneously into a white rabbit. The bacteriocin inoculations were tested and found free of viable bacteriophage. The following dose-schedule was followed:

<u>Week</u>	<u>Inoculations</u>
1	1.0 ml (2 injections)
2	2.0 ml (2 injections)
3	3.0 ml (2 injections)
4	none
5	4.0 ml (2 injections)

One week following the last injection, the rabbit was bled by cardiac puncture. The blood was allowed to clot and the serum collected. The antisera were stored in sterile bottles at -10 C.

A spot test for antigenic specificity (Eisenstark, 1967) was used to determine whether the isolated bacteriocin from HVC 239 was related to any of 16 cholera phages. An agar overlay was seeded with phage-susceptible indicator cells, and a cholera phage sample diluted to give crowded but not overlapping plaques. The mixture was plated over a basal medium, and after solidification of the overlay, a drop of specific antiserum was added to the surface of the plate.

Antiserum not specific for the phage allowed plaques to arise in the area covered by the antiserum. Plaques failed to develop in the antiserum spot where there was an existing homology between the phage and the antiserum.

Eleven phage were screened by this method for any serological relatedness to the HVC 239 vibriocin. Seven antisera against various cholera phage were also spotted on each plate to detect any cross relatedness between the phage. The phage, bacteriocin, and antisera are listed in Table 7.

TABLE 7. Antisera, phage, and bacteriocin used in serological relatedness test.

Antisera	Phage	Bacteriocin
Anti PVc 117	PVc 117	BVc 239
120 SP	120 SP	
120 LP	120 LP	
300	239	
301	300	
307	301	
327	307	
Anti BVc 239	322	
	323	
	Mukerjee I (MI)	
	Mukerjee IV (MIV)	

Host Range of Isolated Bacteriocin and Phages

Vibrio cholerae strains listed in Table 4 were tested for susceptibility to mitomycin C lysates of 29 strains. A ten-fold concentrated HVc 239 mitomycin C induced lysate containing vibriocin was spotted on lawns of 67 vibrio hosts prepared by the overlay technique. These hosts included 59 classical V. cholerae strains, two non-agglutinable V. cholerae strains (NAG vibrios), and six strains of V. cholerae biotype el tor. Following overnight incubation at 37 C, the plates were examined for plaques or zones of inhibition due to either phage or the bacteriocin, respectively. Inhibition due to the vibriocin was graded from one to four plus. Six phage were also spotted on these plates. These were PVc 239, a temperate phage isolated from strain HVc 239 following mitomycin C induction; PVc 322 and PVc 323, temperate phage isolated from the NAG vibrios; PVc 301 and PVc 307, temperate phage from el tor vibrios; and PVc 120 LP, a virulent (intemperate) choleraphage isolated previously as a virulent mutant of the temperate PVc 120 SP.

Differentiation of Bacteriocin from Bacteriophage

Dilution Method. Any lysate showing an inhibitory effect on a sensitive indicator strain was routinely diluted

and spotted again on the indicator host. Inhibition due to bacteriocin fades evenly in greater dilution, whereas phage in greater dilution, appear as distinct plaques.

Electron Microscopic Examination. Lysates showing characteristics of bacteriocin activity by the dilution method were prepared for examination by E.M. as described elsewhere in this paper.

RESULTS

Electron Micrographic Evidence of Defectiveness

Electron micrographic examination of the wild type, small plaque temperate phage, PVC 120 SP, showed phage with binal symmetry, having typical icosahedral head structures and tail components. Some tail components, however, were defective in that more than one sheath segment covered the long tail tube. Figure 1 shows the appearance of normal PVC 120 SP phage in two forms; the first, in which the head contains the nucleic acid, and the sheath is in the extended form, and the second, where the head is empty, and the sheath is contracted. Figures 2 and 3 show this phage with one, two, and four sheath segments, and Figure 4 shows an elongated tube with eight sheath sections, and another, which may be attached to a head component, with seven sheath segments.

The approximate size of these components were calculated to be as follows:

Head components:	58-60 mμ
Sheath segments:	38-40 mμ
Tube structures:	73-340 mμ

PVC 120 LP, the virulent mutant phage, was found to be typical in its icosahedral head component, but aberrant in that no typical tail structures were visible in the micrographs (Figure 5). On several particles favorably

Figure 1. Electron micrograph of normal PVC 120SP. Phage appear in two forms. One in which the head component contains the nucleic acid and the tail sheath is in the extended form (upper left), and another in which the head is devoid of nucleic acid and the sheath contracted (lower right). X 250,000.

Figure 2. Electron micrograph of defective PVC 120SP. One and two sheath segments are visible. X 240,000.

