Role of the bacteriophage encoding cholera toxin (CTXΦ) as a component of the ecosystem for toxigenic *V. cholerae*

M. Phil. Thesis

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A THESIS SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIRMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY

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Declaration

This thesis has been submitted to the University of Dhaka in partial fulfillment of the requirements for the degree of Master of Philosophy (M. Phil.) in Biochemistry. No part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institutes of learning.

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CONTENTS

ABSTRACT

Cholera is severe watery diarrhoea caused by toxigenic strains of the gram-negative bacterium *Vibrio cholerae*. Epidemiological surveillance of cholera and molecular analysis of strains collected during epidemics have demonstrated clonal diversity among epidemic strains, and frequent emergence of new clones of toxigenic *V. cholerae*. The genetic basis and mechanisms involved in the origination of the new epidemic clones have not been adequately explained. Profuse watery diarrhea which is characteristic of cholera, is caused by an enterotoxin, cholera toxin (CT) produced by *V. cholerae* when it colonizes the small intestine. The genes for CT are carried by a filamentous bacteriophage (CTXΦ) which exists as a prophage in the chromosome of toxigenic *Vibrio cholerae*. In the present study factors involved in the induction and propagation of CTXΦ was studied to understand the mechanism of emergence of new toxigenic strains of *V. cholerae*. Of 141 clinical and environmental strains of *V. cholerae* O1 or O139 analyzed to study the induction of CTXΦ, 4.25% (6 of 141) of the isolated *V. cholerae* strains spontaneously produced a detectable level of extracellular CTXΦ particles in the culture supernatants whereas another 34.04% (48 of 141) produced CTXΦ particles when induced with mitomycin-C. Analysis of 146 nontoxigenic strains of *V. cholerae* for susceptibility to CTXΦ, using a genetically marked derivative of the phage, CTX-KmΦ showed that all strains which were positive for the phage receptor toxin coregulated pilus (TCP) were infected by the phage either *in vitro* or inside the intestines of infant mice. The phage genome integrated into the chromosome of infected *V. cholerae* cells forming lysogens. Comparative analysis of rRNA gene restriction patterns revealed that the lysogens derived from nontoxigenic progenitors were either closely related or distinctly different from previously described clones of toxigenic *V. cholerae*. To understand the nature of possible environmental factors associated with the propagation of CTXΦ, the effect of temperature, pH, salinity and exposure to direct sunlight on the induction of CTX prophage, and the transmission of the phage to potential recipient strains were studied. Exposure of toxigenic *V. cholerae* strains to direct sunlight (mean intensity of 42,000 Lux for 30 min) resulted in ~10,000 fold increases in the

titers of cell free CTXΦ. Variation in temperature, pH or salinity of the culture did not have a substantial effect on the induction of the prophage, but these factors influenced the stability of CTXΦ particles. Exposure of mixed cultures of CTXΦ lysogens and potential recipient strains to sunlight significantly increased both the in vitro and in vivo (in rabbit ileal loops) transduction of recipient strains by CTXΦ. The resulting transductants produced biologically active CT both in vitro and in rabbit ileal loops. This finding suggests a possible mechanism explaining the origination of toxigenic *V. cholerae* strains from nontoxigenic progenitors. The results of this study have been discussed on the possible role of CTXΦ as a component of the ecosystem, which supports frequent emergence of new epidemic strains of *V. cholerae* in developing countries.

ABBREVIATIONS

Ace	: Accessory cholera enterotoxin
ace	: Accessory Cholera Enterotoxin Gene
ACF	: Accessory colonization factor
acfD	: Gene for AcfD
ADPR	: ADP-ribose moiety
attRS	: 18 bp sequence, attachment site on either side of CTX element and RS1, also present in non toxigenic strains
ctxA	: Gene for A subunit Cohlera Toxin
ctxAB	: Gene for A and B subunit of Cholera Toxin
cep	: Core-encoded pilin Gene
Cep	: Core-encoded pilin
CT	: Cholera Toxin
CTXΦ	: Filamentous Bacteriophage that encodes Cholera Toxin
CTX element	: Chromosomally integrated copy of CTXΦ
CFTR	: Cystic Fibrosis Transmembrane Regulator
FV	: Filamentous bacterial viruses
Ff	: F pilus specific filamentous phage
If	: I pilus specific filamentous phage
int	: Integrase gene
MFRHA	: Mannose-fucose-resistant cell associated hemagglutinin
MSHA	: mannose sensitive hemagglutinin
OMPs	: Outer membrane proteins
orfU	: Gene for Open reading frame of unknown function
PEG	: Polyethylene Glycol
RFLP	: Restriction Fragment Length Polymorphism
RF	: Replicative form of the bacteriophage
RS1	: 2.7 kb Repetitive Sequence 1
RS2	: 2.4 kb Repetitive Sequence 2

SDS	: Sodium Dodecyle Sulfate
SXT	: Sulfamethoxazole, trimethoprim
TCP	: Toxin-coregulated pilus
ToxR	: A 32 kDa transmembrane protein, master regulator of virulence
toxR	: Gene for ToxR protein
ToxS	: 19 kDa transmembrane protein that stabilize ToxR monomers
ToxT	: Another Regulatory factor, a 32 kDa protein
toxT	: Gene for ToxT
TcpA	: A subunit of TCP
tcpA	: Gene for TcpA
tcpH	: Gene for TcpH
tcpI	: Gene for TcpI protein
VNC	: Viable but non-culturable
Zot	: Zonula occludens toxin
zot,	: Zonula occludens toxin Gene

Chapter 1

INTRODUCTION

1. Introduction

Cholera is one of the well-known enteric infections characterized by watery diarrhoea caused by *Vibrio cholerae*, which can occur both sporadically and as large outbreaks. The disease is an acute dehydrating diarrhea caused principally by the potent enterotoxin, cholera toxin, produced by O1 and O139 serogroups during pathogenesis. Epidemics of cholera cause worldwide deaths estimated at 120,000 and many more cases each year, of which the vast majority occurs in children (116). Cholera is endemic in Southern Asia and parts of Africa and Latin America, where outbreaks occur widely and are particularly associated with poverty and poor sanitation. In areas of endemic infection cholera outbreaks occur in a seasonal pattern. In Bangladesh also, cholera is an endemic disease, and seasonal outbreaks occur almost every year. Epidemiological surveillance of cholera and molecular analysis of *V. cholerae* strains isolated from different outbreaks have revealed clonal diversity among toxigenic *Vibrio cholerae* and frequent emergence of new epidemic clones (26-27). Many aspects of the disease and the pathogen remain unknown, particularly the mechanism of emergence of new epidemic strains, and the ecology of toxigenic *V. cholerae*, which maintains the seasonal pattern of outbreaks in areas of endemic infection.

1.1 NOMENCLATURE OF *VIBRIO CHOLERAE*

A wide variety of Gram-negative, polar-flagellated rod-shaped bacilli were classified as belonging to the genus *Vibrio* in early classifications. Some criteria had been established during the mid-sixties for the taxonomy of the genus *Vibrio.* International Sub-committee on Taxonomy of Vibrios recommended a provisional definition according to which the majority of species previously classified as *Vibrio* are excluded from the genus. Taxonomic studies on the related organisms have indicated a close relationship between the three genera, *Vibrio, Aeromonas*, and *Plesiomonas*. On the basis of biochemical characteristics, it is possible to differentiate members of the genus *Vibrio* from those of allied genera (115). According to the currant classification genus *Vibrio* contains several bacterial species, of which *V. cholerae*, *V. parahaemolyticus* and *V.*

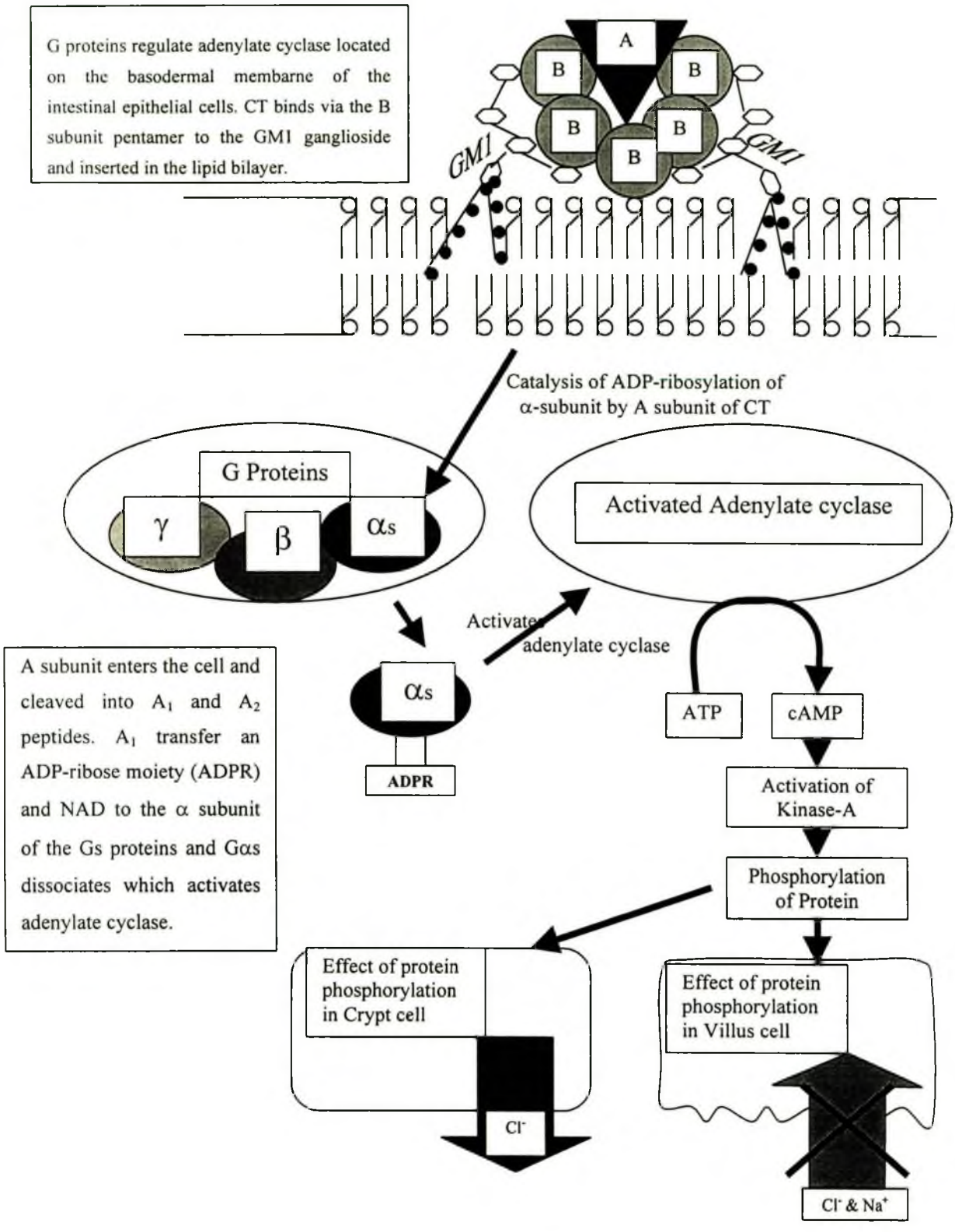
vulnificus are the most important pathogens of humans. Whereas *V. parahaemolyticus* is an important cause of diarrhoeal illness, *V. vulnificus* infections can range from self-limiting gastroenteritis and wound infections to severe necrotizing infections of soft tissues and fatal septicemia (114). *V. mimicus* has also been implicated in diarrheal disease, and some strains of *V. mimicus* may produce cholera enterotoxin or an ST-like toxin (71). Many unknown vibrios have also been isolated from seawater and sea-fish and may sometimes be confused with vibrios that infect humans. At least 155 known groups of *V. cholerae* are identified on the basis of its somatic antigens (O-antigens) which are called serovars or serogroups (7, 90-92). The serogroup O: 1 has two major serotypes, Ogawa and Inaba; a Hikojima serotype has been reported rarely. These serotypes can be further distinguished into two biotypes, classical and El Tor based on some biochemical properties, and susceptibility to bacteriophages (84).

All the 154 known serogroups of non-01 vibrios (92) possess biochemical and morphological characteristics very similar to those of the cholera vibrio but are non-agglutinable with polyvalent O: 1 antiserum. Such vibrios are identified by agglutination with the antisera raised against their own. Non-O1 serogroups of *V. cholerae* had been mostly associated with sporadic cases of diarrhea and extraintestinal infection (48). In 1993, a large cholera-like outbreak in Bangladesh and India was caused by a *V. cholerae* non-O1 strain (1). This organism did not belong to any of the 138 'O' serogroups but belonged to a new serogroup, which was later designated as O139 (91). Since then *V cholerae* O139 has been persisting as a second etiologic agent of cholera. Although the two serogroups, O1 and O139, have been found to be associated with epidemics of the disease, there are also strains of these serogroups which do not produce CT, and are not involved in epidemics.

1.2 PATHOGENESIS OF CHOLERA

Cholera ensues from the oral ingestion of *V. cholerae*. Although the elaboration of cholera toxin is directly responsible for the manifestation of diarrhoea, cholera pathogenesis relies on a number of elements acting synergistically to promote virulence.

Figure 1.2: Model of action of cholera toxin



After surviving passage through the acidic environment of the stomach, cholera vibrios synthesize different factors to resist the cleansing mechanisms in the bowel and proliferate on the intestinal epithelium. Colonization of brush borders in the small intestine is mediated by a pilus colonization factor, known as toxin-coregulated pilus (TCP), and this constitutes a crucial component of the infection strategy (103).

While the organism is in the duodenum and jejunum in alkaline environment furnished with nutrient and bile salt, multiplication occurs with a doubling time of 20 to 30 minutes. Actively motile vibrios penetrate the mucous layer and attach to the brush border of the intestinal epithelium where after successful colonization they secret a potent enterotoxin. The toxin is a protein of 84000 daltons with five B subunits and one A subunit (Figure 1.2). The toxin starts to exert its effect by irreversibly binding to the cell surface receptor GM1. The toxic moiety the A subunit is linked to the B aggregate and gains entry once binding has occurred. The toxin activates adenylate cyclase by causing ADP-ribosylation of the Gαs-protein, which stimulates the cycle. Elevated cAMP level in turn activates protine kinase A, which opens the luminal CFTR Cl^- channel and inhibit the Na^+/H^+ exchanger by protein phosphorilation. The net result is voluminous, life threatening intestinal electrolyte and fluid secretion in cholera patient. Figure 1.2 shows the model for the mechanism of action for cholera toxin (54).

1.3 EPIDEMIOLOGY OF CHOLERA

Waterborne disease Cholera and the close association of *V. cholerae* with surface waters and the population interacting with the contaminated waters suggest the importance of the study of water ecology. Clonal diversity of epidemic strains, a high degree of clustering of cases by location and season, highest rates of infection in children 1 to 5 years of age in endemic areas, antibiotic resistance patterns that frequently change from year to year, and protection against the disease being afforded by improved

sanitation/hygiene and preexisting immunity are the main epidemiological features of cholera.

1.3.1. CHOLERA PANDEMICS**:** Cholera is thought to be a disease of ancient history, with clear written description dating before 500BC. Generally it is accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 (78). Six pandemics arose from the Indian subcontinent, usually from the Ganges delta and spread to reach other continents affecting many countries and extending over many years. Only the seventh pandemic was originated in Indonesia. The sixth pandemic and presumably the fifth pandemic were caused by *V. cholerae* O1 of the classical biotype. Available information on these and earlier pandemics have been reviewed by Kaper *et.al.* (53). The present global spread (seventh pandemic) has been due to the El Tor biotype first recognize in 1911 at the El Tor quarantine station in Persian Gulf. The seventh pandemic is the most extensive of the pandemics in geographic spread and in duration, and the causative agent is *V. cholerae* O1 of the El Tor biotype. The pandemic, which began in 1961 from the island of Sulawesi in Indonesia spread to other islands thereby affecting the entire Southeast Asian archipelago by the end of 1962 (50). During 1963 to 1969, the pandemic spread to the Asian mainland and affected Malaysia, Thailand, Burma, Cambodia, Vietnam, India, Bangladesh and Pakistan. Soon after El Tor cholera reached Pakistan, Afghanistan, Iran, Iraq, and nearby republics within the Soviet Union experienced outbreak of cholera. By 1970, El Tor cholera invaded the Arabian Peninsula, Syria, and Jordan, and a limited outbreak occurred in Israel (39). The seventh pandemic reached the sub-Saharan West Africa in the early seventies and caused explosive outbreaks resulting in more than 400,000 cases with a high case fatality, due mainly to a lack of background immunity in population, and inadequacies in the health care infrastructure (39). In this epidemic, cholera spread along waterways along the coast and into the interior along rivers, and further disseminated into the interior of the Sahelian states by land travel fostered by nomadic tribes. Of the 36 countries that reported cholera in 1970, 28 were newly affected countries and 16 were in Africa (53).

In January 1991, epidemic that began in Peru was the explosive spread of seventh pandemic that reached South America in the form of an epidemic. It was the reemergence of cholera at this continent after more than a century (62,76,82). The epidemic was also spread to the neighboring Equador, and then in Colombia. Low socioeconomic populations lacking proper drinking water and sanitation facilities were most affected in each of these countries (76). A small outbreak occurred in Santiago the capital of Chile (62), and cholera began to travel along the pacific coast of South America to progressively enter more countries in South and Central America by April 1991. According to the estimation of the Pan American Health Organization there were 750,000 cases of cholera in the Americas with 6,500 deaths during 1991-1992 (76). In July 1994, one of the worst cholera outbreaks occurred in Goma, Eastern Zaire (96). Conflicts between tribes in neighboring Rwanda had displaced nearly a million people to Zaire and were sheltered in refugee camps. During a three week period, outbreak of cholera in the poverty- stricken camps led to the death of an estimated 12,000 Rawandan refugees (96). The seventh pandemic is ongoing and it continues to cause seasonal outbreaks in many developing countries, especially in Bangladesh and India.

Epidemic cholera was reported in different places in India and Bangladesh in the late 1992 and in 1993 (12, 81). The causative agent was a *V. cholerae* non-O1 strain with the typical clinical syndrome of cholera, which was later serogrouped as O139 and was recognized as the second causative agent of cholera after the O1 serogroup of *V. cholerae*. Since then the O139 *V. cholerae* has spread throughout India, and outbreaks or cases have been reported in Pakistan, Nepal, China, Thailand, Kazakhastan, Afghanistan, and Malaysia (14,102). Imported cases have been reported from the United Kingdom and the United States (80). Outbreak of cholera due to this new serogroup can be considered as the initiation of eighth pandemic that remains to affect more countries.