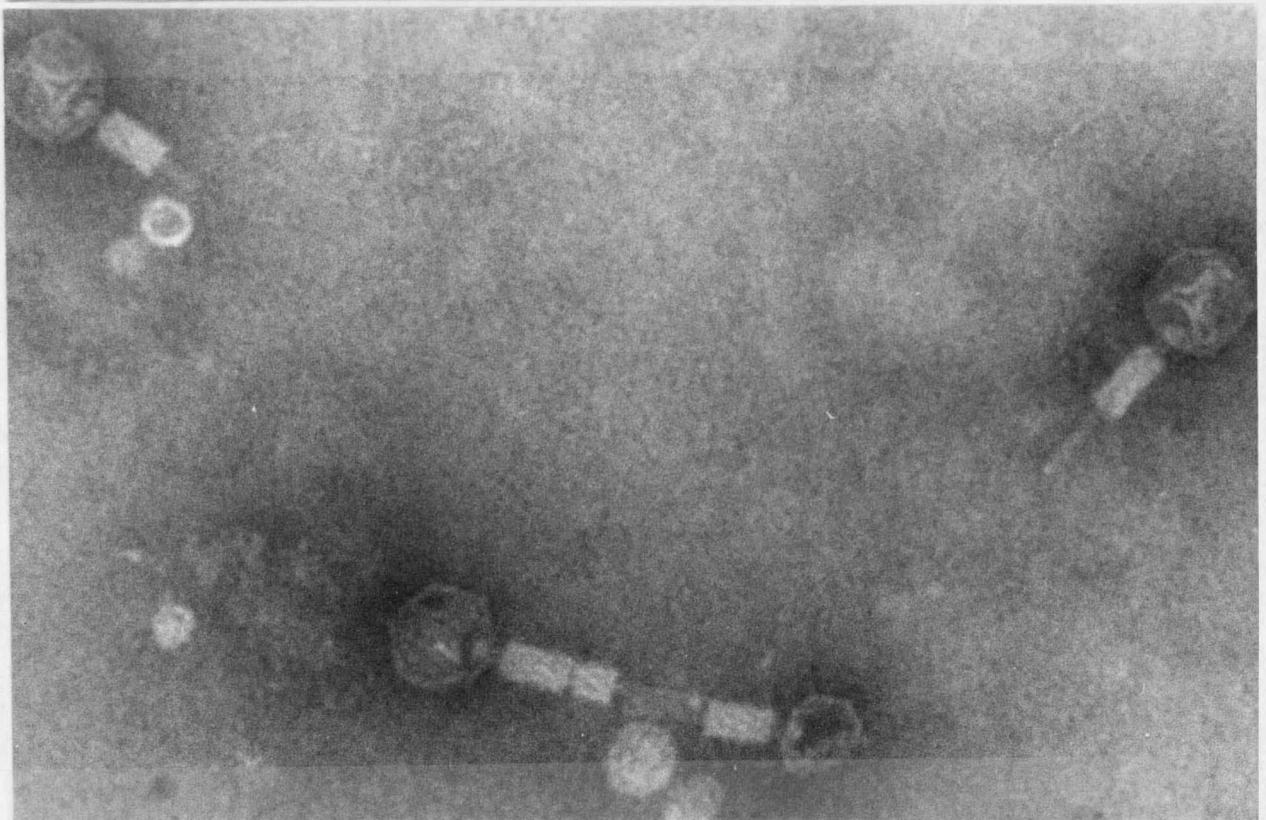
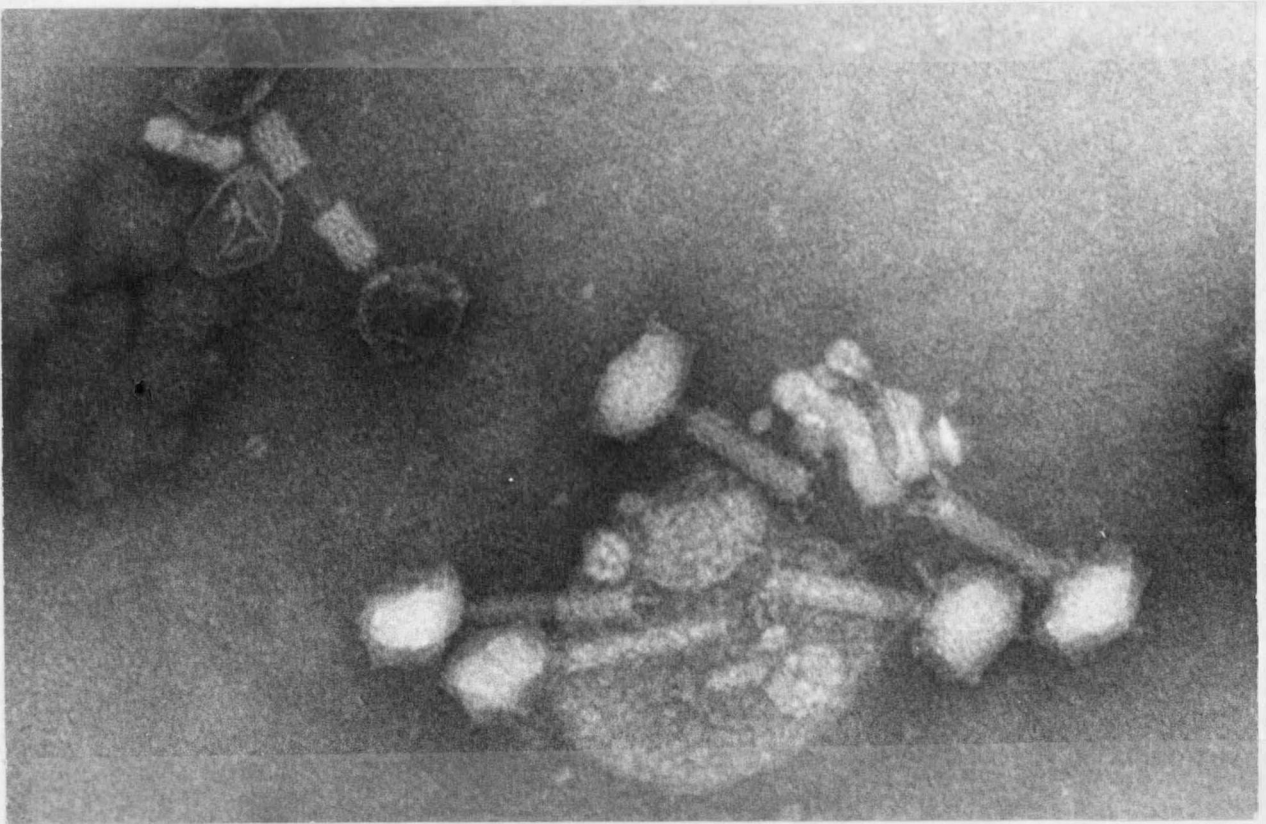


Figure 3. Electron micrograph of defective PVC 120SP.
Four tail sheaths surround the central tube.
X 250,000.

Figure 4. Electron micrograph of defective PVC 120SP.
An elongated tube with eight sheath segments
is visible. Another is visible with seven
sheath segments which may be attached to a
head component. X 225,000.

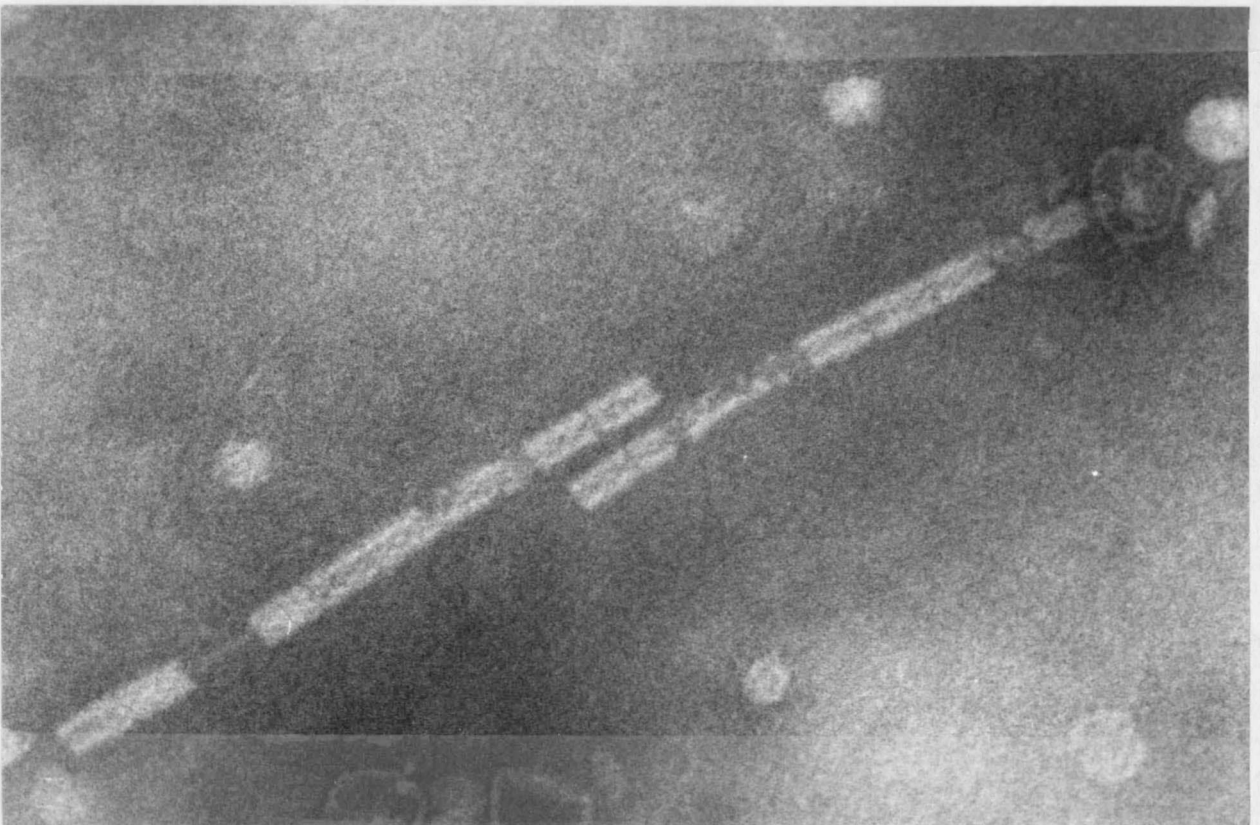
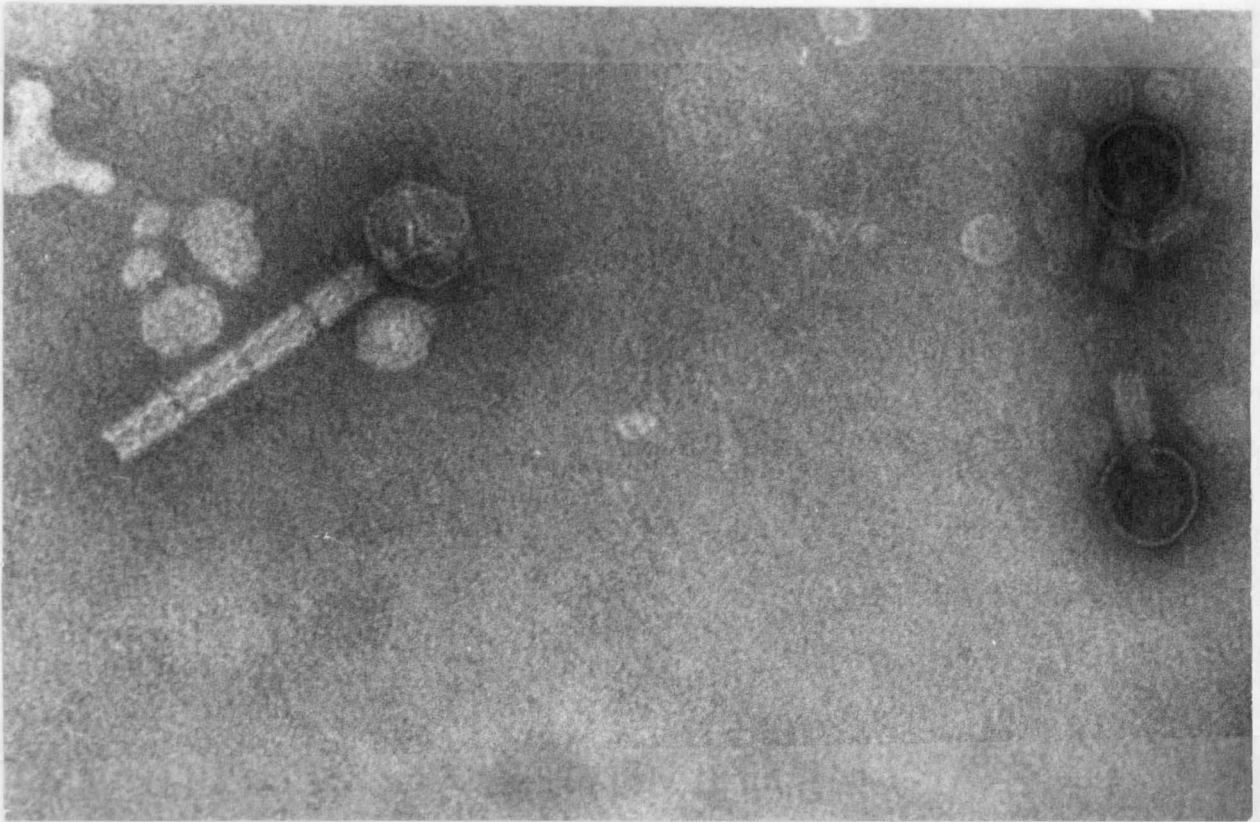


Figure 5. Electron micrograph of PVc 120LP.
Virulent mutant of PVc 120SP with
no apparent typical tail structures.
X 250,000.

Figure 6. Electron micrograph of PVc 120LP.
Phage particles favorably orientated
which suggest the presence of short
tail pins. X 250,000.