1.3.2. EPIDEMIOLOGY OF CHOLERA IN BANGLADESH**:** International Centre for Diarrhoeal Disease Research (ICDDR, B) and the former Pakistan Seato Cholera Research Laboratory has been carried out Systematic surveillance of cholera in Bangladesh during a period of over 35 years (65,67,86,94,95). Epidemic outbreaks of

cholera in Bangladesh usually occur twice during a year, with the highest number of cases just after the monsoon during September to December that has been supported by a number of studies (65,67,86,94,95). Studies also found somewhat smaller peaks of cholera cases during the spring between March and May. Classical Inaba serotype was the causative agent in more than 90% of cholera cases in Bangladesh until 1970, and by 1972, 85% of all cases were due to classical Ogawa serotype (58). In the middle of 1973, the El Tor biotype of *V. cholerae* O1 appeared in Bangladesh, and since then this biotype had completely replaced the classical biotype. The classical biotype reemerged in Bangladesh in 1982 (58, 85). However, it coexisted with the El Tor vibrios until 1992. *V. cholerae* O1 was the most frequently (40%) isolated enteropathogens during the epidemics according to the data obtained from interventions of diarrhea epidemics in nearly 400 rural sub-districts by ICDDR, B medical teams between 1985 and 1991 (95). The 1991 epidemic alone was estimated to have produced between 210,000 and 235,000 cases and over 8,000 deaths (95).

Emergence *V. cholerae* O139 occurred during 1992 and 1993 causing explosive epidemic throughout Bangladesh, India and neighboring countries (12,13,81,102). New strain totally displaced the existing *V. cholerae* O1 strains, including both classical and El Tor biotypes that co-existed only in Bangladesh, in the beginning. During 1994 and till the middle of 1995, in most northern and central areas of Bangladesh, including the capital city Dhaka, a new clone of *V. cholerae* O1 of the El Tor biotype replaced the O139 vibrios. Whereas in the southern coastal regions the O139 vibrios continued to exist (27, 97). During the second half of 1995 and in 1996, nearly four years after the initial detection of O139 vibrios, cases due to both *V. cholerae* O1 and O139 were detected in various regions of Bangladesh. Recent surveillance conducted by the epidemic control preparedness programme of ICDDR, B revealed the coexistence of *V. cholerae* O1 and O139 in Dhaka as well as in many rural districts of the northern, central and southern regions of the country that were affected by outbreaks of cholera between June and December of 1996 (28). Cholera surveillance in Bangladesh has shown that until 1992 *V. cholerae* O1 belonging to both the biotypes caused regular epidemics and since then both

V. cholerae O1 and O139 continue to be a significant cause of infection and morbidity, although the frequency of infection varies from year to year in different regions of the country (2,95, 99).

1.3.3. ANTIBIOTIC RESISTANCE**:** An outbreak of cholera occurred due to multiple drug resistant *V. cholerae* O1 in Matlab, a rural sub-district of Bangladesh, in 1979 (34, 35). After screening of isolates from the outbreak it was observed that 16.7% of the isolates were found to be resistant to five antibiotics: tetracycline, ampicillin, kanamycin, trimethoprim- sulfamethoxazole, and streptomycin, and another 10% of the isolates were resistant to any four of these antibiotics including tetracycline. A conjugative antibiotic resistance plasmid was identified in these isolates and the plasmid was transferable to an *E. coli* K-12 recipient. Epidemiological assessment of the outbreak suggested that the outbreak began from the introduction into the area of a single multiple drug resistant strain of *V. cholerae* O1 (35). By 1986, the drug resistance pattern changed, and screening of *V. cholerae* O1 isolated from cholera patients in January 1986 in Dhaka showed that none of these isolates was resistant to tetracycline, streptomycin, chloramphenicol, amoxicillin or nalidixic acid (74). During 1988 and 1989, nearly all classical *V. cholerae* isolated in Bangladesh were resistant to tetracycline, whereas strains belonging to the El Tor biotype were sensitive to the drug (93). Reemergence of tetracycline resistant El Tor strains was observed after almost a decade, during the 1991 epidemic in Bangladesh (95). In addition to other antibiotics 70% of strains isolated were resistant to tetracycline. The O139 serogroup of *V. cholerae*, which emerged during 1992-1993, were sensitive to tetracycline (12). Although this new serogroup of toxigenic *V. cholerae* has shown a trend of increased resistance to trimethoprim-sulfamethoxazole, it has remained more susceptible to ampicillin and tetracycline than the O1 serogroup of *V. cholerae*. Waldor and coworkers (111) reported the presence of a 62-kb self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *V. cholerae* O139. Recent isolates of *V. cholerae* O139 are mostly sensitive to SXT. However, considering the rapidly changing pattern of antibiotic resistance observed among toxigenic *V. cholerae* isolated

from different outbreaks, it appears that there is substantial mobility in genetic elements encoding antibiotic resistance in *V. cholerae*.

1.3.4. MOLECULAR EPIDEMIOLOGY**:** Recent developments in DNA analysis techniques have introduced several new typing methods and have enabled to study the epidemiology of *V. cholerae* on a larger global perspective, which was limited before the seventies for the lack of suitable typing systems. (11, 17, 22, 23, 25, 26, 51, 106, 107). These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the *ctxAB* genes and their flanking DNA sequences, which are part of a larger genetic element (CTX element), indicated that the U.S. Gulf coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh pandemic isolates (31). RFLPs in conserved rRNA genes have also been used to differentiate *V. cholerae* strains into different ribotypes. Analysis of isolates from the Latin American epidemic in 1991 showed that these were related to the seventh pandemic isolates from other parts of the world and that Latin American cholera epidemic was an extension of the seventh pandemic (22, 106, 107). Analysis of toxigenic El Tor strains by multilocus enzyme electrophoresis has also been used to group the El Tor strains into major clonal groups. The clones seem to reflect broad geographical and epidemiological associations. The clonal diversity and epidemiological associations of toxigenic *V. cholerae* have been reviewed by Wachsmuth and coworkers (108). Comparative molecular analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from the El Tor strains by genetic changes in the serotype specific gene clusters (25, 108).

Clonal diversity among strains isolated during different epidemics was revealed by molecular analysis of epidemic isolates of *V. cholerae* between 1961 and 1996 in Bangladesh (26-28). Transient appearance and disappearance of more than 6 ribotypes among classical vibrios, at least 5 ribotypes of El Tor vibrios and 3 different ribotypes of *V. cholerae* O139 was demonstrated by these studies. Different ribotypes often showed different CTX genotypes resulting from differences in copy number of the CTX element

and variations in the integration site of CTX element in the chromosome (27, 28). These studies indicated that there had been a continual emergence of new clones of toxigenic *V. cholerae* that replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population.

1.4 VIRULENCE ASSOCIATED GENES IN *V. CHOLERAE*

The complex process of cholera pathogenesis involves a number of genes encoding virulence factors which aid the pathogen in its passage to reach the epithelium of the small intestine, colonize the epithelium, and produce cholera toxin (CT) that disrupts ion transport by intestinal epithelial cells. The major virulence genes in *V. cholerae*, required for pathogenesis exist in clusters. These include the CTX genetic element, which is the genome of a lysogenic bacteriophage designated CTXΦ (112) that carries the genes encoding CT, and the TCP pathogenicity island which carries genes for a pilus colonization factor known as toxin coregulated pilus (TCP)(42). TCP pathogenicity island includes a groups of virulence genes, a regulator of virulence genes, a transposase gene, specific (*att*-like) attachment sites flanking each end of the island, and an integrase with homology to a phage integrase gene. The structural feature of this pathogenicity island suggests that it have also been derived from a bacteriophage (55, 60). As the major virulence gene clusters in *V. cholerae* appear to have a phage origin, it can be speculate that the horizontal transfer of gene clusters may be a possible mechanism for the origination of new pathogenic clones of *V. cholerae*.

Since colonization is a prerequisite to establishing a productive infection by *V. cholerae* the existence and role of possible other factors responsible for colonization has also been investigated. This includes the mannose-fucose-resistant cell associated hemagglutinin (MFRHA) the mannose sensitive hemagglutinin (MSHA), and some outer membrane proteins (OMPs) of *V. cholerae* (31,38,49, 87). Although some of these factors including MFRHA, MSHA and OMPs are suspected to have a role in enhancing adhesion and colonization when tested in animal models, the exact role of these factors in the virulence of *V. cholerae* in humans is still uncertain. Studies to date have shown that

the major virulence genes of *V. cholerae* required for pathogenesis are the genes involved in the production of TCP and CT.

1.4.1. THE TCP PATHOGENICITY ISLAND**:** Early works established that the genes encoding TCP were clustered, and were present in clinical isolates of O1 El Tor and classical vibrios but not in environmental isolates of *V. cholerae* O1, with the exception of a few strains from the Gulf Coast of the United States (104). Expression of CT and TCP are co-regulated by the ToxR regulatory system, which includes the ToxT protein (20). The genes encoding ToxT and TCP are located in the same choromosomal region (9) together with other ToxR-regulated genes including those for a potential accessory colonization factor (ACF) (21). Molecular analysis has revealed that although the major subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly require the products of a number of other genes located on the chromosome adjacent to the *tcpA* gene, and these constitute the *tcp* gene cluster (75). At least 15 open reading frames are found in the *tcp* cluster, which is located, immediately downstream of the *tagD* gene. The *tcpH* and *tcpI* genes are two ToxR-regulated genes that influence TcpA synthesis. Inactivation of *tcpH* results in decreased pilin synthesis, whereas inactivation of *tcpI* leads to increased synthesis of TcpA. It has been suggested that regulators such as TcpI that acts downstream of ToxR and ToxT may function to fine tune the expression of the TCP virulence determinant throughout the pathogenic cycle of *V. cholerae* (40). Immediately adjacent and downstream to the *tcp* cluster the *acf* gene cluster is located. The exact nature of the colonization factor is not clear, but *acfD* is one of the four open reading frames (*acfABCD*) encodes a lipoprotein. Further analysis revealed the presence of a putative integrase gene (*int*) and a putative *att*-like 20-bp attachment site adjacent to the TCP/ACF gene cluster (60). The entire region of nearly 40-kb flanked by the *att*-like sequences and including the TCP/ACF gene clusters, the integrase and a transposase gene appear to constitute a pathogenicity island. A recent report suggested that the TCP pathogenicity island is the genome of a bacteriophage (55).

1.4.2. THE CTX GENETIC ELEMENT**:** One or more copies of CT genes (*ctxAB*) are carried by toxigenic *Vibrio cholerae* strains. A and B subunits of CT are encoded by two separate but overlapping open reading frames. A putative toxin, known as zonula occludens toxin (Zot) is also produces by *V. cholerae,* which increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens (6). A 44.8-kDa polypeptide is potentially encoded by the 1.3 kb open reading frame of the *zot* gene that is located immediately upstream of the *ctxA* gene (6). Accessory cholera enterotoxin (Ace), a third toxin that has been described is capable of inducing fluid accumulation in rabbit ligated ileal loops (105).

The genes encoding *ace*, *zot, ctxAB*, a core-encoded pilin (*cep*), and an open reading frame of unknown function (*orfU*) are located on a 4.5 kb "core region", flanked by one or more copies of a repetitive sequence called RS1 (77). Together these DNA units comprise the CTX element, which had originally been perceived as a transposon-like genetic element. It has recently been discovered that the CTX genetic element is the genome of a lysogenic filamentous bacteriophage designated CTXΦ, and that genes in the core region of the CTX element particularly *zot* and *orfU* are crucial for the morphogenesis of the phage (112).

1.4.3. CHOLERA TOXIN CONVERTING BACTERIOPHAGE **(CTXΦ):** Toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles under appropriate condition (112). The phage can be propagated in recipient *V. cholerae* strains in which the CTXΦ genome either integrates chromosomally at a specific site forming stable lysogens or is maintained extrachromosomally as a replicative form (RF) of the phage DNA (112). Cultures of *V. cholerae* harboring the RF of CTXΦ produce high titers of the phage in their supernatants. The bacteriophage has been shown to use the TCP as a receptor, and hence expression of TCP by the bacterium is a prerequisite for its susceptibility to the phage.

The CTXΦ genome has two regions, the "core" and the RS2 (113). Genes with related functions are clustered in the genome of CTXΦ in a way similar to those of other

filamentous phages. Analysis of phage morphogenesis revealed that most of the genes of the "core region" are essential for the formation of the CTXΦ particles and hence for its propagation as an infectious phage. The open reading frames in RS2 were designated as *rstR, rstA2* and *rstB2*, and these were found to encode products required for the integration, replication and regulatory functions of CTXΦ (113). The deletion of a portion of the genes encoding CT by marker exchange, however, did not affect the morphogenesis of the bacteriophage. It appears that the *ctxAB* genes do not participate directly in the formation of phage particles, but these genes are important for the phage to provide a survival advantage to its host bacteria in the gastrointestinal environment (113).

1.5. FILAMENTOUS BACTERIOPHAGES

Filamentous bacterial viruses (FV) are long deoxyribonucleoproteins aboout 5.5 nm in diameter. Their deoxyribonucleic acid (DNA) is a single-stranded ring containing fewer than 10,000 nucleotides. There are no free ends on infectious virus DNA that is accessible to exonuclease (66). Single-stranded DNA used as a template for DNA polymerase forms a double-stranded form, which is seen to be a ring in electron micrographs (66). Their hosts are gram-negative bacteria. They adsorb at the tips of the threadlike bacterial appendiges known as the pili. At least two species of these viruses can be distinguished, corresponding to the two known types of sex pili.The replication cycle of the filamentous DNA phages is similar to that of the isometric phages. Infection is initiated by adsorption of the end of a virion to the terminus of a pilus. Tow morphologically and serologically distinct types of sex pili are known: F pili, such as those produced by the bacteria harboring the sex factor F; and the I pili, such as those produced by the bacteria harboring the colicin factor I. The molecular steps involved in infection of *E. coli* F pilus specific filamentous phages (Ff phages) such as f1 and M13 have been well characterized. The process begins when a domain of minor coat protein (pIII) located on one end of the phage particle binds to the tip of the conjugative F pilus of *E. coli* (47). This interection between pIII and F is thought to result in pilus retraction, which draws the phage through the bacterium's outer membrane. Subsequent phage

translocation through the periplasmic space requires the t*olQRA* gene products (41,101). Recent studies have demonstrated that peroplasmic part of the TolA binds to pIII and thereby servs as the coreceptor for phage enrty into the bacterium. Filamentous phages that infect hosts other than *E. coli*, little is known concerning the molecular aspects of phage enrty. DNA, and virions are extruded through the cell envelope without loss of cell viability. Except for a few enveloped phages, these are the only phages whose egress does not require lysis (41,101).

Partly because of this nonlytic mode of release, it is possible to grow high-titer cultures of the virus. A raw titer of 5 x 10^{12} plaque-forming units/ml is not uncommon. The growth curve of FV shows a rapid increase of virus after a latent period, as for other bacterial viruses. But virus titer continues to increase after the initial rise, at a constant rate of 200 to 2,000 viruses per bacterium per bacterial doubling (depending on conditions), until after the bacteria enter the stationaly phase (66).

Purified concentrated preparations of FV become turbid after standing for a few days because of the formation of birefringent paracrystals (66). FV is resistant to many agents which inactivate other viruses, as indicated by the fact that in most preparations over half of the physical particles are infectious (66). Plaque-forming ability is resistant to deoxyribonuclease and ribonuclease and is moderately resistant to Pronase and trypsin; however, it is sensitive to the proteolytic enzymes Nagarse, ficin, subtilisin, and papain (66).

Plaque-forming ability is not sensitive to freezing and thawing or to heating below about 80°C. The virion is relatively sensitive to shear or to ultrasonic treatment, because of its morphology. The virion is sensitive to chloroform (66). Thus, it is preferable to use ether instead of chloroform to inactivate bacteria in a mixture of bacteria and FV. The virion is sensitive to SDS, although the degree of sensitivity depends on the strain; it is insensitive to sodium deoxycholate and Tween 80 below 1% detergent concentration (66). The virion is stable between pH 2.5 and pH 10.5. Bellow pH 11.9, the rate of inactivation is much more dependent on pH. Purified viral protein and viral DNA can be re aggregated to irregular DNA-protein complexes. Reconstitution to infectious virions is

possible at a low level (10-7of he DNA molecules) if 3% starter protein is left on the DNA (66).

Infection of a filamentous phage to the host is a pool of two independent processes: adsorption and penetration. Ff adsorbs specifically to the F pili and If adsorbs specifically to the I pili. There are only a few of these pili per bacterium corresponding to the two or three adsorption sites. The adsorption site apparently can be used only once, since infected cells can not be superinfected, although they can be co-infected. The adsorption rate is maximal at 0.1 M NaCl and similar ionic strength of several other salts. Adsorption rate is same between pH 5.0 and pH 8.0. There is no special requirement for divalent cations. Adsorption rate increases between 0^{o} and 40^{o}C and then drops off sharply at higher temperature. A temperature of 0^{o}C, 0.01 M KCN, and 0.01 M sodium azide does not prevent adsorption; thus it requires no cell metabolism. Adsorption is irreversible and is prevented by 0.001 M Zn^{++}. On the other hand, penetration is an energy-requiring step; it is prevented by a temperature of 0^{o}, 0.01 M KCN, and 0.01 M sodium azide. There is a 20 fold increase in the rate of penetration between 15^{o} and 45^{o}C (66).

The filamentous phages (especially M13) are popular as cloning vectors because of their high yields, the absence of a packaging limit, and the single-strandedness of the product. Cloning is generally accomplished through cutting and ligation of RF DNA, followed by transfection. Our present consideration is about the typicality of one the recent discovery in the history filamentous bacterial virus the CTXΦ that encodes cholera toxin.

1.6. ECOLOGY OF *V. CHOLERAE*

V. cholerae has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (15). Although, *V. cholerae* is part of the normal, free-living bacterial flora in riverine and estuarine areas, non-O1 non-O139 strains are more commonly isolated from the environment than are O1 or O139 strains. Moreover, outside of epidemic areas and away from areas that may have been contaminated by

cholera patients, environmental isolates of *V. cholerae* O1 have been found to be mostly CT negative. The major pathogenic genes in *V. cholerae* are clustered in several regions of the *V. cholerae* chromosome and the structure of these pathogenic gene clusters indicates that these are capable of being propagated horizontally (55,60, 77,112). This is suggestive of the possibility that environmental strains of *V. cholerae* may develop the ability to adapt to the intestinal environment through acquisition of the virulence genes. In view of the available information on the epidemiology of cholera, the lysogenic conversion by a bacteriophage encoding cholera toxin, and the survival and enrichment of *V. cholerae* under in-vivo and in-vitro conditions, it is apparent that the ecosystem for *V. cholerae* should consists of a number of components. These include (a) the bacterium, (b) the aquatic environment, (c) CTXΦ and other unidentified genetic elements involved in the transfer of virulence genes, and (d) the intestinal environment of the host population.

1.6.1. ENVIRONMENTAL SURVIVAL AND PERSISTENCE OF *V. CHOLERAE*: The physicochemical conditions for the survival of *V. cholerae* O1 suggests the possibility of the survival of this organism in an estuarine environment and other brackish waters (16,62). However, the nature of the survival and persistence of toxigenic *V. cholerae* O1 or O139 in aquatic ecosystem and the factors involved in the conservation of the CTX element (the lysogenic form of CTXΦ) and other pathogenic genes in the aquatic environment is not clear. The survival may be dependent on several factors, such as occurrence of particular physico-chemical conditions, specific association of the bacteria with aquatic plants or animals, and/or the existence of specific ecological association involving several components of the aquatic environment. Vibrios are assumed to be converted to a viable but non-culturable (VNC) form under the stress conditions that cannot be recovered by standard culture techniques, and such VNC forms are able to produce infection and can revert into the culturable form (16). The public health and ecological importance of the possible survival forms such as VNC depends on whether these forms are re-convertible to live infectious bacteria. There is, however, very little evidence to conclusively establish that the possible non-culturable phenomenon is

reversible. Hence there is considerable scope to further investigate the role of postulated VNC forms of *V. cholerae* through carefully controlled studies.

It has also been suggested that during inter-epidemic periods toxigenic *V. cholerae* exists in an unexplained ecological association with aquatic organisms possibly in the VNC form until the next epidemic season, when environmental factors triggers the dormant bacteria to multiply and lead to cholera outbreaks (45, 46). However, differences in genetic or phenotypic properties have been often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics (72, 74). Analysis of rRNA gene restriction patterns of *V. cholerae* strains has also shown clonal diversity among epidemic strains (26-28, 88). These events have raised questions whether seasonal epidemics are caused by periodic appearance of the same strains of *V. cholerae* or due to a continual emergence of new toxigenic clones from nontoxigenic progenitors. Hence further studies are also required to understand more definitive roles of environmental factors in the emergence and reemergence of toxigenic *V. cholerae*.

1.6.2. ENRICHMENT OF TOXIGENIC *V. CHOLERAE* IN THE INTESTINAL ENVIRONMENT**:** Considerable understanding of the mechanism how CT causes diarrhea is available, but why *V. cholerae* should infect and elaborate the lethal toxin in the host system is yet to clarify. Whether the role of the toxin is to simply cause diarrhea and thus disseminate the organism to its next victim or could the toxin be providing a more crucial function for the enrichment and continued existence of the bacteria, is seems worth wondering. By means studies directed towards the development of attenuated *V. cholerae* mutants altered in toxin production for use as live oral cholera vaccines, the role of CT in the intestine was investigated. In 1971, Howard (44) reported the isolation of nontoxigenic mutants of the classical strain 569B by mutagenesis with nitrosoguanidine. The mutants were unable to induce a secretory response in the rabbit intestinal loop model, and did not survive or multiply in the intestinal environment. During 1974-75, Finkelstein et al (30) and Holmes et al (43) observed that the ability of various mutants to grow in the intestinal environment correlates with the ability of the mutants to induce a residual secretory response in the infant rabbit model. A variety of different toxin

deficient mutants of *V. cholerae* tested in rabbit and infant mouse models also suggested that the mutants showed enhanced killing and mechanical clearance in the intestinal environment compared to the toxigenic parental strain (4, 5). It seems possible that many of these early mutants were altered in TCP as well as CT expression, perhaps by carrying mutations in regulatory genes involved in the expression of CT and TCP. The selection for their reversion in vivo may have, therefore, been driven by the need to up regulate the expression of TCP more than that of CT. The characterization in vivo of site-specific *ctxAB* mutants constructed by in vitro recombinant DNA methods provided the most convincing evidence that the toxin is beneficial to growth in the intestinal environment. It was demonstrated that the *ctx* mutants colonized rabbit intestines about 10 to 100-fold less efficiently than the parental strain (68). The details of the mechanisms involved have been reviewed by Mekalanos (68).

Causation of cholera in humans is the natural process of enrichment of toxigenic *V. cholerae*, and is facilitated by the virulence factors such as CT and TCP. Studies so far suggest this phenomena and partly explains the benefit imparted to the pathogen during the disease in humans. In order to understand the general epidemiological behavior of *V. cholerae*, which includes seasonal pattern of epidemics, transient appearance and disappearance of different clones, and emergence of new epidemic clones, it is important to study the interactions among the bacteria, genetic elements mediating the transfer of virulence genes, the human host and possible environmental factors.

1.7. AIM OF THIS STUDY

The aim of this study was to investigate the role of the bacteriophage encoding Cholera Toxin (CTXΦ) as a component of the ecosystem for toxigenic *Vibrio cholerae*. This included examination of physicochemical factors involved in the induction and propagation of CTXΦ and to understand the mechanism of emergence of new toxigenic strains of *V. cholerae* from nontoxigenic environmental strains.

1.7.1. SPECIFIC OBJECTIVES:

i) To study the induction of the CTX prophage in toxigenic strains of *V. cholerae* O1 and O139.

ii) To study the effect of natural parameter such as temperature, pH variation, salinity, and sunlight on the induction of CTX phage.

iii) To study the stability of cell free phage particles in different environmental conditions.

iv) To study the susceptibility of the non-toxigenic strains to CTXΦ.

v) Study for the distribution of the virulence genes among the non-toxigenic *V. cholerae.*

vi) To study the propagation of CTXΦ from toxigenic *V. cholerae* to non-toxigenic progenitors.

vii) To characterize the toxigenic and non-toxigenic *V. cholerae* strains isolated from the environment and cholera patients by genetic fingerprinting,

Chapter 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 LABORATORY APPARATUS

All Petri dishes and plasticwares used in microbiological work were of sterile disposable type provided by either Sterilin or Gibco. Eppendorf tubes and micropipette tips were obtained from Sigma, and were sterilised by autoclaving at 121°C for 20 min. The gel kits used for agarose gel electrophoresis were obtained from BRL Life technologies. Unless otherwise stated, all mini scale centrifugation were performed in an Eppendorf microfuge and large-scale centrifugation were performed in a Sorvall RC-5C super speed centrifuge using the SS-34 rotor. The intensity of sunlight was measured using a digital light meter (VWR Scientific, South Plainfield, N.J.). Salinity of the water samples was measured by a Hand Refracto meter (ATAGO S-10, SALT: 0-10%, Japan). All other laboratory equipment was of standard types and is described in the relevant sections.

2.2 LABORATORY REAGENTS

The sources of any special reagent are stated in the relevant method section. All other standard laboratory chemicals were of Analar Grade and supplied by either Sigma or Gibco BRL Life Technologies.

2.3 STOCK SOLUTIONS

a). 1 M Tris-Cl: 121.1g tris base [tris (hydroxymethyl) aminomethane] was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl, and the final volume was made up to 1l with distilled water. The solution was sterilized by autoclaving and stored at 4^0C.

b). 3 M NaCl: 175.32g of NaCl was dissolved in distilled water to a final volume of 1L. The solution was autoclaved and stored at room temperature.

c). 0.5 M EDTA: 186.1g of $Na_2EDTA,2H_2O$ (disodium ethylene diamine tetraacetate) and 20g NaOH pellets were added to 800ml distilled water and dissolved by stirring on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and the final volume was made up to 1l with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

d). 3 M sodium acetate: 40.81g of $Na_2(CH_3COO)$, $3H_2o$ was dissolved in 80ml distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4^0C.

e). RNase A solution: Molecular biology grade pancreatic RNase (RNase A, Sigma) was dissolved at a concentration of 10mg/ml in 10mM Tris-Cl (pH 7.5), 15 mM NaCl. The soltion boiled for 10 minutes to destroy any contaminating DNase, and allowed to cool slowly to room temperature. It was stored at –20°C.

f). Proteinase K soltion: Proteinase K (type XI, Sigma) was dissolved in sterile distilled water at a concentration of 20mg/ml and stored at –20°C.

g). Kanamycine solution: Kanamycine sodium salt (Sigma) dissolved in water to a final concentration of 50mg/ml. The solution was sterilized by filtering through a 0.22μm Millipore filter and stored at -20°C.

h). Ampicillin solution: Sodium salt of ampicillin (Sigma) was dissolved in water at a concentration of 25mg/ml. The solution was sterilized by filtering through a 0.22μm Millipore filter and was stored at –20°C.

i). TE buffer (pH 8.0): TE was prepared by diluting concentrated stocks of 1M tris-Cl (pH 8.0) and 0.5 M EDTA to a final concentration of 10 mM Tris-HCl and 1 mM EDTA. It was stored at 4°C.

j). TES buffer (pH 8.0): TES was prepared by diluting stock solutions to a final concentration of 20 mM Tris-HCl, 10 mM NaCl, and 0.1 mM EDTA.

k). Buffer-saturated phenol: Redistilled molecular biology grade phenol (BRL) was melted at 68°C and 0.1g of 8-hydroxy-quinoline was added per 100ml of phenol. The phenol was then extracted several times with an equal volume of buffer [1 M tris-Cl (pH 8.0), followed by 0.1 M tris- Cl (pH 8.0) containing 0.2% β-mercaptoethanol] , until the pH of the aqueous phase reached more than 7.6. the phenol was stored under equilibration buffer (TE, pH 8.0) at 4°C in the dark for a maximum period of 2 months.

l). Phenol/chloroform/isoamyl alcohol solution: This solution was prepared by mixing of buffer-solution phenol, chloroform, and isoamyl alcohol at volume ratio of 25:24:1

respectively. The phenol/chloroform/isoamyl alcohol solution was stored in the dark at 4°C under equilibration buffer (TE, pH 8.0).

m). Electrophoresis buffer: 10x concentrated tris-borate-EDTA (TBE) buffer (pH 8.3) was prepared by dissolving 54g tris base, 27.5g boric acid and 3.7g $Na_2EDTA,2H_2O$, in distilled water to a final volume of 1l, and stored at room temperature.

n). Gel-loading buffer for DNA: 6x concentrated loading buffer consisted of 0.025% bromophenol blue, 0. 025% xylene cyanol, and 2.5% Ficoll (type 400) in water. It was stored at room temperature.

o). Ethidium bromide solution: Ethidium bromide was dissolved in distilled water at a concentration of 10mg/ml and stored at 4°C in the dark.

p). Phosphate buffered saline (PBS)(pH 7.2-7.3): 10X concentration of PBS stock was prepared by dissolving 10.96g Na2HPO4 and 3.15g NaH2PO4.H2O in 1L of distilled water. Working 1XPBS was prepared by diluting the stock and adding 8.5g NaCl in 1L of working solution.

q). Standard saline citrate (SSC): 20X concentrated standard saline citrate was prepared by dissolving 175.3g of NaCl and 88.2g of trisodium citrate in distilled water. The pH was adjusted to 7.0 and the final volume was made upto 1l. it was sterilized by autoclaving and stored at room temperature.

r). Denhardt's solution: 50x concentrated Denhardt's solution was prepared by dissolving 5g Ficoll (type 400), 5g polyvinyl pyrrolidone, and 5g bovine serum albumin (Pentax Fraction V, Sigma) in distilled water to make a final volume of 500ml. This solution was dispensed into 25ml aliquots and stored frozen at –20°C.

s). Denatured salmon sperm DNA: Sodium salt of salmon sperm DNA (type III, Sigma) was dissolved in distilled water at a concentration of 10mg/ml by stirring vigorously on a magnetic stirrer. The DNA was sheared by passing several times through an 18-gauge hypodermic needle. It was denatured by boiling for 10min followed by quick chilling on ice and was stored at -20°C.

2.4 MICROBIOLOGICAL MEDIA

a). LB (Luria-Bertani) medium: 10g Bacto-tryptone, 5g Bacto-yeast extract and 10g NaCl were dissolved in 1L of distilled water, and the pH was adjusted to 7.4 with a

few drops of 1M NaOH. The medium was sterilized by autoclaving at 121°C for 20 min and stored at 4 °C. Occasionally antibiotics were added into this medium.

b). LA (Luria agar) medium: 10g Bacto-tryptone, 5g Bacto-yeast extract and 10g NaCl were dissolved in 1L of distilled water, and the pH was adjusted to 7.4 with a few drops of 1M NaOH. Then 15g Agar was added and dissolved with brief heating. The medium was sterilized by autoclaving at 121°C for use and poured onto sterile plates. Occasionally antibiotics were added into this medium.

c). APW (Alkaline peptone water): (10g Bacto-peptone, and 10g NaCl were dissolved in 1L of distilled water, and the pH was adjusted to 9(±0.2) with 1M NaOH. The medium was sterilized by autoclaving at 121°C for 20 min and stored at 4 °C.

d). AKI medium: 10g Bacto-peptone, 4g Bacto-yeast extract and 5g NaCl were dissolved in 1L of distilled water, and the pH was adjusted to 7.4 (±0.2) with a few drops of 1M NaOH. The medium was sterilized by autoclaving at 121°C for 20 min and stored at 4 °C.

e). GA (Gelatin Agar) medium: 10g Trypticase, 10g NaCl, 30g Gelatin, and 15g Agar were dissolved in 1L of distilled water, and the pH was adjusted to 7.4 (±0.2) with a few drops of 1M NaOH. The medium was sterilized by autoclaving at 121°C for use and poured onto sterile plates. Occasionally antibiotics were added into this medium.

f). TTGA or Monsur's medium: 10g Trypticase, 10g NaCl, 5g Sodium torocholate, Na_2CO_3 1.2-1.3g, 30g Gelatin, and 16g Agar were dissolved in 1L of distilled water, and the pH was adjusted to 8.5-9.0 with a few drops of 1M NaOH. The medium was sterilized by autoclaving at 121°C for use and poured onto sterile plates.

2.5 ANIMALS

For in vivo assays 5-days old Swiss Albino mice and adult New Zealand White rabbits was obtained from the breeding facility of Animal Resource Branch of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B).

2.6 ISOLATION OF PLASMID DNA

Plasmid DNA was isolated by the method of Birnboym and Doly modified by Maniatis (64). Isolation was carried out from midi preparations.

Working Solutions:

Solution I: 50 mM Glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. The solution was prepared from the corresponding stock solutions and autoclaved and stored at –20°C.

Solution II: 0.2 N NaOH, 1% SDS. The solution was prepared from sodium hydroxide pellets and 10% SDS.

Solution III: Potassium acetate solution, was prepared 11.5 ml glacial acetic acid, 28.5ml distilled water, and 60 ml of %M potassium acetate. The resulting solution was 3M with respect to potassium and 5M with respect to acetate, and the resulting pH was 4.8.

Method: Cells pellet from 50 ml O/N culture was collected in tube(s) by centrifugation at 8000 RPM and at 4°C in a Sorvall RC 5C suppressed centrifuge. Cell pellet was dissolved in 2 ml of solution I and kept at room temperature for 5 minutes. Then the tube(s) were transferred on ice and 4 ml of solution II was added and incubated on ice for another 5 minutes. In this step the contents of the tube was mixed by gently inverting the tube. Finally solution III was added and the content was mixed on a vortex. The tubes were placed on the vortex at inverted position. The tubes were then placed on ice for final 5 minutes and centrifuged at 18000 RPM in Sorvall RC 5C centrifuge. Supernatant was collected and extracted with phenol/chloroform/isoamyl alcohol followed by a precipitation with double volume absolute ethanol. The pellet was dried in vacuum drier and dissolved in 400 µl TE, and treated with RNase. Finally plasmid DNA was prepared after extraction with both phenol and phenol/chloroform/isoamyl alcohol followed by double volume ethanol precipitation.

2.7 ISOLATION OF TOTAL DNA FROM BACTERIA

Total DNA was isolated in midi preparations.

Working Solutions:

Solution I: 50 mM Glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. The solution was prepared from the corresponding stock solutions and autoclaved and stored at –20°C.

EDTA: 0.5 M EDTA solution.

Tris-HCl (pH 8.0): 0.5 M Tris-HCl.

Lysozyme: Powder 10 mg.

Proteinase K: 20-mg/ml stock solution.

Proteinase K buffer: 0.5 M EDTA and 0.5 % SDS.

Method: Cells pellet from 50 ml O/N culture was collected in tube(s) by centrifugation at 8000 RPM and at 4°C in a Sorvall RC 5C suppressed centrifuge. Cell pellet was dissolved in 2 ml of solution I on a vortex. Then 750 µl 0.5 M Tris-HCl, 750 µl 0.5 M EDTA, and 10 mg lysozyme was added to the tube, the contents of the tube was mixed by gentle pipetting and incubated at 40°C. After 5 minutes incubation, 2.5-ml proteinase K buffer and 25 µl proteinase K was added to the tube ad mixed by gentle pipetting and shaking. This step was followed by an incubation period of more then 30 minutes at 45°C. Then the preparation was extracted with phenol and then with phenol/chloroform/isoamyl alcohol followed by a precipitation with double volume absolute ethanol. During ethanol precipitation one tenth volume of 3 M sodium acetate (pH 5.2) was added to the contents of the tube to facilitate the precipitation. DNA was then pelleted and dried in vacuum drier and dissolved in 400 µl TE, and treated with RNase. Finally DNA was prepared after extraction with both phenol and phenol/chloroform/isoamyl alcohol followed by double volume ethanol precipitation as before.

2.8 ISOLATION OF BACTERIOPHAGE AND PHAGE DNA

Phage or phage DNA was generally isolated from 50-ml culture.

Working solutions:

PEG/ NaCl solution: 20% polyethylene $glycol_{6000}$ and 10% NaCl.

TES buffer: mentioned earlier.

Dnase I: 169U/µl, Gibco BRL life Technologies.

RNase A: mentioned earlier.

Method: Culture supernatant was sterilized by filtration through 0.22-µm-pore-size filters (Millipore Corp., Bedford, Mass.). Sterile filtrate was mixed with one-fourth volume of a PEG/ NaCl solution and centrifuged at 12,000 X g to precipitate the phage particles. The precipitate was dissolved in TES buffer (pH 8.0). During the different infection study this preparation was used as phage preparation, and to isolate

phage DNA this preparation was subjected to digestion with pancreatic DNase I (100U/ml) and RNase A (50µg/ml) at 37°C for 1 hr to remove possible nucleic acids carried over from lysed bacterial cells. The solution was then extracted with phenol-chloroform to disrupt possible phage particles, and the total nucleic acids were precipitated with ethanol.

2.9 BACTERIAL STRAINS AND PHAGES

Naturally occurring toxigenic *V. cholerae* strains used in the present study included 141 strains belonging to the 01, 0139, and non-01 non-0139 serogroups, and the strains were isolated from cholera patients or environmental surface water in Bangladesh (see Table 2.1). The non-toxigenic *V. cholerae* strains used in the transduction studies isolated from five different countries, obtained from either surface water samples or patients. The study included non-toxigenic 0139 strains, E1 Tor strains, and non-O1, non-O139 strain isolated in Bangladesh or India or Saudi Arabia. Before use, biochemical or serological methods (117) confirmed the identities of the strains, and the presence or absence of CTX element was tested with specific DNA probes. The genetically marked phage IG-KmΦ was a derivative of CTXΦ into which a kanamycin resistance (Km^r) determinant was introduced into an intergenic *Not*I site (61). Strain ASF-1 carried a chromosomally integrated copy of the IG-KmΦ genome and was constructed by lysogenic conversion of CT-negative *V. cholerae* 01 E1 Tor strain SA-317 with IG-KmΦ. Strain SM44 was a derivative of toxigenic E1 Tor Strain P27459 in which the CTX element was marked with a Km^r determinant (37). The genetically marked phage CTX-KmΦ was derived from strain SM-44 as described previously (112). Properties of the control bacterial strains and phages used in this study are presented in table 2.1. Supernatant fluids of toxigenic *V. cholerae* strains, exposed to direct sunlight (42,000 lx) for 30 minutes and subsequently grown for 5 hrs, were sterilized by filtration through 0.22-µm-pore-size filter and were used to precipitate possible bacteriophage particles. The precipitate was dissolved in appropriate buffer and treated with DNase I and RNase A to remove

contaminating exogenous DNA or RNA. Total phage or nucleic acids were isolated as described in the text and analyzed using *ctxA* probe.