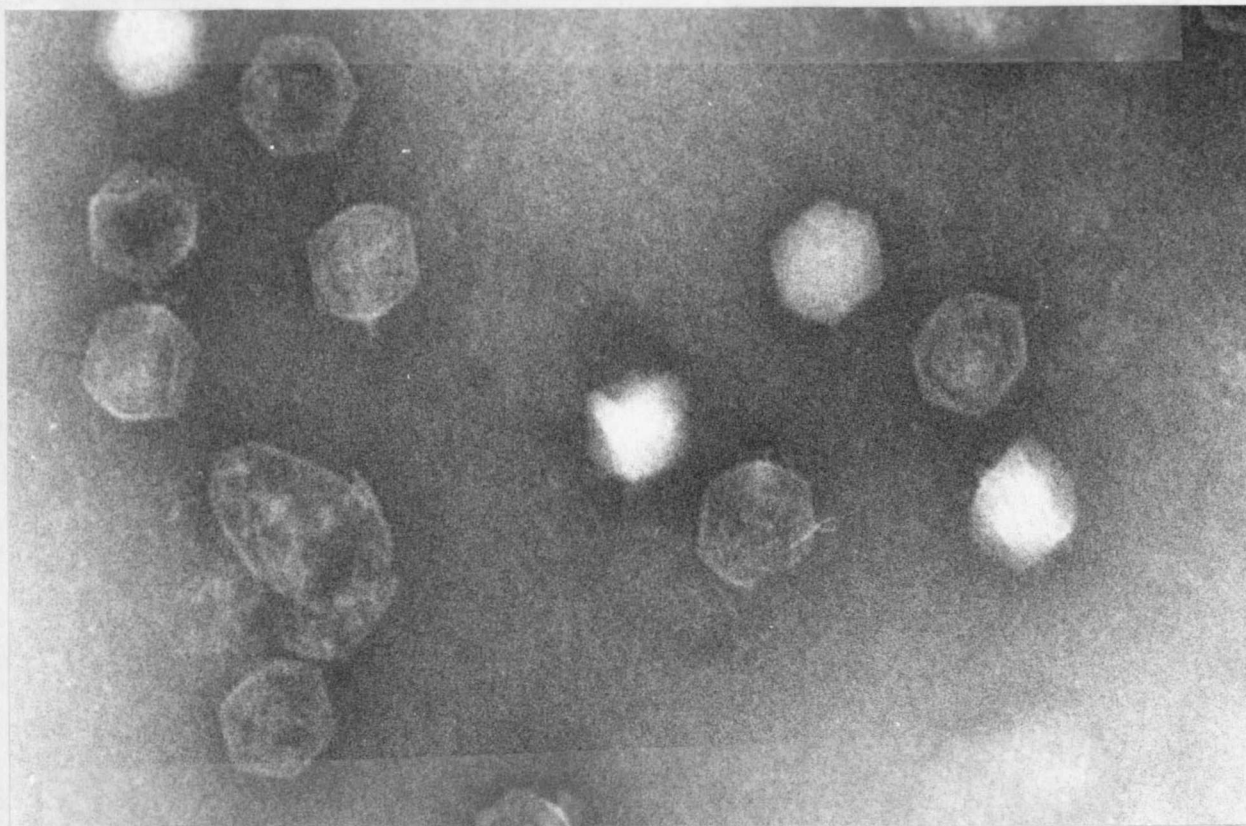
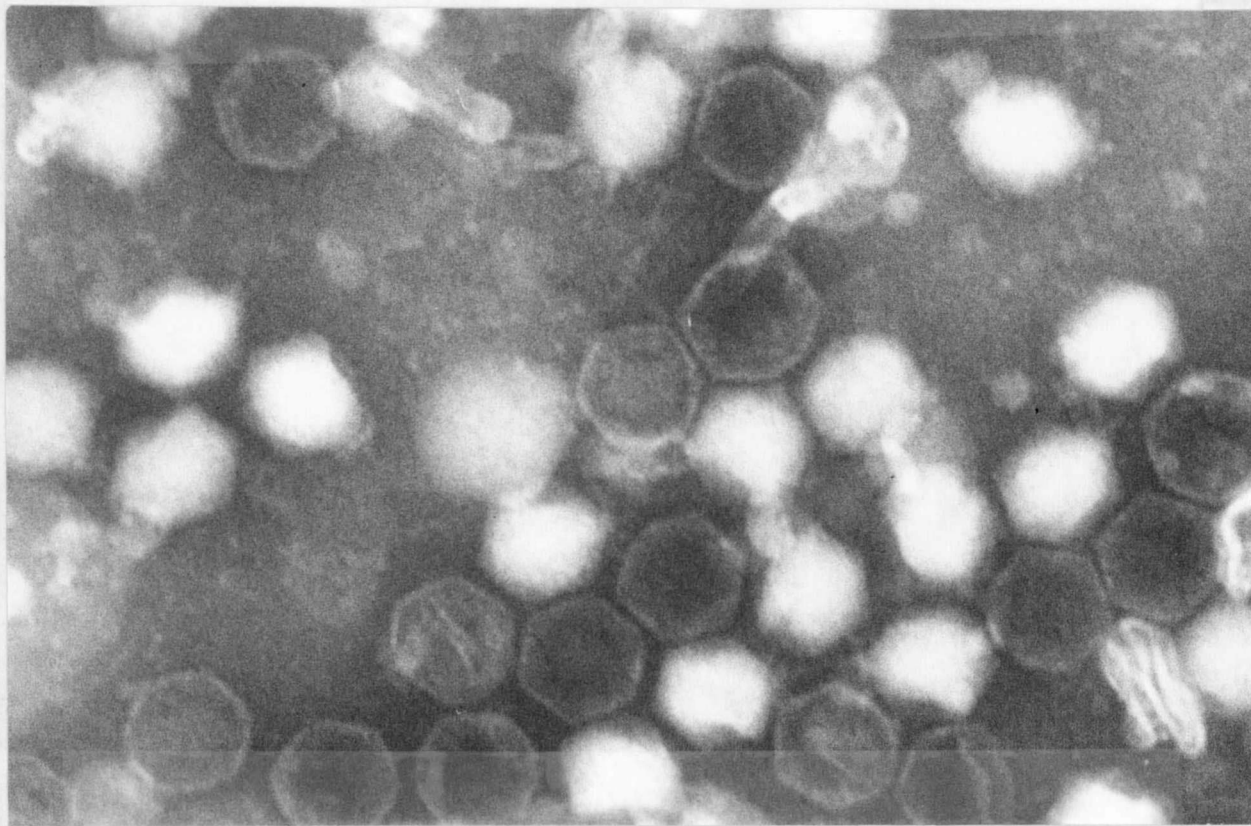
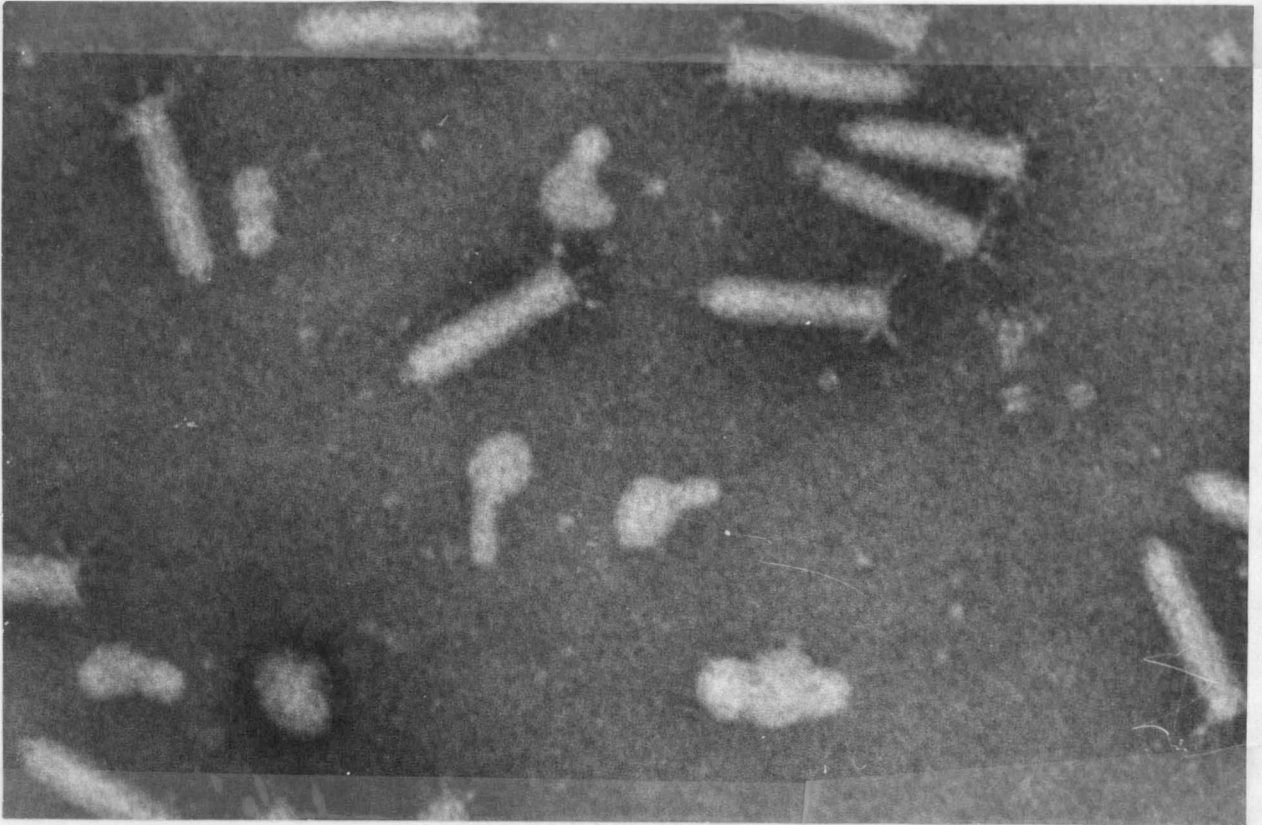