Table 2.1 Characteristics of control strains and phages used in this study

Strain or plasmid	Relevant characteristics	Reference or source
SM-44	Derivative of El Tor strain P-27459, in which the CTX genetic element is marked with a Km^r determinant by marker exchange disruption of the *ctxAB* operon	37
RV-508	Derivative of classical biotype strain 569B, which is known to constitutively express CT, TCP, and other *toxR*-regulated gene products	112
569B	*V. cholerae* O1 classical Inaba strain	Lab. collection
P-27457	*V. cholerae* O1 El Tor Inaba strain	Lab. collection
SA-317, SA-590, SA-406, SA-674 W-03, W-05, W-06, W-07, W-10, W-21	Nontoxigenic ($CTX\Phi^-$) *V. cholerae* O1 El Tor strains isolated in India	Lab. collections
O395	Classical Ogawa streptomycin-resistant strain	Lab. collection
O395(pCTX-Km)	O395 strain carrying the RF of CTX-KmΦ derived from SM-44	112
pIG-Km	Derivative of the RF DNA of wild-type CTXΦ carrying an intergenic Km^r marker	61
ASF-1	Strain SA-317 lysogenized with IG-KmΦ	This study
AOE-12/39, EV-99	Environmental non-toxigenic ($CTX\Phi^-$) *V. cholerae*	Lab collection

2.10 PROBE AND HYBRIDIZATION:

The gene probe used in this study to detect the CTXΦ genome was a 0.5-kb EcoRI fragment of pCVD27 (52). Strand-specific oligonucleotide probes with the sequences 5′ TCTATCTCTGTAGCCCCTATTACG and 5′ CTCAGACGGGATTTGTTAGGCACG, for probing the plus and minus strands, respectively, were also used to detect the presence of single-stranded DNA of CTXΦ, and to distinguish between the replicative-form (RF) double-stranded DNA and single-stranded phage DNA in crude preparations. The 0139-specific probe was a 1.3-kb EcoRI fragment of pCRII-A3 (73,109). The nontoxigenic 0139 strains were also tested for the presence of genes encoding toxin-coregulated pilus (TCP), which is the receptor for CTXΦ, the virulence regulatory gene toxR, and the CTXΦ attachment sequence *attRS*. An 840-bp region internal to the zonula occludens toxin gene (*zot*) amplified by PCR from the recombinant plasmid pBB241 as described (24), and a 2.1-kb *Sph*I-*Xba*I fragment of pCTX-Km (112) containing the entire *zot* and *ace* genes and part of *orfU* was used. The toxR gene probe was a 2.4 –kb BamHI fragment of pVM7 (69), and the 18-bp *attRS* sequence was identified using a synthetic oligonucleotide corresponding to the *attRS* sequence (37). The rRNA gene probe consisted of a 7.5 kb *Bam*HI fragment of the E, coli rRNA clone (8,26). Colony blots or Southern blots were prepared using nylon filters (Hybond; Amersham International plc, Aylesbury, United Kingdom) and processed by standard methods (64,98). The polynucleotide probes were labeled by random priming (29) using a random primer DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.) and [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). Oligonucleotide probes were labeled by 3 tailing using terminal deoxynucleotide transferase and [α-^{32}P]dCTP (Amersham). Southern blots and colony blots were hybridized with the labeled probes and autoradiographed as described previously (24,26).

2.11 PCR ASSAYS

The presence of the TCP pathogenicity island was determined by PCR assays specific for the *tcpA, tcpI*, and *acfB* genes. All oligonucleotides used either as probes or as PCR primers were synthesized commercially by Oswel DNA Service

(University of Edinburgh, Edinburgh, United Kingdom), and PCR reagents and kits were procured from Gibco BRL Life Technologies. The presence of *tcpA* genes specific for the classical and El Tor biotypes was determined by a multiplex PCR assay (57), and the *tcpI* gene was detected by PCR assay as described previously(27). The *acfB* gene was detected by a PCR assay based on the published sequence of *acfB* (21) in which two primers with the following sequences were used: 5′ GGACCAAGCATTATTATCTCT and 5′ AATGATAAACTTACTGATTAA. Thermocycle parameters for the PCR assay consisted of denaturation at 94° C for 2 min, annealing of primers at 60°C for 2 min, and primer extension at 72°C for 3 min. amplification was performed for 25 cycles, and the size of the amplicon (1.9 kb, which is the expected size) was ascertained by electrophoresis in 1 % agarose gels. The identities of all PCR products were further verified with specific oligonucleotide probes. The absence of the relevant genes in the PCR-negative strains was further confirmed by colony blot hybridization with the corresponding PCR-generated amplicons from a positive control strain, 569B, as specific probes.

2.12 INFECTION OF *V. CHOLERAE* STRAINS WITH CTX-KMΦ:

The recipient strain was grown O/N in Luria broth (LB) at 30°C. In the following morning it was subcultured at a ratio of 1:40 and grown for 3-hrs. The cells from the 3-hrs old culture was precipitated by centrifugation and washed in fresh LB. Then approximately 10^6 recipient cells in LB were mixed with 10^5 phage particles. For the in vitro assay, the mixture was incubated for an hour at 30° C, and aliquots of the culture were dilute and plated on Luria agar plates containing kanamycin (50μg/ml) to select for kanamycin-resistant colonies and on plates devoid of kanamycin to determine the total number of colonies.

For the in vivo assay, the same mixtures of phage and recipient cells were used to gastrointestinally inculate into ileal loops of adult New Zealand White rabbits or 5-day old Swiss Albino mice obtained from the breeding facility of the Animal Resources Branch of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), and prepared as described previously (19). For each strain and phage combination, at least five loops of different rabbits or five mice were

inoculated. Animals were sacrificed after 16 hrs, and their inside of the loops or intestinal content was washed out with 1 ml of 10 mM phosphate-buffered saline (pH 7.4) and collected in the same tube. Dilutions of the intestinal fluids were analyzed to screen for the presence of Km^r *V. cholerae* colonies. The ratio of Km^r colonies to total colonies was calculated and expressed as the percentage of recipient cells carrying the phage genome.

2.13 STABILITY STUDIES OF CTXΦ:

To examine the stability of CTXΦ under different conditions of temperature, pH, and salinity, CTX-KmΦ was isolated from a culture of O395 (pCTX-Km). Approximately $5x10^6$ phage particles were transferred into a series of test tubes containing 10 ml of LB adjusted to different pH and salinity. Control tubes containing normal saline or distilled water were also inoculated. The tubes were stored at desired condition and aliquots of medium were periodically removed and analyzed for the remaining infectious CTX-KmΦ particles. To study stability at different pH, phage preparation was incubated at different pH between pH 2 and pH 12.6, at 37°C. Remnants were counted after an incubation of 1 hour, 24 hours, and 7 days. Stability of the phage was expressed as the percentage of initial inoculum of infectious particles. Effect of salinity on the stability was studied in two different sets of experiments. The first experiment was carried out by adding different amounts of NaCl in distilled water that contained a defined amount of the phage preparation added into it, and was incubated at room temperature and at 37°C. Aliquots of samples were tested after certain day's interval, and the stability of phage was expressed in days, that took to show the availability of last viable phage particle. In a similar way another experiment was carried out with Turag river water of pH 7.53 with supplemented salinity.

2.14 EFFECT OF SALINITY (NaCl) ON INFECTION OF CTXΦ TO THE HOST STRAINS:

To study the effect of salinity on transduction, different concentrations of NaCl from 0.0 M to 0.46 M (0.0% to 2.7%) was added to LB and the infection of the

recipient cell, RV-508, was carried out in those media. Infection of the recipient cells with phage was studied by the standard method described earlier.

2.15 EFFECT OF pH ON INFECTION OF CTXΦ TO THE HOST STRAINS:

To study the effect of pH on transduction, the pH of LB was adjusted to different values between pH 2 and pH 12.6. Infection of the recipient cell, RV-508, was carried out in those media. Infection of the recipient cells with phage was studied by the standard method described earlier.

2.16 EFFECT OF VARIATIONS IN pH, SALINITY AND TEMPERATURE ON PHAGE INDUCTION:

To study the effects of pH, NaCl concentration (salinity), and temperature on the induction of CTX prophage, a series of test flasks containing LB was adjusted to different pHs (6.5, 7.5, 8.0, and 8.5), salinity levels (0.5,to 2.0%, in increments of 0.5%), or combinations of different pHs and salinities. Series of flasks containing 50 ml of LB with defined pH and salinity were inoculated with 5x 10^4 cells of strain SM44 and incubated at four different temperatures, 25, 30, 37, or 40°C, with shaking. The induction of the prophage was monitored by periodically examining aliquots of culture supernatants for the presence of CTX-KmΦ during the following 12h, as described above.

2.17 INDUCTION OF LYSOGENY WITH MITOMYCIN C:

V. cholerae strains were grown in Luria broth (LB) at 30°C to an absorbance at 540 nm of 0.2. The cells were collected by centrifugation, washed, and resuspended in fresh LB. The suspension was divided into aliquots, to which Mitomycin C (Sigma Chemical Co., St. Louis Mo.) was added at a concentration of 20ng/ml and incubated for 6 hours at 30°C. The culture supernatants were analyzed for extracellular phages carrying the CTX as described above. Strains grown in a similar way but without mitomycin C were used as controls.

2.18 INDUCTION OF CTXΦ PRODUCTION WITH SUNLIGHT:

All strains tested for induction by sunlight were grown in Luria broth (LB) at 37°C to an absorbance at 540-nm (A_{540}) of 0.2. The cells were collected by centrifugation, washed, and resuspended in fresh LB. Five milliliters of the suspension, containing approximately 5x 10^4 bacterial cells, was spread on a sterile petri dish and exposed to direct sunlight for a specified time (30 to 45 min). The intensity of the light was measured using a digital light meter (VWR Scientific, South Planfield, N.J.). Control plates were kept under normal light in a shaded area during the same time period. The treated cells were inoculated into test flasks containing 50 ml of fresh LB. All flasks were incubated at 37°C with shaking, and aliquots of culture were periodically removed and analyzed for the presence of extracellular CTXΦ. Briefly, to detect Km^r determinant-labeled CTXΦ particles derived from strains SM-44 and ASF-1, the culture supernatant was sterilized by filtration through 0.22-μm-pore-size filters (Millipore Corp.,Bedford, Mass.), and the filtrate was titrated for infectious phage particles by incubating aliquots of the supernatant with the classical strain RV508 and then selecting for colonies resistant to kanamycin (50 μg/ml). To detect the induction of the CTX prophage in wild-type strains, preparations of the culture supernatants containing CTXΦ DNA were identified by Southern blot hybridization using specific probes. Aliquots of LB mixed with serial dilutions of 0395(pIG-Km) were used as positive controls to test the detection limit of the hybridization assay. An aliquot of the culture obtained for the phage assay was also diluted and plated to determine the number of viable cells.

2.19 PROPAGATION OF LYSOGENIC BACTERIOPHAGE:

Propagation CTXΦ was studied using two different donor strains, SM44 and ASF-1, and two nontoxigenic 0139 strains, AOE12-39 and Env-99 as recipient (Table2.1). The donor and recipient strains were grown separately, washed, and resuspended in fresh LB. Approximately 2.5x 10^6 cells of the donor and the recipient strain were mixed in 5 ml of LB medium and exposed to direct sunlight (~42,000 lx) for 30 min. For the in vitro assay, 1 ml of the same medium was incubated at 30°C for

16 h. Aliquots of the culture were analyzed for transduction of the recipient strain by using appropriate antibiotics and DNA probes. To detect the transduction of the nontoxigenic 0139 strains, dilutions of the mixed culture were grown on Kanamycin plates, and colony blots prepared from the plates were hybridized with the 0139-specific DNA probe to detect Km^r 0139 derivatives.

For the in vivo assays, l-ml aliquots of the sunlight-treated cells were inoculated into the ileal loops of adult New Zealand White rabbits obtained from the breeding facility of the Animal Resources Branch of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), and prepared as described previously (19). After 16 hrs, the rabbits were sacrificed and contents of the ileal loops were collected. The inside of the loops was washed out with 1 ml of 10 mM phosphate-buffered saline (pH 7.4) and collected in the same tube. Dilutions of the ileal loop fluids were analyzed by plating in appropriate antibiotic plates and using DNA probes. The ratio of Km^r-transduced colonies to total number of colonies derived from the recipient strain was calculated and expressed as the percentage of recipient cells carrying the phage genome.

2.20 ANALYSIS OF THE INTEGRATION OF THE PHAGE GENOME INTO THE CHROMOSOME:

Representative colonies were picked, grown in LB containing kanamycin (50 μg/ml), and further analyzed for the presence of the phage genome. Total DNA or plasmid DNA was extracted from overnight cultures by standard methods (64) and purified using microcentrifuge filter units (Ultra-free-Probind; Sigma). Integration of the phage genome into the chromosome of the recipient cells was studied by comparative Southern blot analysis of total DNA and plasmid preparations from infected and native strain by using the *ctxA* probe. The ability of the CTX lysogens derived from parental nontoxigenic 0139 strains to produce CT was determined by the G_{M1} ganglioside-dependent enzyme-linked immunosorbent assay and the rabbit ileal loop assay, as described previously (19).

2.21 ANALYSIS OF rRNA GENE RESTRICTION PATTERNS:

Comparative analysis of the *V. cholerae* strains by their rRNA gene restriction patterns (ribotype) was carried out as described previously (25,26,27,28) to study clonal relationships among the strains. Briefly, 5-μg aliquots of total DNA were digested with the appropriate restriction enzyme and electrophoresed in 0.8% agarose gel, and the DNA fragments were blotted onto nylon membranes (Hybond; Amersham). The genomic blots were hybridized with the rRNA gene probe, and autoradiographs were developed as described previously (25-28). rRNA gene restriction patterns produced by the newly formed lysogens derived from nontoxigenic progenitors were compared with previously reported ribotype patterns derived from toxigenic *V. cholerae* strains isolated from outbreaks of cholera in different countries (19, 25-28, 79).

Chapter 3

RESULTS

3. Results

3.1 INDUCTION OF CTXΦ LYSOGENS:

3.1.1 PHAGE INDUCTION WITH MITOMYCIN C

Of a total of 141 *V. cholerae* strains obtained from culture collection and from fresh culture positive stools, 6 strains spontaneously produced a low but detectable level of CTXΦ whereas another 48 strains (34.04%) produced extracellular CTXΦ when induced with mitomycin C. These 54 strains contained 12 isolates from culture positive stools. Strains that produced extracellular CTXΦ included 7 (19.44%) of 36 El Tor strains and 5 (45.45%) of 11 O139 strains obtained from freshly collected stools and 22 (43.13%) of 51 El Tor strains and 20 (46.51%) of 43 O139 strains analyzed from previous collections. A complete result is presented in table 3.1.1. As expected for the filamentous phage that gave Southern blot hybridization with the strand specific probe, the phage DNA was found to be single stranded and hybridizes with the probe specific for the plus strand (Figure is not presented in this case, because a similar type of figure will be found in the case sunlight induction).

3.1.2 INDUCTION OF CTX PROPHAGE BY SUNLIGHT:

Two strains, SM44 and ASF-1, harboring Km^r determinant-marked CTX prophages were initially used to study parameters influencing prophage induction. To examine the effect of sunlight on the induction of CTX prophages in SM-44 and ASF-1, colonies were selected that spontaneously produced a small number of phage particles (<50/ml). No significant difference in the CTX-KmΦ transducing activity of the culture supernatants was noted when the pH, salinity, or temperature of the cultures was varied. However, when the cultures were exposed to direct sunlight (mean intensity of 42,000lx) for 30 min prior to incubation, the number of phage particles increased more than 10,000-fold (Table 3.1.2a). No increase was noted in control cultures, which were stored in a well-lighted area (average of 3,180 lx), but away from direct sunshine. This showed that direct sunlight is a significant natural inducer of the CTX prophage. Examination of the production of phage particles in sunlight-induced cultures at different stages of cell growth showed a steady increase

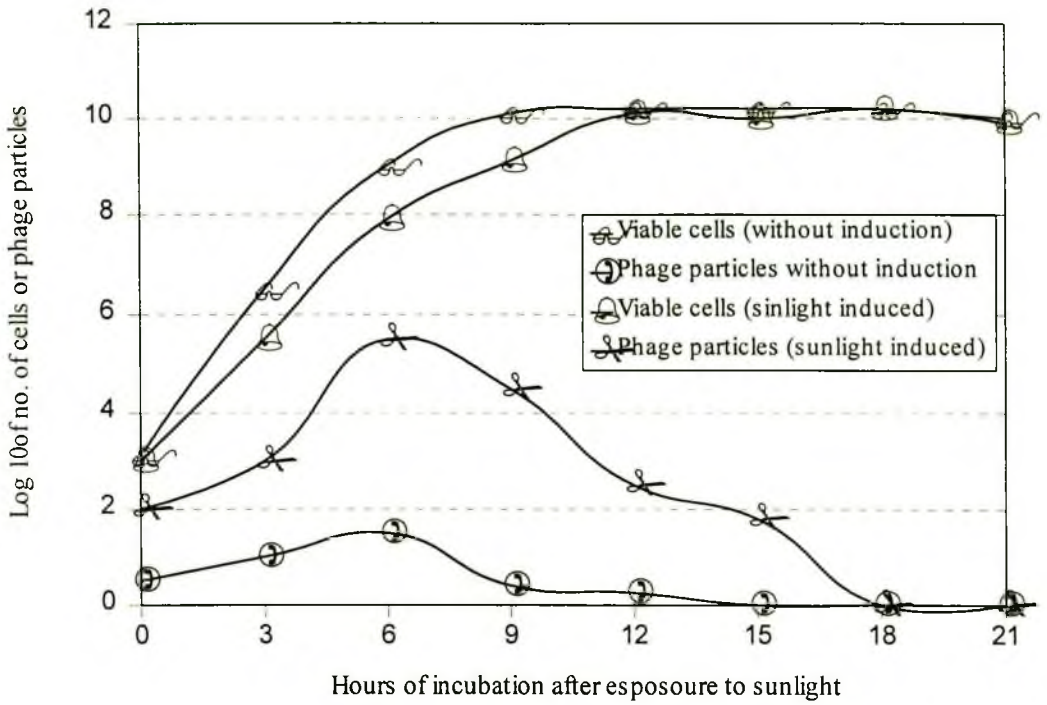
of phage particles for nearly 6 hrs. This was followed by a sharp decline, which corresponded, to the stationary phase of the culture (Figure 3.1.2a).