Figure 7. Electron micrograph of BVC 239. Particles with activity associated with vibriocin, appearing in the extended form with phage tail appendages. X 250,000.



50% COTTON

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orientated, what appears to be short tail "pins" are visible (Figure 6).

Mitomycin C lysates of strain HVC 239 contained particles with activity associated with the vibriocin particles of Farkas-Himsley and Seyfried (1962b). This activity was noted as a zone of partial inhibition of the indicator strain when the lysate was spotted on an indicator lawn. In ultracentrifuged preparations, activity was limited to the pellet, and therefore the activity was associated with these particles. Particulate structures resembling phage tail components were visible in the preparations, and there was little evidence of other phage-associated materials by electron microscopy. Figure 7 shows these particles which were calculated to be about 100 m μ in length, and which appear to be in the extended form with appendages.

Induction and Screening Methods

The colony overlay technique of Wahba (1965) was used to screen for bacteriocinogeny in two experiments.

In the first, 11 strains were tested in a "checker board" manner. That is, with 11 tests the strains were tested against the same 11 strains used as indicator cells. The organisms were one strain of classical V. cholerae,

two strains of non-agglutinable V. cholerae (NAG vibrios), and six strains of V. cholerae el tor.

Ten of the 11 strains tested showed inhibition of all indicator strains, and one strain was negative on all indicators. Inhibition was noted as a circular zone of markedly reduced indicator growth, and the size of the zone directly corresponded to the size of the colony producing the inhibitory substance. There was no evidence of any diffusible agent involved with the inhibition. The 10 strains also showed inhibition when the indicator strain was the same as the test strain. For example, HVC 41 test strain inhibited HVC 41 used as an indicator. On the plates showing inhibition, generally not all colonies were inhibitory, and positive and negative producers were easily distinguished. Colonies producing inhibition ranged from 25% to 100% of total colonies, with most positive strains having 80-100% of colonies showing the inhibition. Of the six el tor cholera strains used, five were known to be lysogenic. Phage involvement was noted when phage-susceptible classical cholera vibrios were used as indicators.

In a second test, 80 strains were tested against a non-agglutinable vibrio indicator host. NAG vibrios have been found resistant to cholera phage, and thus phage involvement

by producer strains was eliminated. Included in this test were 72 strains of classical V. cholerae, two strains of NAG vibrios, and six strains of V. cholerae el tor.

Of the 80 strains tested, 53 (66%) were found to produce indicator inhibition. No diffusible agent was noted, and the size of the zone of inhibition again was the same size as the colony producing the inhibitory agent. Examination of the progeny of colonies producing this inhibitory substance showed daughter cells that were both positive and negative for this agent. Colonies negative for production of the agent generally produced progeny incapable of producing inhibition of indicators in subsequent testing. All attempts to separate this agent from the cells failed.

In a modification of this test, citrate-phosphate buffer was added to the basal and overlay agar to a concentration of 0.5% (Chakrabarty, 1970). Zones of inhibition were slightly more transparent, but their size remained constant.

The mitomycin C induction method of Jayawardene and Farkas-Himsley (1969a) was used to test a total of 29 strains in two experiments. The cultures induced included 26 strains of classical V. cholerae, one strain of V.

cholerae el tor, and two strains of NAG vibrios.

First, 13 strains were induced and their lysates were spotted on 82 strains used as indicators. In a second experiment, the lysates of 16 additional strains were spotted on 30 indicators selected from the 82 used previously. The results are given in Table 8. The results were condensed to show the action of the 29 induced strains on the 30 selected indicator hosts.

Four mitomycin induced cultures produced inhibition of indicators suggestive of bacteriocin involvement. Three of the four lysates gave extremely weak inhibitory reactions and also several discrete phage plaques were noted within the zones of inhibition. Therefore, further investigations were not conducted on these three lysates. However, the HVc 239 lysate showed a somewhat stronger inhibition, visible to a dilution of 1:4, and further investigations were designed to partially characterize this agent.

Mitomycin C induction and screening of the lysates of two NAG vibrios, HVc 322 and 323 revealed the presence of bacteriophage in these lysates. Further examinations of these phages were planned since non-agglutinable vibrios have never been reported as lysogenic. During subsequent mitomycin C induction of HVc 239, a phage was isolated from

TABLE 8. Results of mitomycin C inductions.

Selected Indicator Strains	Induced Strains																												
	36	39	41	49	63	70	76	89	91	93	94	98	99	198	199	201	236	237	239	241	299	301	302	313	315	321	322	323	329
63													φ						φ			φ			φ				
67																						φ			φ				
77			φ			φ																φ							
80			φ																			φ							
81																				φ	φ		φ						
91		φ	φ			φ														φ		φ							
93			φ			φ		φ												φ		φ							
94		φ	φ			φ		φ												β		φ				φ			
98		φ						φ							φ					β	φ		φ			φ			
99															φ					β	φ		φ			φ			
140		φ	φ			φ		φ							φ					β		φ			φ				
198								φ					φ									φ			φ				
199		φ				φ		φ					φ									φ			φ				φ
201								φ					φ									φ			φ				
236		φ	φ			φ														φ	φ		φ			φ	φ		φ
237																					φ		φ						φ
239	neg			neg			neg			neg			neg			neg				neg			neg		neg				neg
241			φ																										
299			φ			φ		φ														φ							
299SM													β																φ
301		β																		β									φ
301SM		β																		β		φ							φ
302			φ			φ							φ		φ		φ					φ		φ					
314						φ		φ			φ						φ			β	φ		φ		φ				
316		φ				φ		φ					φ				φ					φ		φ		φ			
317		φ				φ	φ	φ					φ				φ					φ		φ		φ			φ
318		φ						φ					φ									φ		φ		φ			
320					φ												φ			φ		φ		φ		φ			
321		φ	φ			φ											φ			φ	φ		φ		φ	φ		φ	
322																										φ			
323																													β

φ = suspected phage
β = suspected bacteriocin

this host, as well as the agent responsible for indicator inhibition.

Table 9 summarizes the bacteriocin detection methods. Seven methods gave no evidence of indicator inhibition under the specific test conditions. The negative results do not necessarily indicate a faulty detection method. A vibriocinogenic host may not have been included in the test, the indicator system may not have been suitable, or specific conditions for the production of bacteriocin may not have been present.

Vibriocin Characterization

Thermal Stability. A concentrated BVC 329 preparation showed no apparent loss of activity following heating at 65 C for 30 min. The agent was sensitive to boiling, however, losing activity after less than one minute of boiling.

Sensitivity to Freezing. Repeated freeze-thaw treatment had no apparent effect on the inhibitory effect of a concentrated vibriocin preparation on sensitive indicator cells.

Trypsin Sensitivity. The addition of trypsin to the basal and overlay agar used to suspend the indicator cells had no effect on the vibriocin or host activity. There was also no apparent loss of activity when trypsin was added to

TABLE 9. Summary of the bacteriocin detection methods.

Method	Reference	No. of Strains Tested	No. of Indicator Strains	No. Positive for Indicator Inhibition
1. Colony overlay technique	Wahba, 1965	11 80	11 1	10 53
2. Mitomycin C induction	Jayawardene & Farkas-Himsley, 1969a	13 16	82 30 (selected)	4
3. Streak plate method	Chakrabaraty <u>et</u> <u>al.</u> , 1970	8	8	0
4. Overlay seeded with producer and indicator strains	Takeya and Shimodori, 1969	8	4	0
5. Use of SMs and SMr strains	Farkas-Himsley & Seyfried, 1962b	6	6	0
6. Phage lysis of UV indirect strains		3	10	0
7. Agar well technique	Datta and Prescott, 1969a	10	4	0
8. Eh reduction method	Farkas-Himsley & Seyfried, 1962b	11	11	0
9. Ammonium sulfate in the growth medium	Liu and Hsieh, 1969	3	1	0

the vibriocin preparation prior to spotting on the indicator lawn.

Serological Relatedness to Choleraphage. The results of the spot test for antigenic specificity are given in Table 10. The table includes first the effects of the antivibriocin serum on 11 phage and on the vibriocin itself, then the results of seven anti-phage sera on the one vibriocin preparation and the 11 phage preparations.

Host Range of Isolated Vibriocin and Phages. The results of the host range study are shown in Table 11. Grading the degree of indicator inhibition due to BVC 239 revealed V. cholerae el tor to be the most sensitive to the agent. Lysogenic el tor vibrios were resistant to the phage also spotted on the indicator lawn by virtue of a functional immune system. Non-agglutinable vibrios were resistant to the vibriocin and all phages. Classical cholera strains were both sensitive and resistant to the bacteriocin and showed varied susceptibility patterns to the bacteriophages.

TABLE 10. Results of spot test for antigenic specificity.

Antigens		Antisera							
		Anti-vibriocin	Anti-Phage						
		BVc 239	PVc 117	120SP	120LP	300	301	307	327
Vibriocin	BVc 239	+	+	-	+	+	+	+	+
Phage	PVc 117	+	+	+	-	+	+	+	+
	120SP	+	+-	+	-	+	+	+	+-
	120LP	-	-	-	+-	-	-	-	-
	239	-	+-	+-	-	+	+	+	-
	300	+-	-	-	+	+	+	-	-
	301	+	+-	+-	+-	+	+	+	+
	307	+-	-	-	-	+	+	+-	-
	322	+-	-	-	-	+	+	+	-
	323	+-	+-	+-	+-	+	+	+	+-
	M I	+-	-	-	-	+	+-	+-	-
	M IV	-	-	-	+	-	-	-	-

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+ = inactivation
 - = no inactivation
 +- = partial inactivation

TABLE 11. Host range of isolated vibriocin and phages.