In 25 out of 32 wild-type (nonmarked) strains induced with sunlight (Table 3.1.2b), CTXΦ was detectable in culture supernatants by Southern blot hybridization using a plus strand-specific oligonucleotide probe (Figure 3.1.2b). The bands corresponding to the single-stranded phage DNA was absent in 30 of 32 preparations derived from supernatants of wild-type strains grown without sunlight induction. In control assays, approximately 10^3 transducing particles produced a visible band.

Table 3.1.1: Analysis of toxigenic *Vibrio cholerae* O1 and O139 strains isolated between 1969 and 1997 in Bangladesh for the induction of lysogenic phage encoding cholera toxin.

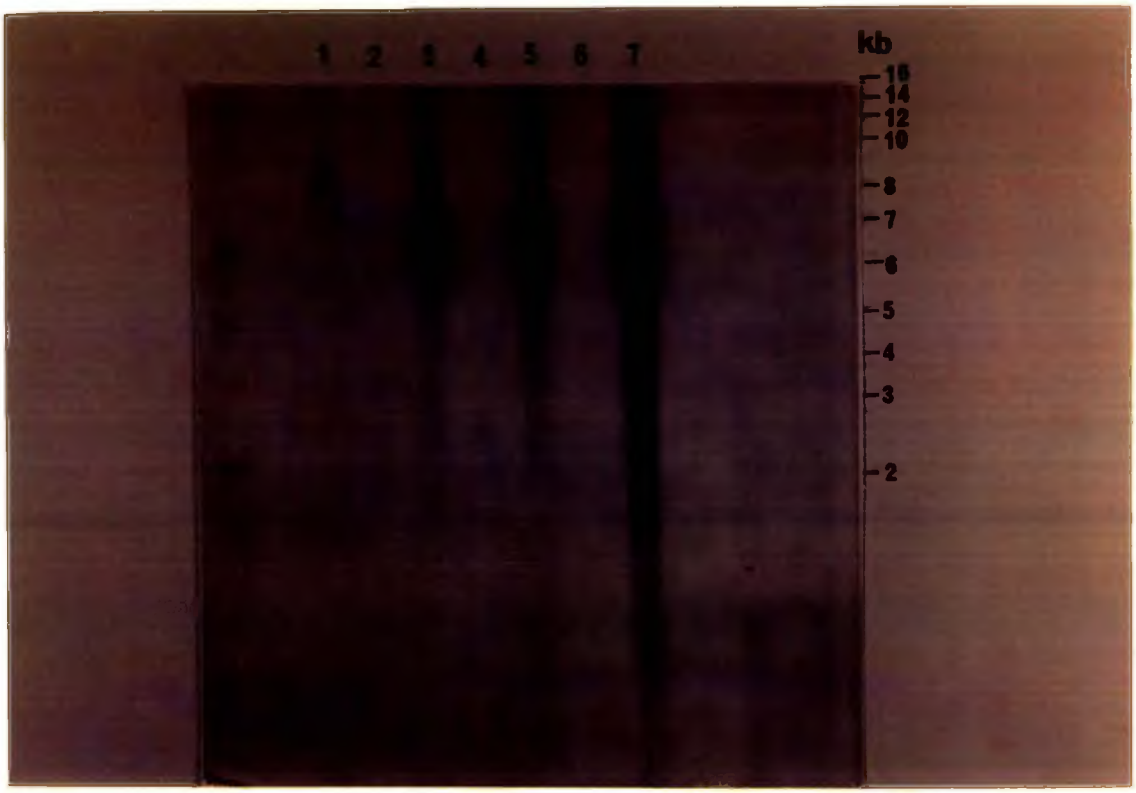
Strain	Year of Isolation	Source	No. of strains analyzed	No. of strains producing CTXΦ	
				Induction with mitomycin C	Without mitomycin C
V. cholerae O1 biotype El Tor	1969-1976	Patient	5	4	0
	1990-1992	Patient	6	3	2
	1995-1997	Patient	67	16	0
	1993-1996	Surface water	9	6	1
V. cholerae O139	1993	Patient	9	4	0
	1995-1997	Patient	38	17	2
	1993-1997	Surface water	7	4	1

Figure 3.1.2a: Effect of sunlight on phage induction:



Kinetics of cell growth and production of extracellular CTXΦ particles by strain SM-44 induced with exposure to direct sunlight (42,000 lx) for 30 minutes. Values are the average of five different observations.

Figure3.1.2b: Induction of CTXΦ from wild type toxigenic strain of *V. cholerae* by sunlight.



Lanes 2, 4, and 6, contains phage DNA preparations from three toxigenic strains grown without exposure to sunlight, whereas lanes 3, 5, and 7contains phage DNA preparations derived from the corresponding sunlight-induced cultures. Lane 1 contains phage DNA preparation from approximately 10^3 IG-KmΦ particles added to LB and used as a control. Numbers indicating molecular size of bands corresponds to a supercoiled DNA ladder (Bethesda Research Laboratories).

Table 3.1.2a: Sunlight induced production of extracellular CTXΦ particles by genetically marked strains of *V. cholerae* carrying a kanamycine resistance determinant in the CTXΦ genome.

Strains	Culture condition[a]	Titer of CTXΦ particles/ml[b]
SM-44	Without exposure to direct sunlight	$0.3x10^2$
	After exposure to direct sunlight	$4.2x10^5$
ASF-1	Without exposure to direct sunlight	$0.1x10^2$
	After exposure to direct sunlight	$3.7x10^5$
SA-317	Without exposure to direct sunlight	0
	After exposure to direct sunlight	0
SA-406(pCTX-km)	Without exposure to direct sunlight	$8.1x10^4$
	After exposure to direct sunlight	$9.1x10^4$

[a]Cells were exposed to direct sunlight for 30 minutes prior to incubation, and supernatant of culture were analyzed after incubation for 5 hrs at 30°C.

[b]Values are average of five different observations.

Table 3.1.2b: Analysis of naturally occurring toxigenic strains of *V. cholerae* of environmental or clinical origin for sunlight-induced production of extracellular CTXΦ.

Serogroup and/or biotype	Yr. of isolation	Source	No of strains analyzed	No of strains producing extracellular CTXΦ	
				Induced by sunlight	Without induction
O1, El Tor	1995-1998	Patient	9	8	1
	1998-1999	Surface water	3	3	0
O139	1995-1998	Patient	11	9	1
	1995-1999	Surface water	4	4	0
Non-O1, Non-O139	1995-1998	Surface water	5	0	0

3.2 STABILITY OF CTXΦ PARTICLES:

3.2.1 STABILITY OF CTXΦ AT DIFFERENT SALINITY:

Two sets of experiment were carried out to determine the stability of CTXΦ at varying salinity. In the first experiment it was observed that phage particles are significantly stable at concentrations above 0.9% w/v NaCl at room temperature (Figure 3.2.1a). Phage particles remained infectious for more than 4 weeks when stored at room temperature in normal saline (0.9% w/v NaCl). Below this salinity there was a quick decline in the number of infectious particles, and at 0% salinity phage particles were stable not more than four days. Higher stability was observed at higher salinity values. In another set of experiment different concentrations of salt (NaCl) was supplemented in river water of pH 7.53. It was observed that at 3% w/v salinity infectious particles were available for around two months, whereas no infectious particles was found after 19 days in the same water without supplemented salinity (Figure 3.2.1b). In every case CTXΦ showed higher stability in salty water compared to the control (0% and 0.9% NaCl).

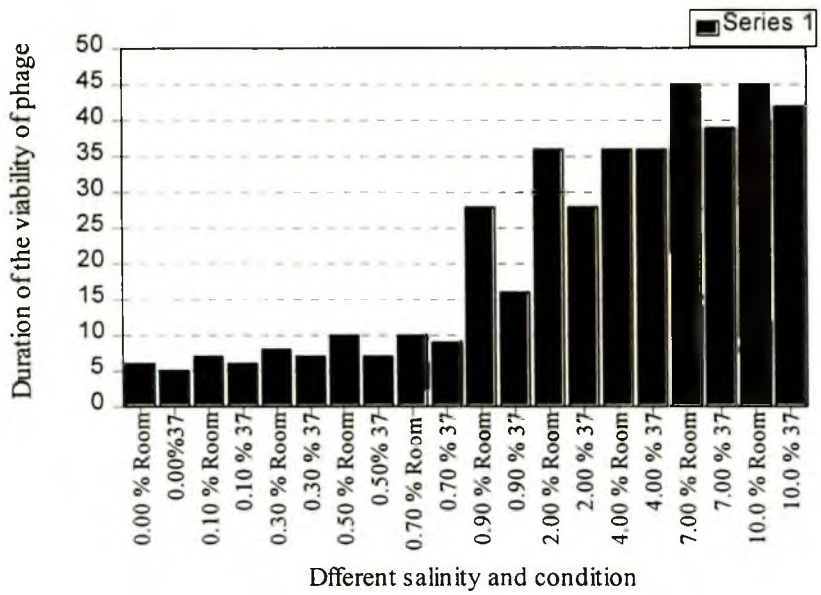
3.2.2 STABILITY OF CTXΦ AT DIFFERENT TEMPERATURE:

CTXΦ was found to be stable for more than one year at 4°C with 80% of its initial number of infectious particles during storage. Higher stability was observed at room temperature than at 37°C in every cases of the stability study with varying salinity (Figure 3.2.1a, 3.2.1b).

3.2.3 STABILITY OF CTXΦ AT DIFFERENT pH:

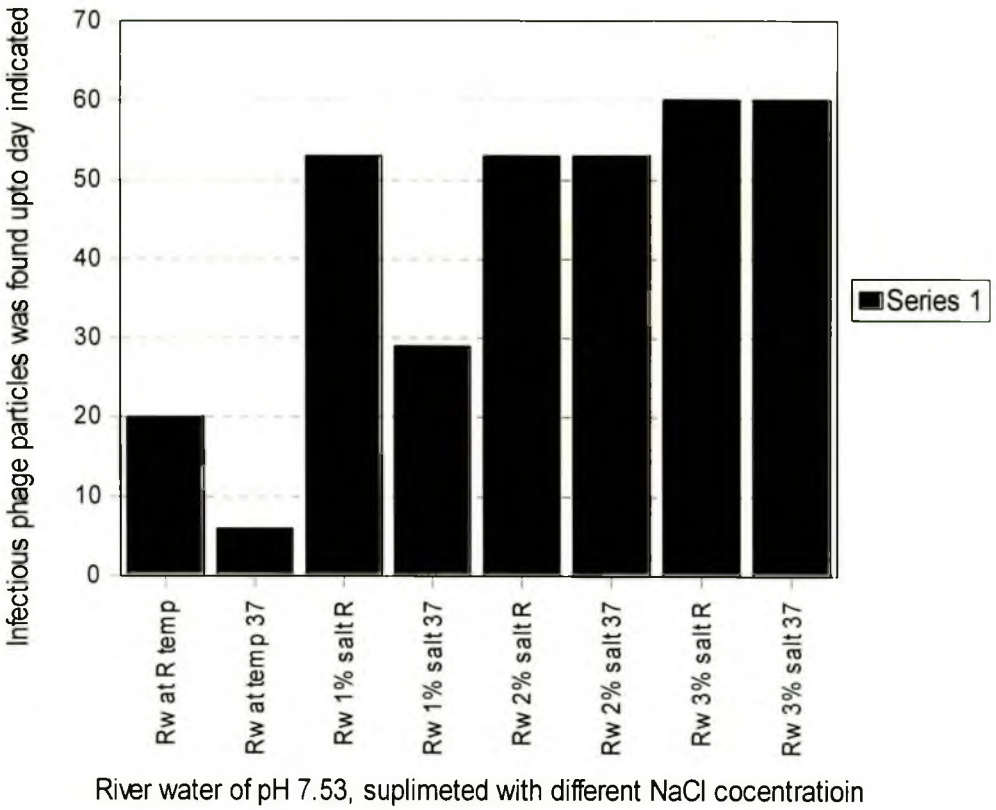
The effects of pH on the stability was studied by adding a defined number of CTXΦ particles into the medium and the pH of the medium was adjusted at desired pH values. Significant stability between pH 3.5 and pH 10.0 was observed after 1 hour of incubation (Figure 3.2.3). After an incubation of 24 hours, more than 50% of the initial count was still present, and at about pH 8.5 maximum stability was observed. 90% of the initial count was found to remain after 7 days at pH 8.5.

Figure 3.2.1a: Stability of CTXΦ at different salinity (NaCl) concentration



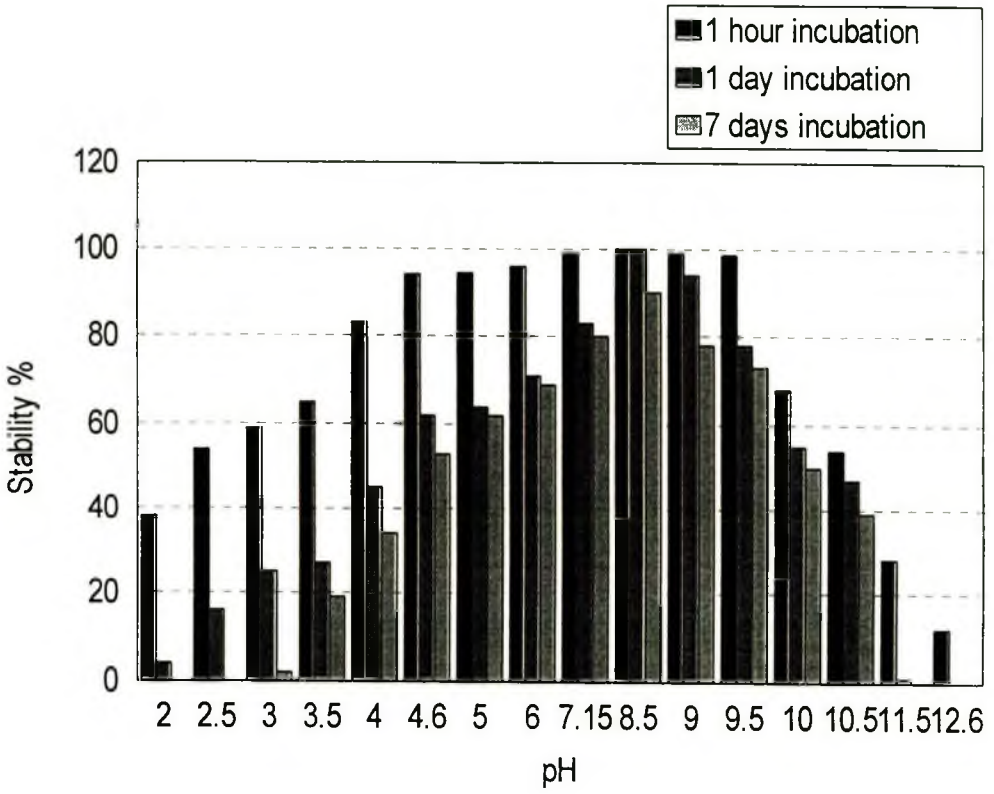
The effect of salinity on the stability of CTXΦ was studied after incubation of the phage in different salinity levels (0%-10% NaCl) at room temperature and at 37° C. Aliquots of phage was removed and tested for the presence of viable phage particles. Genetically marked CTX-KmΦ (see text for details) was used for this study.

Figure 3.2.1b: Stability of CTXΦ in river water adjusted to different salinity:



Effect of salinity on the stability of CTXΦ was assayed using genetically marked phage CTX-KmΦ(please see text for details). The effect of salinity was assayed at pH 7.53, which was the normal pH of River water (Rw). Experiment was carried out at salinity of 0%, 1%, 2%, and 3% at room temperature ($25^{o}\pm2$) and at 37^{o} C.

Figure 3.2.3: Stability of CTXΦ at different pH:



Effect of pH on the stability of CTXΦ, assayed after incubation of 1 hour, 1 day, and 7 days. Phage preparations were incubated in LB adjusted to different pH. Experiment was carried out with genetically marked phage CTX-KmΦ (see text for details). Values are the average of three different observations.

3.3 TRANSDUCTION OF RECIPIENT STRAINS:

3.3.1 IN VITRO SYSTEM

Of the 146 nontoxigenic strains exposed to the phage, 10 strains belonging to the 01 serogroup and 1 non-01, non-0139 strain were infected by the phage. However, the 01 strains that carried the TCP genes were infected more efficiently than the TCP-negative, non-01 strain (Figure 3.3).

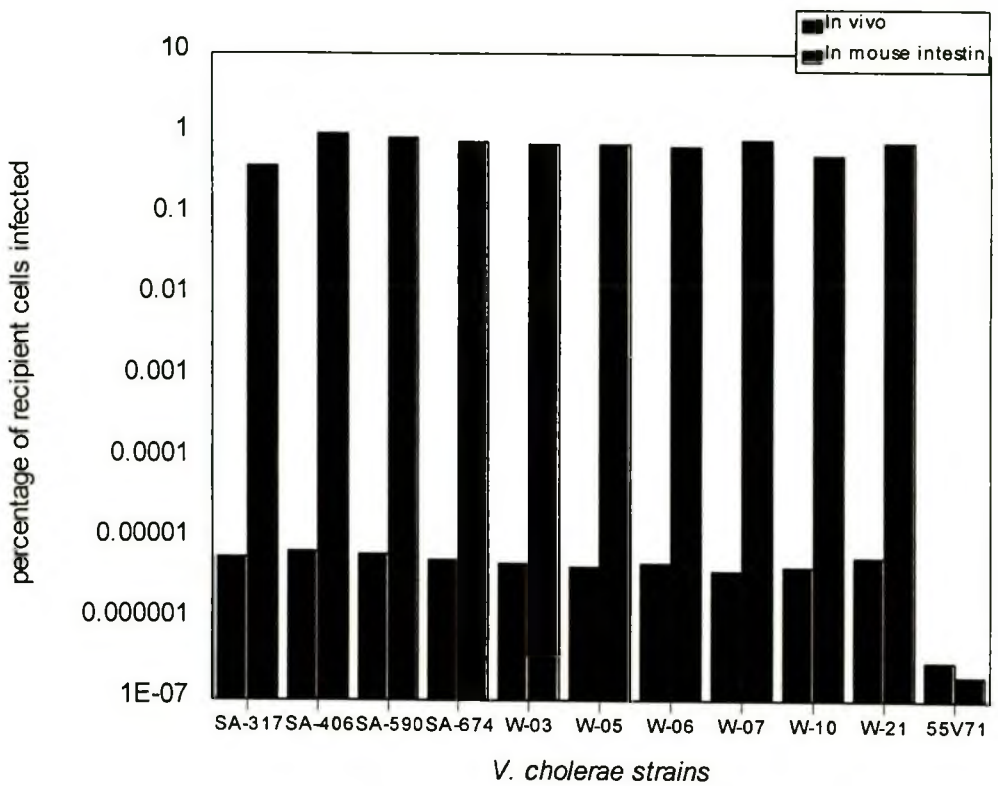
3.3.2 IN VIVO SYSTEM

Since TCP expressed more efficiently in vivo, the infant mouse model was used as in vivo system to test the susceptibility of the 11 strains, which were infected in the initial in vitro screening. When the TCP-positive strains were tested in the mouse intestine, the proportion of infected cells recovered was higher than in the in vitro assay. In the case of single non-O1 strain that was devoid of TCP, the infant mouse assay did not produce a higher level of infection than the in vitro assay (Figure 3.3).