		BVc 239 Bacteriocin	PVc 239 Temperate	330 A02 Temperate	330 A01 Temperate	323 Temperate	322 Temperate	301 Temperate	307 Temperate	120LP Virulent
<u>El tor Hosts</u>										
HVc	250	4+	-	-	-	-	-	-	-	-
	300	4+	-	-	-	-	-	-	-	-
	301	4+	-	-	-	-	-	-	-	-
	307	4+	-	-	-	-	-	-	-	-
	324	4+	-	-	-	-	-	-	-	-
	330	4+	2+	3+	3+	2+	2+	3+	3+	-
<u>Other Hosts</u>										
	241	3+	3+	3+	3+	3+	3+	4+	4+	4+
	318	3+	3+	3+	3+	3+	3+	4+	4+	4+
	320	3+	3+	3+	3+	3+	3+	4+	4+	4+
	67	2+	4+	2+	1+	-	-	4+	4+	4+
	82	2+	3+	-	1+	2+	2+	4+	4+	4+
	299	2+	4+	3+	3+	4+	4+	3+	4+	4+
	316	2+	3+	3+	3+	3+	3+	2+	2+	2+
	319	2+	2+	-	3+	2+	3+	-	-	4+
	321	2+	3+	3+	3+	3+	3+	4+	4+	4+
	328	2+	3+	1+	1+	1+	2+	3+	2+	2+
	36	1+	3+	1+	2+	2+	3+	4+	4+	4+
	77	1+	4+	1+	-	4+	4+	4+	4+	4+
	85	1+	3+	-	-	3+	3+	4+	4+	4+

TABLE 11 (Continued)

	BVC 239	PVC 239	330 A02	330 A01	323	322	301	307	120LP
166	1+	3+	3+	3+	3+	2+	4+	4+	4+
174	1+	4+	3+	4+	4+	4+	4+	4+	4+
198	1+	3+	3+	1+	3+	3+	4+	4+	4+
199	1+	3+	1+	-	3+	3+	4+	4+	4+
201	1+	3+	3+	-	1+	2+	4+	4+	4+
237	1+	3+	3+	3+	3+	2+	4+	4+	4+
322*	-	-	-	-	-	-	-	-	-
323*	-	-	-	-	-	-	-	-	-
39*	-	-	-	-	-	-	-	-	4+
41	-	4+	4+	4+	4+	4+	4+	4+	1+
49	-	2+	1+	2+	2+	2+	4+	4+	4+
52	-	3+	-	-	1+	1+	4+	4+	4+
53	-	-	-	-	-	-	-	-	3+
55	-	4+	-	-	1+	3+	4+	4+	4+
63	-	4+	1+	-	1+	3+	4+	4+	4+
70	-	3+	1+	-	2+	3+	4+	4+	4+
72	-	4+	4+	2+	2+	3+	4+	4+	4+
76	-	4+	2+	2+	3+	3+	4+	4+	4+
81	-	3+	-	1+	3+	3+	4+	4+	4+
87	-	3+	2+	2+	2+	3+	4+	4+	4+
91	-	3+	-	2+	2+	3+	4+	4+	4+
93	-	2+	-	-	1+	2+	4+	4+	4+
94	-	4+	-	-	2+	4+	4+	4+	4+
98	-	3+	2+	1+	1+	3+	4+	4+	4+
99	-	-	-	-	-	-	-	-	4+

TABLE 11 (Continued)

	BVc 239	PVc 239	330 A02	330 A01	323	322	301	307	120LP
122*+	-	-	-	-	-	-	-	-	3+
130*+	-	-	-	-	-	-	-	-	2+
140	-	2+	-	1+	1+	2+	4+	4+	4+
152	-	4+	-	-	3+	3+	4+	4+	4+
164	-	1+	-	-	1+	1+	4+	4+	4+
170	-	4+	3+	1+	4+	4+	4+	4+	4+
184	-	2+	2+	-	1+	1+	4+	4+	4+
189	-	3+	2+	1+	2+	2+	4+	2+	4+
225	-	-	-	-	-	-	-	-	4+
233	-	3+	3+	3+	3+	2+	4+	4+	4+
236	-	4+	3+	4+	4+	4+	4+	4+	4+
239+	-	-	-	-	-	1+	-	-	4+
302	-	2+	1+	-	1+	1+	4+	4+	-
303	-	2+	1+	2+	1+	1+	4+	4+	4+
305	-	3+	-	-	2+	3+	4+	4+	4+
309	-	3+	3+	3+	3+	3+	NT	NT	NT
310	-	4+	4+	4+	2+	2+	4+	4+	4+
311	-	3+	1+	2+	3+	3+	4+	4+	4+
313	-	4+	3+	3+	2+	3+	4+	4+	4+
314	-	3+	1+	3+	3+	3+	4+	1+	4+
315	-	1+	-	-	-	-	2+	4+	4+
317	-	3+	3+	3+	3+	3+	4+	4+	4+
329	-	-	-	-	-	-	-	-	4+

* found to be lysogenic upon mitomycin C induction
+ bacterocin/defective phage producer

DISCUSSION

Two general types of bacteriocins exist which have a somewhat similar mode of action on sensitive host cells. Apart from this similarity, the differences between the two types are great. Structurally, the two types are quite unrelated. For each, the genetic determinant, physical properties, and the method of production and release differ enough to cause speculation as to their relationship to each other and to other substances. Yet, both groups are frequently included in one family; bacteriocins.

The purpose of this investigation was to find any correlations between bacteriocins of Vibrio cholerae and defective choleraphage. The evidence for the relatedness is presented in two ways. First, structural evidence shows similarities between vibriocin and bacteriophage or bacteriophage associated structures of V. cholerae, and secondly, serological data reveal the homologies existing between choleraphage and an isolated vibriocin.

Evidence of structural defectiveness was found by electron micrographic examination of choleraphage preparations. The multiple tail sheath segments of PVC 120 SP closely resembled particles associated with a bacteriocinogenic strain of V. cholerae as reported by Lang et al. (1968).

The particles associated with PVC 120 SP, however, were found both free of other phage components and connected to "empty" bacteriophage head components. Polysheath and polycore components somewhat like those associated with defective PVC 120 SP have been reported by Higerd, Baechler, and Berk (1969) in studies of pyocin, a particulate, high molecular weight bacteriocin of Pseudomonas aeruginosa. The pyocin structures differed from the defective cholera-phage in that polysheath segments were not found associated with a polycore or phage-polycore structure. Also, the polycore or elongated tube was not shown with sheath segments surrounding the tube, or found in association with other bacteriophage structures, as were the polycore and polysheaths of the defective choleraphage PVC 120 SP.

Electron micrographic examination of mitomycin C lysates of another V. cholerae strain, HVC 239, revealed particles resembling bacteriophage tails. These particles had activity associated with particulate bacteriocins of V. cholerae called vibriocin. They also structurally resembled the vibriocin particles from another strain of V. cholerae (Jayawardene and Farkas-Himsley, 1968), the pyocin particles of P. aeruginosa (Bradley, 1967), and colicin 15 particles of E. coli (Endo et al., 1965).

The serological relatedness study suggests a correlation between the isolated vibriocin and certain cholera phage. Nine of ten temperate phages were inactivated by anti-vibriocin 239 serum. Interestingly, the only temperate phage not affected by the serum was PVc 239, a temperate phage isolated from the same "vibriocinogenic" host during a later mitomycin C induction. The bacteriocin was inactivated by antisera against six different temperate cholera phage, and unaffected by antisera against a virulent cholera phage. It would appear from the evidence that a serologically unrelated bacteriocin and bacteriophage were induced from the same host, that HVC 239 is therefore doubly lysogenized, and that the products of induction may be either phage, vibriocin, or both.

Although the vibriocin isolated from HVC 239 structurally resembles the vibriocin of Jayawardene and Farkas-Himsley, differences were found in regard to its production and character. Jayawardene and Farkas-Himsley (1969a) isolated a vibriocin following mitomycin C induction of Vibrio comma, strain 9168. Its biosynthesis was found to occur only in complex medium, and could not be achieved in a simple growth medium. The vibriocin was sensitive to trypsin, a character usually associated with low molecular

weight bacteriocins. Streptomycin resistant (SMr) mutant cells were found to be predominantly sensitive to the agent. In contrast, HVC 239 vibriocin was found to be produced in a minimal medium, was insensitive to trypsin, and SMr mutant cells used as indicators were not found to be sensitive to BVC 239 unless the SMs wild type strain was also sensitive.

There are great differences in the reported success of demonstrating bacteriocins of Vibrio cholerae. The Wahba technique (Wahba, 1965), involving cell to cell contact, has been successful in showing indicator cell inhibition, but all attempts by various methods to isolate an agent involved with this cell to cell inhibition have reportedly failed. These results have prompted a cell-associated bacteriocin hypothesis (Takeya and Shimodori, 1969). In this study, many vibrios were found to be capable of producing indicator inhibition by the Wahba technique. Unexpectedly, inhibition was evident even when the producer and indicator strain were identical. Strains producing this inhibition were found not to produce a diffusible agent, nor were any agents detectible in the medium following any of several induction methods used on the positive cells. Possible explanations for these phenomena are that the inhibition is not caused by a typical

bacteriocin, or that perhaps that the inhibition is due to some other unrelated factor. The characteristic of indicator inhibition by some colonies was apparently genetically controlled since cells positive for inhibition by this method were either positive or negative in subsequent testing, whereas negative cells were generally negative in subsequent testing. It is obvious that this phenomenon needs further study before any concise conclusions can be made.

Recently, the relationship between lysogeny and pathogenicity in vibrios was studied by Parker, Richardson, and Romig (1970). Of 27 strains induced with mitomycin C, one strain produced typical bacteriophages, and 12 produced material which could be indentified as bacteriophage tails. One of these 12 strains was used by Jayawardene and Farkas-Himsley as a vibriocin producer. In 25 of 27 strains, a correlation was shown between the production of bacteriophage related material and pathogenicity. Male and female (P+ and P-) cells, following induction, produced these bacteriophage-associated tail structures. If these structures are bacteriocins, Bhaskaran's findings (Bhaskaran, 1964) that vibriocin was always associated with donor or P+ cells may have arisen from the observation of a different type of bacteriocin or a completely different bacteriocidal

principle. This evidence, apart from its interesting and important clinical significance, also suggests the relationship of vibriocins to defective bacteriophage.

Host range studies with phage and the isolated bacteriocin showed several strains of V. cholerae hosts immune to all six temperate phage used in the test. This implies the presence of an active immune repressor substance whose production is directed by the presence of a prophage. Fifteen of 67 hosts showed this immunity. Five of these were known lysogenic el tor strains from which phage could easily be isolated. Two strains of non-agglutinable (NAG) vibrios were included which were not known to be lysogenized, as were eight strains of classical cholera vibrios. Upon mitomycin C induction, phage were isolated from both strains of NAG vibrios, never before reported as lysogenic, and phage were also isolated from six of seven classical cholera strains showing the immunity. Bacteriocin activity was suspected in four of the classical cholera lysates. The HVc 239 lysate, however, gave considerably stronger inhibition than others and therefore was selected as the vibriocin producing strain for further experiments. In these procedures, mitomycin C was shown to induce phage not spontaneously released from host cells under normal

conditions, and also to induce phage-related materials associated with defective phage.

The sensitivity of the V. cholerae el tor hosts to the vibriocin suggests that these agents could possibly be developed for use in cholera vibrio typing schemes. However, the action of the vibriocin on the classical cholera hosts as well prevents the development of such a scheme at this time.

The structural evidence of defectiveness and serological data relating cholera phages to vibriocin are presented in this thesis. These relationships, combined with the facts that high molecular weight bacteriocins are insensitive to trypsin, are induced by effective inducers of prophage, and that their action on sensitive cells can be duplicated by phage ghosts point to the relatedness of the vibriocins to defective bacteriophage.

SUMMARY

Defective phages and an isolated vibriocin of Vibrio cholerae were studied. Electron microscopic studies revealed an aberrant structured virulent phage lacking typical tail structures. Structures resembling those reported associate with other vibriocinogenic strains of cholera vibrios were found in lysates which also contained complete phage virions and defective virions with polycore and polysheath structures. Serological relatedness was found between an isolated particle with vibriocin activity and several temperate choleraphages. Upon mitomycin C induction, phage were isolated from several cholera vibrios, and two non-agglutinable vibrios.

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