3.3.3 PRESENCE OF VIRULENCE GENES IN *VIBRIO CHOLERAE*

DNA probe and PCR analysis of a total of 146 CTX-negative strains belonging to the 01 and non-01 serogroups (Table 3.3) showed that 6.85% of the strains were positive for the *tcpA, tcpI*, and *acfB* genes and presumably the entire TCP pathogenicity is land in addition to *toxR* but negative for TCP and att_{RS}; and 2.04% were positive for att_{RS} alone but negative for *toxR* and TCP. All strains simultaneously positive for TCP, *toxR,* and att_{RS} belonged to the 01 serogroup and were clinical origin, whereas none of the non-01, non-0139 strains analyzed in the present study carried the genes for TCP. In addition, three *V. cholerae* 01 strains isolated from seawater in Estonia and Latvia were also negative for TCP. The *toxR* gene was found to be widely distributed among nontoxigenic *V. cholerae* strains and was present in 84.93% of the total strains tested. A complete result is presented in table 3.3. The table also shows the total number of strains of each genotype that are infected with the CTXΦ and lysogenized.

Figure. 3.3: Variation in in vivo and in vitro transduction rate.



Susceptibility of TCP-positive and TCP-negative non-toxigenic *V. cholerae* O1 and non-O1 strains to CTX-KmΦ under in vitro laboratory condition and in vivo in the intestine of infant mice. Strains SA-317, SA-406, SA-590, and SA-674 are TCP-positive O1 clinical strains isolated in Saudi Arabia; strains W-03, W-05, W-06, W-07, W-10, and W-21 are the TCP positive O1 clinical strains isolated in India; strain 55V71 is a TCP negative non-O1, non-O139 environmental isolate of Bangladesh. Values represent the average of three different observations.

Table. 3.3: Distribution of major virulence-associated genes among non-toxigenic *V. cholerae* O1 and non-O1 strains isolated from cholera patients or environmental surface water and susceptibility of the strains to genetically marked derivative of CTXΦ.

V.cholerae serogroup	Countr	Yr of isolat	Source	No. of strains	Presence of[a]								No. of strains	
					ctxA	*zot*	*ace*	*tcpA*	*tcpI*	*acfB*	*toxR*	*attRS*	Infected with CTX-KmΦ	Lysogenized[c]
O1 El Tor biotype	India	1993	Patient	6	-	-	-	+	+	+	+	+	6	6
	Estonia	1995	SW[d]	3	-	-	-	-	-	-	+	-	0	0
	Latvia	1995	SW	1	-	-	-	-	-	-	+	-	0	0
	Saudi Arabia	1997	Patient	4	-	-	-	+	+	+	+	+	4	4
Non-O1, Non-O139	Saudi Arabia	1997	Patient	1	-	-	-	-	-	-	+	-	0	0
	Saudi Arabia	1997	Patient	2	-	-	-	-	-	-	+	+	0	0
	Banglad	1992-1995	SW	3	-	-	-	-	-	-	-	+	0	0
	Banglad	1992-1995	SW	19	-	-	-	-	-	-	-	-	0	0
	Banglad	1994-1996	SW	23	-	-	-	-	-	-	+	+	0	0
	Banglad	1993-1996	SW	84	-	-	-	-	-	-	+	-	1	0

[a] The presence of different genes was detected with DNA probes and PCR assays. The size of the PCR generated amplicon corresponding to the *tcpA* gene was characteristic of that of the El Tor biotype.

[b] Susceptibility to the phage was determined with a genetically marked phage, CTX-KmΦ.

[c] Integration of the phage genome into the host chromosome was determined by Southern blot analysis of genomic and plasmid DNA isolated from native and infected strains.

[d] SW, surface water.

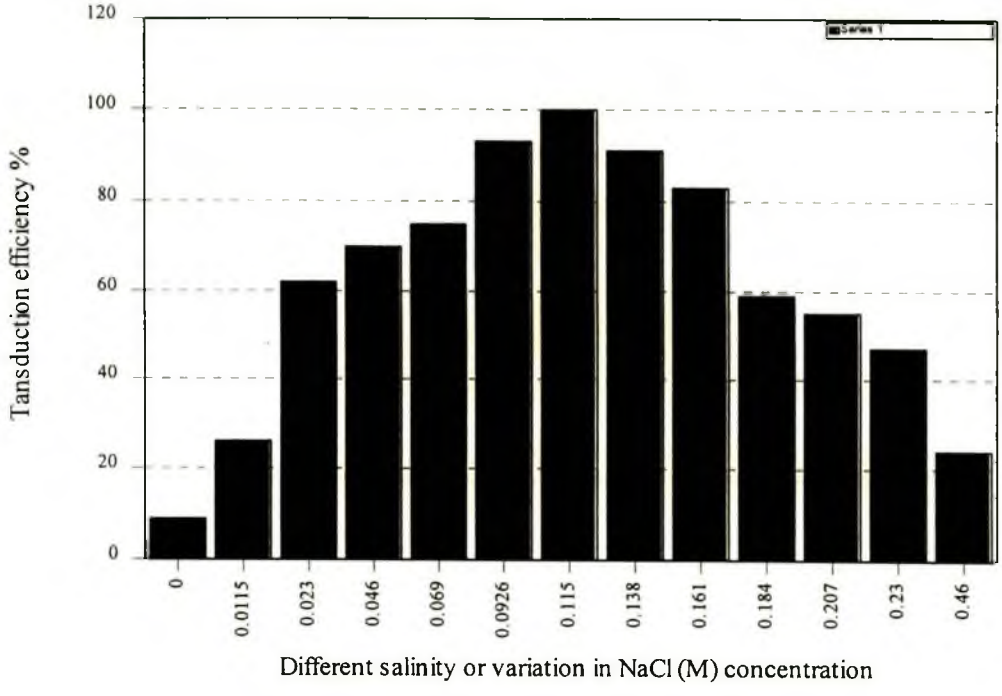
3.4 EFFECT OF SALINITY ON TRANSDUCTION EFFICIENCY:

Significant variation on the transduction efficiency was observed with the variation of salinity. At around 0.1 M NaCl, transduction efficiency was the maximum. Above and bellow this concentration reduction in the transduction efficiency was clearly observed. At 0 salinity transduction efficiency was only 9% of the maximum. As the strain RV-508 constitutively express TCP, the primary receptor of CTXΦ, so the variation in the infection frequency due to the variation in the salinity was solely considered as the property of the filamentous bacteriophage CTXΦ (Figure 3.4).

3.5 EFFECT OF pH ON TRANSDUCTION EFFICIENCY:

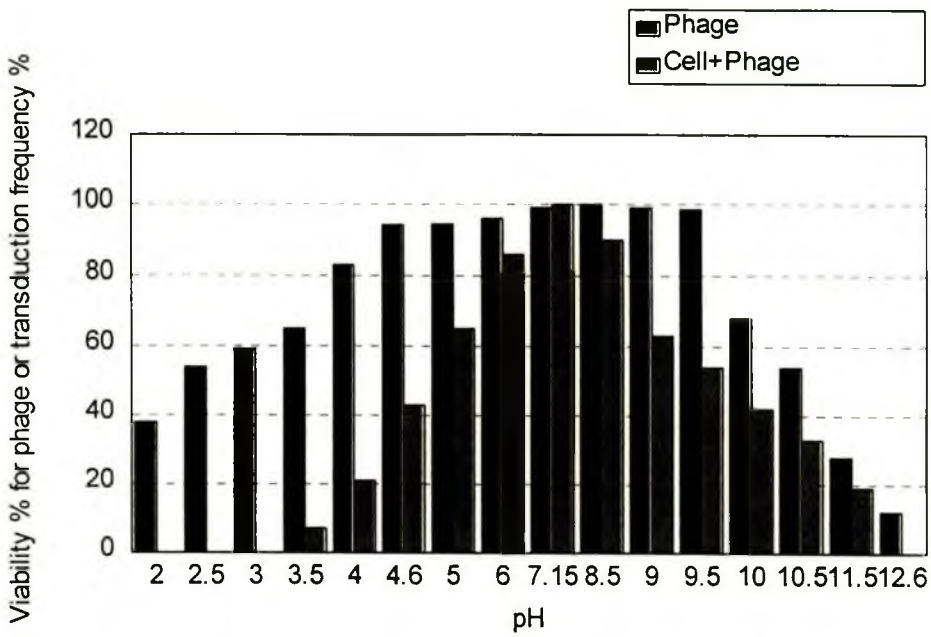
Variation with pH transduction rate was also varied significantly. Transduction rate was maximal at around pH 7.0. CTXΦ infected the recipient RV-508, that constitutively express TCP, over a pH range of 3.5 and 11.5 with varied efficiency. Figure 3.5 shows that above and bellow pH 7.15 rate of transduction gradually declined.

Figure 3.4: Effect of salinity on transduction



The effect of salinity on transduction of CTXΦ after incubation of the phage and recipient cell at different salinity levels, using the genetically marked CTX-KmΦ (please see text for details). Effect of salinity was assayed at pH 8.0.

Figure. 3.5: Effect of pH on transduction



Effect of pH on the transduction of CTXΦ, assayed after incubation of recipient cell + phage and phage alone at different pH for an hour. Preparations were incubated in LB adjusted to different pH. Experiment was carried out with genetically marked phage CTX-KmΦ (details in text).

3.6 PROPAGATION OF CTXΦ:

CTXΦ was found to propagate at a lower rate (0.06-0.12%) without induction, between donor (SM-44 and ASF1) and recipient (EV-99 and AOE-12/39) strains in vitro (Table 3.6). Sunlight exposure significantly increased in vitro transduction of the recipient strains. When the mixed cultures were exposed to sunlight, the number of transductants was more than 20-fold higher (3.45 or 3.49%) than the number obtained in the absence of sunlight (Table 3.6). The overall transduction efficiency was higher in the rabbit ileal loops than in vitro, and the in vivo efficiency increased further when the mixed cultures were exposed to sunlight prior to inoculation in rabbits (Table 3.6).

3.7 PRODUCTION CT BY TRANSDUCED STRAINS:

The lysogens of the O139 strains were tested for the production of CT and were found to produce high level of biologically active CT. Results are presented in (Table 3.7). Results of CT production was compared with the CT production by a toxigenic El Tor strain P-27457, which is also shown in the table. CT production was undetectable in non-toxigenic progenitor strain and the strains with pCTX-Km (Table 3.7).

Table 3.6: Transduction of *V. cholerae* strains by CTXΦ derived from sunlight induced lysogens carrying genetically marked CTX prophage

Donor	Recipient	% of recipient cells transduced			
		In vitro		Rabbit Ileal loop	
		Without induction	After induction	Without induction	After induction
SM-44	AOE-12/39	0.06	3.45	11.82	25.21
ASF-1	AOE-12/39	0.12	3.94	7.49	15.12
SM-44	EV-99	0.06	3.45	9.52	25.21
ASF-1	EV-99	0.12	3.94	4.49	17.16

Transductants of O139 strains were examined by screening kanamycin-resistant colonies using an O139 specific DNA probe (please see text for details). Values are the average of five different observations made in different rabbit models. Results are expressed as a percentage of total number of recipient cells recovered from rabbit ileal loop.

Table 3.7: Production of cholera toxin by CTXΦ-infected derivatives of nontoxigenic *V. cholerae* O139 strains.

Strain	Characteristic(s)	CT production	Fluid accumulation in rabbit ileal loops (ml/cm of ileal loop)
P-27457	Toxigenic El Tor, *V. cholerae* O1 Strain	2.65±0.56	2.27±0.42
AOE-12/39	Wild type, CTX negative V. Cholreae O139 strain	UD(<0.01)	0
AOE-12/39(pIG-KM)	Strain AOE-12/39 lysogenized with IG-KmΦ	3.29±0.71	2.53±0.7.
AOE-12/39(pCTX-KM)	Strain AOE-12/39 lysogenized with CTX-KmΦ	UD(<0.01)	0
EV-99	Wild type, CTX negative V. Cholreae O139 strain	UD(<0.01)	0
EV-99(pIG-KM)	Strain EV-99 lysogenized with IG-KmΦ	2.79±0.62	2.45±0.65
EV-99(pCTX-KM	Strain EV-99 lysogenized with CTX-KmΦ	UD(<0.01)	0

[a] Determination by GM1 ganglioside-dependent enzyme-linked immunosorbent assay. Toxin amounts are expressed in micrograms per unit of optical density of the culture at 600nm.

[b] Values are the average of five different observations.

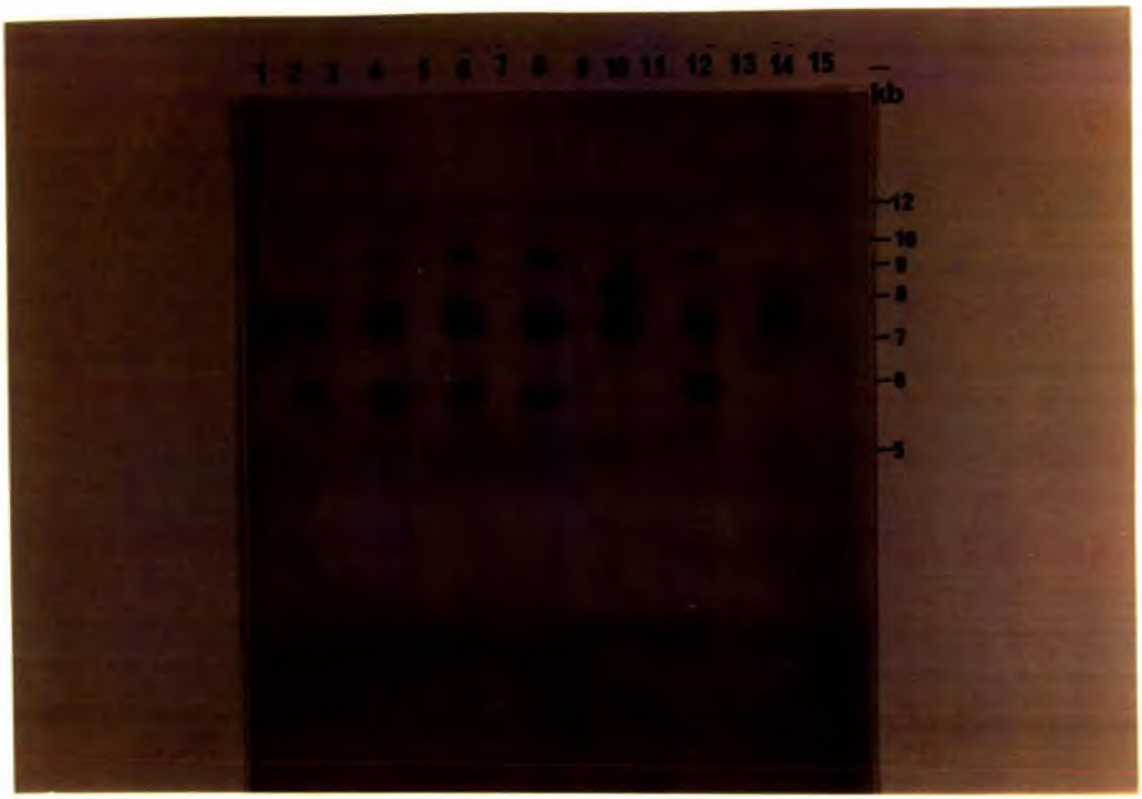
[c] UD, undetectable. The toxin amounts were less then 0.01 μg/ml, which was the lowest concentration of the purified toxin used as a control (details in text).

3.8 ANALYSIS OF THE INTEGRATION OF THE PHAGE GENOME INTO HOST CHROMOSOME:

In all *V. cholerae* 01 strains that were infected by CTX-KmΦ, the phage genome was found to be integrated into the chromosome of the host, forming lysogens (Figure 3.8.1). Hybridization of genomic DNA digested with *Bgl*I from infected and corresponding wild-type progenitor strains with the *zot* probe produced three bands corresponding to the *zot* gene (6). Interpretation of the restriction patterns confirmed that the phage genome integrated into the chromosome of the infected *V. cholerae* cells, and two copies of phage genome were present in tandem repeats in the host chromosome (Figure 3.8.1). Although plasmid preparations from the freshly infected cells showed the presence of pCTX-Km (Figure3.8.1) (band at the middle corresponding to the size of the digested plasmid), the lysogens spontaneously lost the RF of the phage genome, as confirmed by subsequent plasmid preparations (data not shown). In the single non-01, non-0139 strain, which was infected by the phage, the phage genome was maintained as the RF and produced a band corresponding to the linearized pCTX-Km (Figure 3.8.1).

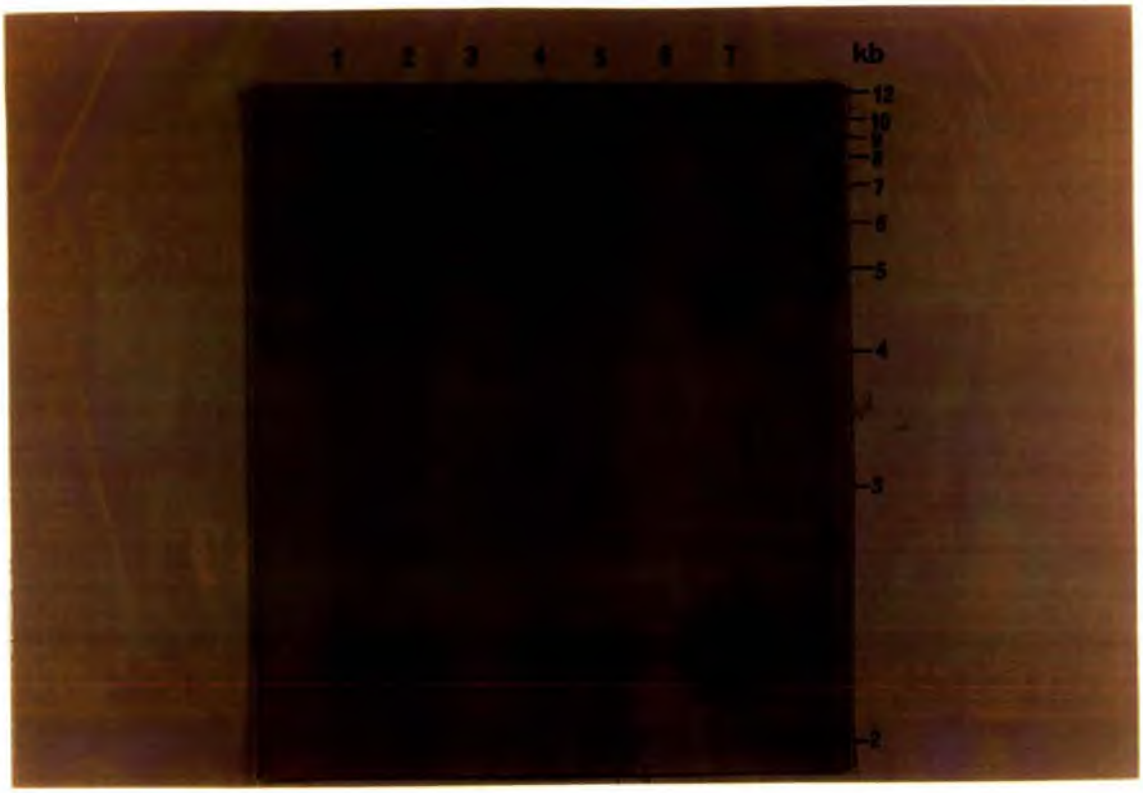
Non-toxigenic O139 strains of the propagation study were also found to carry integrated IG-KmΦ genome into their chromosome. Their DNA was digested with *Bgl*I and after hybridization with CT probe one integrated copy IG-KmΦ genome was found in their chromosome. In case of the pIG-Km plasmid, DNA was hybridized at two bands due to its structural difference with pCTX-Km (Figure 3.8.2).

Figure 3.8.1: Analysis of the genomic DNA of the lysogenized El Tor strains.



Southern hybridization analysis of the genomic DNA and plasmids isolated from *V. cholerae* strains infected with CTX-KmΦ and the native strains. Total DNA and plasmids were digested with *Bgl* I and probed with an 850-bp PCR generated probe for the *zot* gene. Lane 1, pCTX-Km digested with *Bgl* I, lanes 2, 4, 6, 8, 10, 12 and 14 are total DNA of non toxigenic strains Env-002, SA-317, SA-590, W-03, W-05, and 55V71respectively; lanes 3, 5, 7, 9, 11, 13, and 15 are the corresponding derivatives of the non-toxigenic strains infected with CTX-KmΦ. Numbers indicating the molecular size of the bands correspond to a 1-kb DNA ladder (Bethesda Research Laboratories).

Figure 3.8.2: Analysis of the genomic DNA of the lysogenized O139 strains.



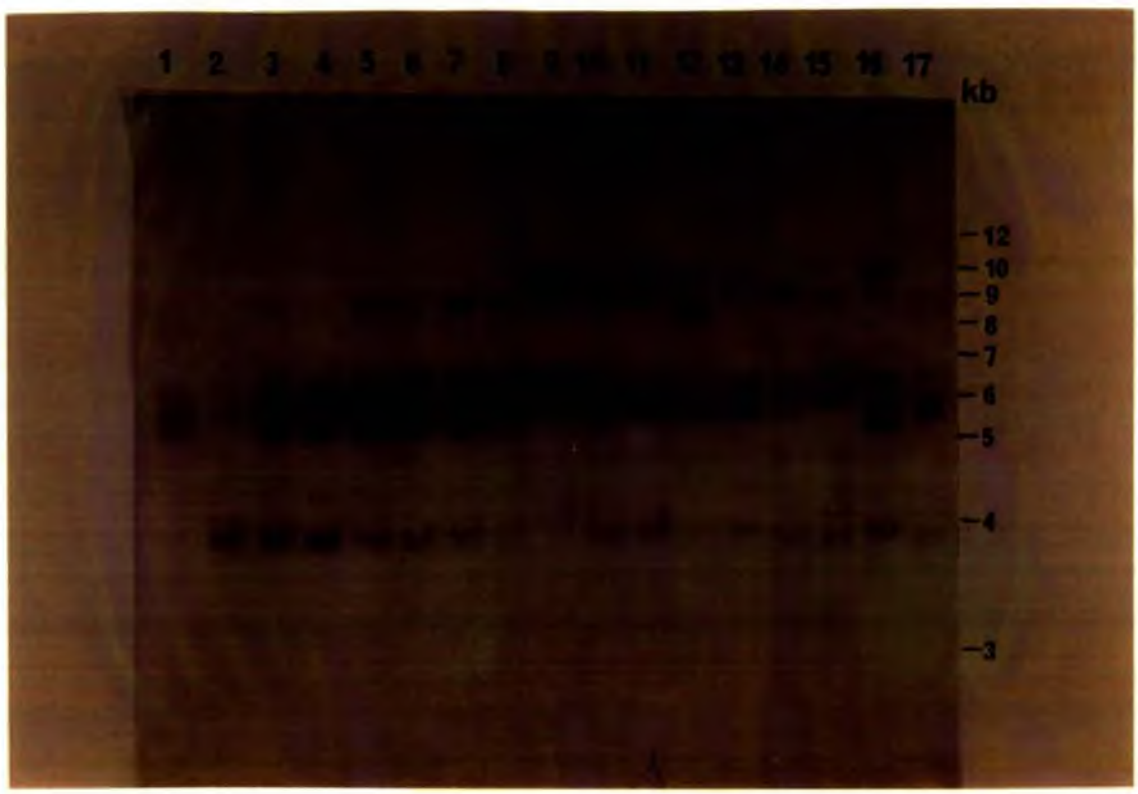
Southern hybridization analysis of the genomic DNA and plasmids isolated from *V. cholerae* strains infected with IG-KmΦ and the native strains. Total DNA and plasmids were digested with *Bgl* I and probed with *ctxA* probe. Lanes 1 and 2 are total DNA of two colonies EV-99 (pIG-Km) and lanes 3, total DNA of the corresponding non-toxigenic O139 strain EV-99. Lane 4 and lane 5 are total DNA of AOE-12/39(pIG-Km) and lane 6, total DNA of the corresponding non-toxigenic O139 strain AOE-12/39. Lane 7, pIG-Km digested with *Bgl* I. Integration of IG-KmΦ DNA into the chromosome of the recipient strains is shown. Numbers indicating the molecular size of the bands correspond to a 1-kb DNA ladder (Bethesda Research Laboratories).

3.9 ANALYSIS OF THE rRNA GENE RESTRICTION PATTERN:

rRNA gene restriction pattern analysis (ribotyping) was performed for the new lysogens derived from nontoxigenic progenitors or the native nontoxigenic O1 El Tor strains and compared with the patterns of those previous epidemic isolates as well as with published data on the ribotype patterns of toxigenic *V. cholerae* strains (18, 26-28,79). Figure 3.9.1 shows *Bgl*I restriction patterns of rRNA genes of nontoxigenic *V. cholerae* 01 strains and selected toxigenic strains of *V. cholerae* 01 (control ribotypes) isolated from epidemic outbreaks of cholera. Lanes 1 and 2, TCP-negative CTX-KmΦ-resistant strains Fin-41642 and Fin-41643, isolated from seawater in Estonia and Latvia, respectively, in 1995; lanes 3 through 7, TCP-positive CTX-KmΦ-susceptible strains W-03, W-05, W-7, W-10, and W-21 respectively, isolated from patients in India in 1993; lanes 8 and 9, TCP-positive CTX-KmΦ-susceptible strains SA-317 and SA-674, respectively, isolated from patients in Saudi Arabia in 1997; lanes 10 through 15, epidemic strains of toxigenic *V. cholerae* isolated in different countries (the strains and their countries and years of isolation are as follows: AK-21445, Bangladesh, 1995;Syria-5, Syria, 1992; AF-9710, Bangladesh, 1990; 62-6-91, Tanzania, 1991; AG-19438, Bangladesh, 1991; and AH-806, Bangladesh, 1992). Nontoxigenic *V. cholerae* 01 strains analyzed in the present study revealed four different *Bgl*I restriction patterns of their rRNA genes (Figure 3.9.1). The four different patterns corresponded to strains collected from four countries---Estonia, Latvia, India, and Saudi Arabia ---whereas all of the 01 strains collected from each of these countries were clonal and produced identical restriction patterns (Fig. 3.9.1). The rRNA gene restriction patterns of the *V. cholerae* 01 strains from Estonia, Latvia, and Saudi Arabia were distinctly different from the restriction patterns of *V. cholerae* previously reported (18, 26-28,79).

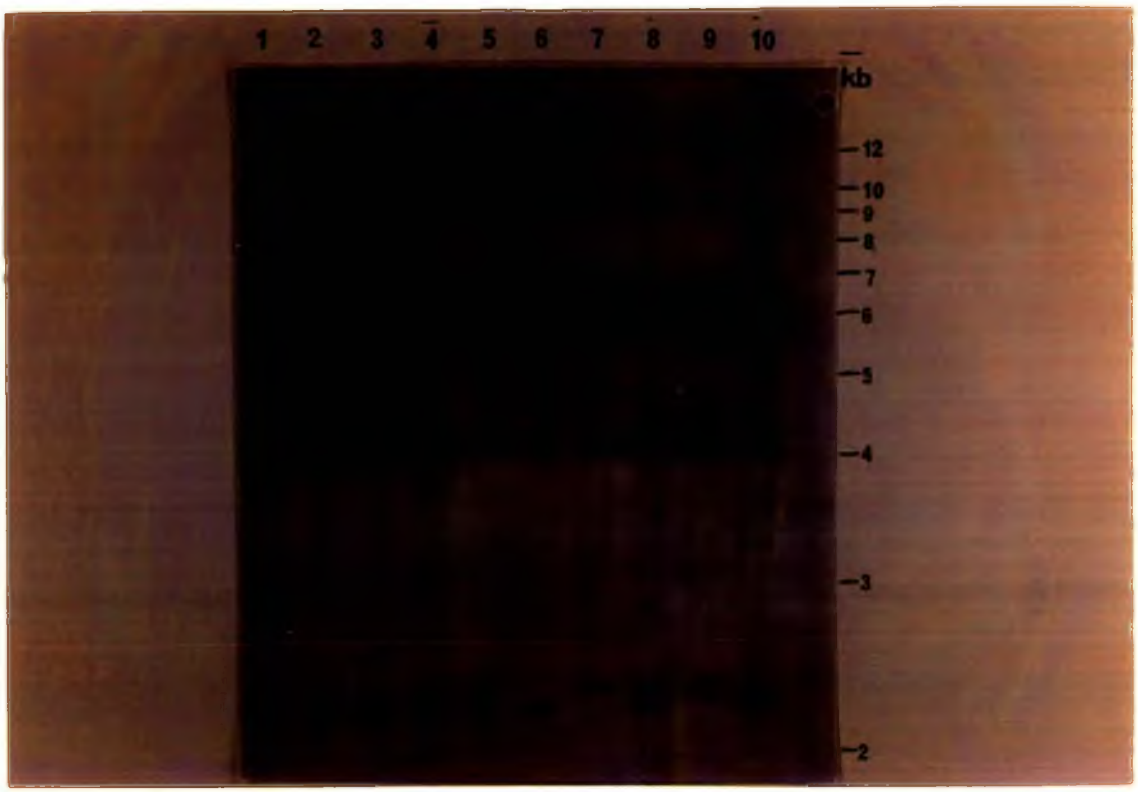
rRNA gene restriction pattern analysis was also carried out for the non-toxigenic O139 and their derivatives produced by lysogenic conversion of IG-KmΦ. Both the strains were of same ribotype and their ribotype was similar to one of the toxigenic ribotype of O139 strains. Figure 3.9.2 shows the different O139 control ribotypes with the ribopypes of the nontoxigenic progenitors or their derivatives.

Figure 3.9.1: Analysis of the ribotypes of the O1 El Tor strains.



*Bgl*I restriction patterns of rRNA genes of nontoxigenic *V. cholerae* 01 strains compared to those of selected toxigenic strains of *V. cholerae* 01 isolated from epidemic outbreaks of cholera. Lanes 1 and 2, Fin-41642 and Fin-41643, isolated from seawater in Estonia and Latvia, respectively, in 1995; lanes 3 through 8, W-03, W-05, W-06, W-07, W-10, and W-21 respectively, isolated from patients in India in 1993; lanes 9 through 11, SA-317, SA-406 and SA-674, respectively, isolated from patients in Saudi Arabia in 1997; lanes 12 through 17, epidemic strains of toxigenic *V. cholerae* isolated in different countries. Numbers indicating molecular sizes of bands correspond to a 1-kb DNA ladder (Life Technologies Gibco BRL; Gaithersburg, MD).

Figure 3.9.2: Analysis of the ribotypes of O139 strains.



*Bgl*I restriction patterns of rRNA genes in *Vibrio cholerae* 0139 strains isolated between 1992 and 1999, and the two non-toxigenic O139 progenitor strains. Lanes 3-7, Ribotype patterns B-I through B-V produced by toxigenic strains. Lanes 8-10, Ribotypes B-I, B-VI, and NB-1, respectively, which were produced by nontoxigenic 0139 strains, and lanes 1 and 2 are the Ribotype pattern of two non toxigenic environmental O139 progenitor strains and their derivatives. Numbers indicating molecular sizes of bands correspond to 1-kb DNA ladder (Life Technologies Gibco BRL; Gaithersburg, MD).

Chapter 4

DISCUSSION

4. Discussion

Toxigenic *V. cholerae* strains that cause cholera, are lysogens of a filamentous bacterial virus, known as CTXΦ, which carries the genes for cholera toxin. CTXΦ is supposed to propagate by infecting susceptible strains of *V. cholerae* by using a pilus termed toxin-coregulated pillus (TCP) as the receptor that binds the phage on to the cell surface. Infection of a susceptible nontoxigenic *V. cholerae* cell by CTXΦ leads to the transformation of non-toxigenic strain into a toxigenic derivative. Propagation of the bacteriophage is directly dependent on its stability and the optimized condition of the environment to infect the susceptible host. Keeping these in mind the effect of temperature, pH, salinity were studied over the stability and infection rate of the CTXΦ. Phage induction is the prerequisite for the propagation. In laboratory chemical inducer can induce lysogenic state and phage production commences. But in nature, it is obvious that some unknown natural phenomena play the role as inducers to induce phage production and hence propagation. The present study was conducted to find out whether the CTXΦ is inducible or not, and if it is inducible what natural phenomena plays the role as inducer. Speculating the probable role of CTXΦ in the origination of new toxigenic strains, stability of cell free phage particles was studied in various laboratory conditions. As the sunlight was detected as significant natural inducer, propagation between marked (kanamycin resistant) donor and non-toxigenic recipient strains was studied. *In vivo* and *in vitro* transduction by CTXΦ was studied with the non-toxigenic strains to find out their susceptibility. All these results along with the available information suggest a clear mechanism for the development of new toxigenic strains with epidemic potential.

CTXΦ is inducible lysogen:

Induction of lysogenic CTXΦ was investigated by culturing the toxigenic *V. cholerae* strains to produce extracellular infectious particles in the presence of a DNA-damaging agent, mitomycin C, and then screening the culture supernatant fluids for the presence of CTXΦ by using DNA probes. Out of 141 toxigenic O1 El Tor and O139

strains analyzed, 54 were found to produce detectable CTXΦ in their culture supernatant. Most of the genes in the core region of the CTXΦ genome play a crucial role in the morphogenesis of the phage that has been shown previously (112). 87 of the 141 strains were unable to produce CTXΦ particles after induction with mitomycin C (Table 3.1.1). The reasons may be that the integrated form of CTXΦ carried by these strains is defective due to possible mutations in one or more genes essential for phage morphogenesis. Both *V. cholerae* 01 and 0139 strains were included to analyze in this study carrying variable number of copies of the CTX element, and a difference was noted in terms of induction of CTXΦ in the strains belonging to these serogroups. It was observed that the phage is more often inducible in 0139 strains (46.29%) than in El Tor strains (33.33%). Generally, 0139 strains are known to carry more than one copy of the CTX genetic element (25, 110), this may account for the higher prevalence of inducible lysogens among 0139 vibrios. In the present study, CTXΦ was induced by the DNA-damaging agent mitomycin C, which is known to induce many temperate bacteriophages. However, six strains including two environmental strains produced a detectable level of extracellular CTXΦ particles, even without treatment with mitomycin C (Table 3.1.1), indicating that unidentified environmental factors or possible mutations in the phage or the host bacteria might have caused induction of the CTXΦ in these strains.

Sunlight is recognized as inducer of lysogenic CTXΦ:

Both wild type toxigenic strains and genetically modified (harboring Km^r determinant) strains were used to study the effect of sunlight on phage induction. Initially two strains, SM44 and ASF-1, harboring Km^r determinant-marked CTX prophages were used to study parameters influencing prophage induction. As the CTXΦ doses not form visible plaques, genetically marked prophages allow quantification of the number of phage particles secreted in supernatants via transduction assays (112). Initially, a large number of individual colonies of strains SM-44 and ASF-1 were picked and tested separately for the production of Km^r-transducing particles. When grown without artificial induction, most colonies tested were found either negative or produced a very small

number of phage particles (between 5 and 127 particles per ml). The number of spontaneous CTXΦ transducing particles produced by SM44 or ASF-1 was significantly lower than the titers previously reported for two other CTXΦ lysogens (59). Presumably, this reflects strain differences.

Selected colonies that spontaneously produced a small number of phage particles (<50/ml) were used to examine the effect of sunlight on the induction of CTX prophages in SM-44 and ASF-1. No significant difference in the CTX-KmΦ transducing activity of the culture supernatants was noted when the pH, salinity, or temperature of the cultures was varied. However, when the cultures were exposed to direct sunlight (mean intensity of 42,000lx) for 30 min prior to incubation, the number of phage particles increased more than 10,000-fold (Table 3.1.2a). No increase was noted in control cultures, which were stored in a well-lighted area (average of 3,180 lx), but covered from sunshine. This showed that direct sunlight is a significant natural inducer of the CTX prophage. At different stages of cell growth showed a steady increase of the production of phage particles for nearly 6 hrs in sunlight-induced cultures was clearly observed (Figure 3.1.2a). This was followed by a sharp decline, which corresponded, to the stationary phase of the culture (Figure 3.1.2a). These findings are in agreement with a previous report (59) that infectious CTXΦ particles are rapidly inactivated during the stationary phase of a culture by a secreted CTXΦ-destroying factor identified as the hemagglutinin protease of *V. cholerae*. The results also support the typical nature of the growth curve of a filamentous bacteriophage CTXΦ (66). It was also confirmed that the rapid decline in CTXΦ titers in stationary-phase cultures of SM44 and ASF-1 was secondary to CTXΦ-destroying factor activity (59). In another study with some other strains it was also observed that CTXΦ remains in the culture after the stationary-phase of growth. Possibly these type of strain may play role in the horizontal transfer of the phage genome significantly (work in progress).

32 wild-type toxigenic *V. cholerae* strains was checked for the presence of CTXΦ in culture supernatants after induction with sunlight and phage was detectable from 25 of

them (Table 3.1.2b) by Southern blot hybridization using a plus strand-specific oligonucleotide probe (Figure 3.1.2b). Wild type strains while grown without sunlight induction, the band corresponding to the single-stranded phage DNA was absent in 30 of the 32 preparations derived from culture supernatants. In control assays, approximately 10^3 transducing particles produced a visible band. Thus, some of these wild-type strains may have produced a small number of phage particles during normal growth that was below this level of detection.

The duration of sunlight exposure as well as the intensity of sunlight had a significant effect on both phage titer and viable cell number. The effect of sunlight was roughly proportional to the product of light intensity and duration of exposure. Exposure to sunlight resulted in cell death, but phage production by the surviving cells was increased. For example, immediately after exposure to sunlight at an average intensity of 42, 000 lx for 30 min, the viable cell count was reduced by approximately 6%, and the total phage production by the surviving cells increased more than 20-fold (Figure 3.1.2a).

The CTXΦ genome is comprised of a core region and an RS2 region (113). With the exception of *ctxAB*, the genes of the core region are thought to be essential for morphogenesis of CTXΦ particles and hence for its propagation as an infectious phage. The open reading frames in RS2, *rstR*, *rstA*, and *rstB*, encode a repressor (RstR) as well as products required for the replication and integration of CTXΦ (113). In lysogens, RstR is thought to repress transcription of *rstA*, a gene required for CTXΦ replication. Our data suggest that RstR repressor function is probably inactivated by direct sunlight. Inactivation of RstR presumably leads to the expression of *rstA* and thereby allows the CTX prophage to enter into a replicative pathway. To begin to address this possibility, the sunlight-exposed cells were examined for the presence of CTXΦ RF DNA. This was accomplished by hybridizing plasmid preparations with a minus strand-specific oligonucleotide probe, which would not hybridize with plus strand found in the CTXΦ genome. The CTXΦ RF DNA was detectable in Southern blots of plasmids prepared from sunlight-exposed cells and not from unexposed cells. This suggests that the

sunlight-induced increases in phage production in strains carrying the CTX prophage were preceded by and mediated by production of the RF DNA of CTXΦ. This is further supported by the observation that phage titers in the culture supernatants of strain SA-406 (pCTX-Km), which carried the RF of the phage genome, did not increase substantially after the culture was exposed to sunlight (Table 3.1.2a). High titers of the phage were detected in the supernatants from cultures of this strain, irrespective of whether the cells were exposed to sunlight or kept in the shade. However, further studies are required to investigate the detailed molecular mechanisms associated with sunlight-mediated induction of the CTX prophage.

Stability is influenced by salinity, pH, and temperature:

In the natural habitat, the physiological state of *V. cholerae* is expected to be influenced by environmental parameters, such as temperature, pH and salinity of the aqueous environment. The possible effect of these parameters on the induction, stability and availability of infectious CTXΦ particles was studied in laboratory microcosms. The effect of salinity was studied over a broad range from 0% to 10% NaCl in distilled water. In this experiment it was revealed that higher salinity have significant effect on the stabilization of phage particles (Figure 3.2.1a). The stabilization effect was much clearer at higher temperature (37°C), because, it was observed that at higher salinity (over 2%), there was comparative increment in the availability of infectious particles for a longer time compared to that of at lower values. The effect of temperature on the stability of phage particles was easily deducible from figure 3.2.1a. A rise in temperature at each salinity concentration was accompanied by a reduced stability of infectious particles (Figure 3.2.1a, 3.2.1b). The effect of salinity was later studied by supplementing different concentration NaCl in the Turag river water of pH 7.53 (Figure 3.2.1b). The result was exactly similar to the previous one and at higher salinity stabilization effect was clearer. Variation in salinity showed various duration in the availability of phage particles, when phage particles were stored in aliquots of 5ml volume. Their stability period was compared with the stability in distilled water and normal saline (0% and 0.9% NaCl), and

there was a clear indication about increased stability in water having salinity. These findings suggest that phage particles may persist in the aquatic environment as infectious agents, depending on these and possibly other parameters. Environmental water samples collected from different parts of the country were also studied for their presence of free phage particles and *V. cholerae*. Seasonal appearance and disappearance of *V. cholerae* was observed, but no phage was found. This does not eradicate the possibility of the presence of CTXΦ in the ecosystem, because of the limitations of our detection range ($\geq 10^3$).

In another experiment the stability of phage was studied over a broad range of pH (pH 2 and pH 12.6), expectedly CTXΦ was found to behave like a typical filamentous bacterial virus (Figure 3.2.3)(66). The virion was found to be stable between pH 2.5 and pH 10.5. Significant transduction efficiency was also observed at pH range from pH 4.5 to pH 9.5. Significant reduction in the recipient cell (RV-508) number was also observed above pH 10.5 and bellow pH 4.5.

CTXΦ infects recipient *V. cholerae* more efficiently in the intestinal environment where virulence factors such as TCP are adequately expressed (112). Effect of pH on the viability and infection frequency of CTXΦ keeps a clear indication about the negative role of the stomach in the conversion of nontoxigenic strain within the gastrointestinal tract of the mammalian host as the pH of the gastric juice is as low as pH 1.5 (63). Negative Role of the stomach is probably nullified by the liquid meal that has a lower gastric emptying time than the solid meal (63). The result of this study showed that CTXΦ is significantly stable over a pH range of 3.5 and 9.5. Bellow this pH range quick degradation of the phage particles occurred with prolonged storage. It was also observed that, transduction does not occur bellow the pH of 3.5. Individuals with low gastric acid production, who are on acid suppressing medication, or who have had gastrectomies are especially vulnerable, since *V. cholerae* is quite sensitive to acid (3). So it is not impractical to speculate that these individuals could be a good *in vivo* system for the transformation of naturally occurring nontoxigenic progenitor strains by the CTXΦ to a

toxigenic one with epidemic potential, and can act as a primary host in the spread of the disease.

Non toxigenic strains are suspected progenitors: in vivo and in vitro demonstrations

Genetically marked derivative of CTXΦ designated CTX-KmΦ (112) was used to study the susceptibility of the strains because the Km^r marker allowed conveniently to detect and analyzed infected cells. Two different methods were used to test the susceptibly of the nontoxigenic strains to CTX-KmΦ. Initially, all the strains were exposed to cell-free phage particles under *in vitro* laboratory conditions. Of the 146 nontoxigenic strains exposed to the phage, 10 strains belonging to the 01 serogroup and 1 non-01, non-0139 strain were infected by the phage. However, the 01 strains, which carried the TCP genes, were infected more efficiently than the TCP-negative, non-01 strain (Figure 3.3).

TCP is the receptor for CTXΦ infection and the efficient expression of TCP occurs *in vivo*, which is a prerequisite for susceptibility to the phage (112), infant mouse model was used to further test the susceptibility of the 11 strains, which were infected in the initial screening. When the TCP-positive strains were tested in the mouse intestine, the proportion of infected cells recovered was higher than the in vitro. The strain that was devoid of TCP, the infant mouse assay did not produce a higher level of infection than the *in vitro* assay (Figure 3.3). In the present study, the portion of infected cells recovered from both *in vitro* and *in vivo* studies possibly included progeny of infected cells as well as fresh infection of uninfected cells with phage particles derived from infected cells during the prolonged incubation period. Nevertheless, for the TCP-positive strains, the proportion of total cells carrying the phage genome was found to be significantly higher when the strains were grown inside the mouse intestine than after an equal length of incubation under laboratory conditions. Although these results supported previous reports (112) that recipient strains were more efficiently infected under conditions favorable to the expression of TCP, this study also demonstrated that at least one strain which did not carry the genes for TCP was infected by the phage, even though at a low efficiency

(Figure 3.3). The TCP-negative non-01, non-0139 strain was distinctly susceptible to the phage in repeated *in vitro* assays, producing a small number of infected colonies, while the rest of the 135 TCP-negative strains were completely resistant to infection by the phage when assayed under identical conditions. It is not clear what determined the different phage sensitivities of these TCP-negative strains. These results indicated that in addition to the TCP-mediated mechanism, there might be a second mechanism for CTXΦ infection. In agreement with this observation, a study in India has reported the presence of CT-positive but TCP-negative *V. cholerae* strains belonging to non-01 serogroups from both patients and the environment (33).

Unequal distribution of the virulence genes among non toxigenic V. cholerae:

Toxigenic *V. cholerae* strains isolated from cholera patients simultaneously carries genes for CT, TCP, and ToxR in most cases (27,28). In order to identify CTX-negative strains which are possible progenitors or intermediates in the origination of new toxigenic strains, the distribution of genes encoding TCP and ToxR and the CTXΦ attachment sequence (att_{RS}) was studied among nontoxigenic strains of *V. cholerae* with either DNA probes or PCR assays. Since the genes encoding TCP have been suggested to be part of a large genetic element consisting of clusters of genes, studies were carried out to look for the simultaneous presence of the *tcpA, tcpI,* and *acfB* genes to screen for the presence of the TCP pathogenicity island (56,60). DNA probe and PCR analysis of a total of 146 CTX-negative strains belonging to the 01 and non-01 serogroups (Table 3.3.2) showed four different types of patterns: (i) strains positive for *toxR* but negative for TCP and att_{RS};, (ii) strains positive for *toxR* and att_{RS} but negative of the TCP pathogenicity island, (iii) strains positive for the *tcpA, tcpI*, and *acfB* genes and presumably the entire TCP pathogenicity island in addition to *toxR* and att_{RS}, and (iii) strains positive for att_{RS} alone but negative for *toxR* and TCP. All strains simultaneously positive for TCP, *toxR,* and att_{RS} belonged to the 01 serogroup and were of clinical origin, whereas none of the non-01, non-0139 strains analyzed in the present study carried the genes for TCP. In addition, three *V. cholerae* 01 strains isolated from seawater in Estonia and Latvia were

also negative for TCP. Previous studies (83, 89, 104) have reported the absence of TCP in *V. cholerae* 01 or non-01 strains, which do not produce CT. The present study demonstrated the existence of TCP-positive, CT-negative *V. cholerae* strains, although such strains were less prevalent than TCP-negative strains. The present study identified 10 isolates of *V. cholerae* 01 from Saudi Arabia and India, which were TCP positive and CT negative, these results did not reflect the real prevalence of TCP-positive, CT-negative strains. Since this study did not analyze a statistically defined proportion of strains from patients or environments in different countries. It can be assume that the real prevalence of TCP-positive, CT-negative strains should be considerably lower, taking into consideration previous studies in which no such strain was isolated from the environment or from patients (33,83,104). Nevertheless, the demonstration of the existence of TCP-positive, CT-negative strains in the present study is significant for understanding possible mechanisms involved in the origination of novel toxigenic strains of *V. cholerae*. The reason for the observed low prevalence of CT-negative but TCP-positive strains may be that these strains do not cause full-blown cholera and hence are not adequately enriched through interactions with the mammalian host. Previous studies have shown that CT- positive *V. cholerae* 01 strains are more enriched in the intestinal environment than the corresponding CT negative mutants (5). An alternative explanation for the low prevalence of CT negative but TCP-positive strains may be that such strains are rapidly converted to toxigenic strains by CTXΦ either inside the host intestine or in the environment. The *toxR* gene was found to be widely distributed among nontoxigenic *V. cholerae* strains and was present in 84.93% of the total strains tested. This was probably because ToxR is involved in the regulation of a number of other genes in addition to those encoding TCP and CT (70) and may be part of a common regulatory mechanism possessed by different serogroups of *V. cholerae*. Although toxR and att_{RS} sequences were shared by both 01 and non-01 strains, the possession of genes encoding TCP was confined to strains of the 01 serogroup. This also agreed with previous studies (33,83,89) in which *V. cholerae* non-01, non-0139 strains have been very rarely found to possess the genes for TCP.

In vitro transduction is affected by pH and salinity variation:

Variation in pH and salinity not only affect the stability of the CTXΦ, but also the transduction rate. This study have found that at a salinity of 0.115 M, and at a pH of 7.15, the transduction was maximum (Figure 3.4 & 3.5). The results are in accordance with the property of a typical filamentous bacteriophage (66). The strain, RV-508, that constitutively expresses TCP, the receptor for CTXΦ was used in this study. As the strain constitutively expresses TCP, infection rate is dependent on other parameters that can influence the adsorption and penetration of the phage particles into the cell. So, it can be strongly said that, the variation in the infection frequency is due to the variation in the salinity and pH is the sole characteristics of the filamentous bacteriophage CTXΦ. These data also suggest that appropriate salinity, pH and other conditions if satisfy the prerequisites for the expression of cell surface receptors for successful coalition CTXΦ with nontoxigenic *V. cholerae* in the ecosystem, then transformation of nontoxigenic strain to toxigenic one can be brought about.

Induction with sunlight facilitates the propagation CTXΦ:

The propagation of CTXΦ between strains was examined when mixed cultures of potential donor and recipient strains were exposed to sunlight. Transfer of kanamycin resistance to the recipient strains monitored transduction of the Km^r determinant-marked CTX prophages, carried by strains SM-44 and ASF-1. Normally it is not possible to select the transduced Km^r cells from the donor population, as both of them will be resistant for the same antibiotic. To over come this problem the strains EV-99 and AOE-12/39, which are resistant for both sulfamethoxazol-trimethoprim and streptomycin was selected. Transduced cells were selected with triple antibiotic resistance and finally verified with their presence of O139 specific DNA sequences, as these are the two non-toxigenic O139 strains. Transduction was assayed both under *in vitro* laboratory conditions and *in vivo* inside the ileal loops of adult rabbits. Previously infant mice was used to study *in vivo* transfer of CTXΦ, but it was observed that the rabbit ileal loop model is also useful for *in vivo* CTXΦ transduction assays. Sunlight exposure

significantly increased *in vitro* transduction of the recipient strains. When the mixed cultures were exposed to sunlight, the number of transductants was more than 20-fold higher than the number obtained in the absence of sunlight (Table 3.6). The overall transduction efficiency was higher in the rabbit ileal loops than in vitro, and the *in vivo* efficiency increased further when the mixed cultures were exposed to sunlight prior to inoculation in rabbits (Table 3.6). TCP, the receptor for CTXΦ, is known to be expressed more adequately *in vivo* (112). Induction studies clearly showed a marked increase in extracellular CTXΦ, so it is most likely that in addition to adequate expression of TCP, the increased efficiency of CTXΦ transduction *in vivo* was due to the availability of high titers of transducing particles in the inoculum which was preexposed to sunlight.

Genome of the CTXΦ stably integrates into the chromosome of non-toxigenic progenitors:

V. cholerae 01 strains were infected by CTX-KmΦ, the phage genome gets integrated into the chromosome of the host, forming lysogens (Figure 3.8.1). Genomic DNA was digested with *Bgl*I from infected and corresponding wild-type progenitor strains and the fragments after hybridization with the *zot* probe produced three bands for *Bgl*I digests corresponding to the zot gene (6). Interpretation of the restriction patterns confirmed that two copies of phage genome were integrated in tandem repeats into the chromosome of the infected *V. cholerae* cells (Figure 3.8.1). Although plasmid preparations from the freshly infected cells showed the presence of pCTX-Km, the lysogens spontaneously lost the RF of the phage genome, as confirmed by subsequent plasmid preparations (data not shown). The integrated form of the phage, however, was stable, and the lysogens did not lose the prophage when grown or stored in the absence of kanamycin. In the single non-01, non-0139 strain, which was infected by the phage, the phage genome was maintained as the RF and produced a band corresponding to the linearized pCTX-Km (Figure 3.8.1). This strain produced high titers of infectious phage particles in the supernatant fluids of the culture (mean, 2.7×10^6 particles/ml),as determined by titration with strain RV508 as the recipient. Previous studies (61, 112)

described the induction and transmission of CTXΦ in the intestines of infant mice based on observed transduction of recipient strains. In the present study, the presence of at least one non-01, non-0139 strain in the environment, which was capable of harboring the RF of CTXΦ and producing infectious phage particles, indicated that some environmental non-01 strains may have a role in supporting the replication and propagation of CTXΦ in the environmental habitat. A recent demonstration of the presence of CT-positive but TCP-negative non-01, non-0139 strains in the environment (33) supports this assumption. It is also interesting that a previous study in India reported an increased incidence of toxigenicity among freshwater isolates of *V. cholerae* non-01, non-0139 strains during a cholera outbreak caused by *V. cholerae* 0139 (32). This emphasizes the importance of further studies to understand the propagation of CTXΦ and its role in the ecology of *V. cholerae*.

Two nontoxigenic 0139 strains were transduced by a genetically marked phage, IG-KmΦ that carried a functional *ctxAB* operon in the present study. Subsequent analysis of the transductants DNA by digestion with *BglI* followed by Southern Hybridization it was observed that the phage genome was integrated into the 0139 chromosome, forming stable lysogens (Figure 3.8.2). These lysogens of the 0139 strains were tested for production of CT and were found to produce high levels of biologically active CT (Table 3.7). Thus, the lysogenic conversion of two nontoxigenic 0139 strains into toxigenic derivatives is successfully demonstrated, and this process was significantly enhanced by sunlight (Table 3.6).

Analysis of rRNA gene restriction patterns.

Molecular analyses of *V. cholerae* strains collected during different epidemics have shown clonal diversity among strains and a continual emergence of new epidemic clones in cholera endemic regions (26-28). Conal diversity was demonstrated based on restriction fragment length polymorphisms (RFLPs) in conserved rRNA genes in the previous studies. Present study performed rRNA gene restriction pattern analysis (ribotyping) of the new lysogens derived from nontoxigenic progenitors or the native

nontoxigenic strains and compared these patterns with those of previous epidemic isolates as well as with published data on the ribotype patterns of toxigenic *V. cholerae* strains (18,26-28,79). It was done with the restriction enzyme *Bgl*I, which was previously used by others for comparative analysis of the ribotypes. Nontoxigenic *V. cholerae* 01 strains analyzed in the present study revealed four different *Bgl*I restriction patterns of their rRNA genes (Figure 3.9.1). The four different patterns corresponded to strains collected from four countries---Estonia, Latvia, India, and Saudi Arabia ---whereas all of the 01 strains collected from each of these countries were clonal and produced identical restriction patterns (Figure 3.9.1). Strains from Saudi Arabia and from India were positive for the TCP pathogenicity island and were lysogenized by the CTX-KmΦphage, whereas those from Estonia and Latvia were negative for TCP and were resistant to the phage. The rRNA gene restriction patterns of the *V. cholerae* 01 strains from Estonia, Latvia, and Saudi Arabia were distinctly different from the restriction patterns of *V. cholerae* previously reported by other investigators who analyzed a large number of toxigenic *V. cholera* strains isolated in different countries (18,26-28,79). This data provided evidence that clonal diversity among epidemic strains of *V. cholerae* may have arisen, at least partly, due to the emergence of new toxigenic strains from nontoxigenic *V. cholerae* which are possibly undergoing a continuous genetic reassortment. This study also suggested that in an area where cholera is endemic, clinical and environmental surveillance for the presence of nontoxigenic *V. cholerae* carrying the genetic determinants for TCP ToxR and the att_{RS} sequence may be a means for predicting the origination of new toxigenic *V. cholerae* strains. The ribotype pattern of the new CTX-KmΦ lysogens derived from the nontoxigenic southern India strains in this study, however, was very similar to that of a clone of toxigenic *V. cholerae* strains isolated from cholera outbreaks in 1987 in Guinea-Bissau (18), situated along the West African coast. This suggested that the lysogenic conversion by CTXΦ demonstrated in the present study under laboratory conditions might indeed have been occurring efficiently in the ecological habitat.

The origins of strains belonging to the newly emerged epidemic serogroup of 0139 strains have been investigated in several previous studies (28, 101, 109). These studies suggested that the 0139 serogroup probably originated from a toxigenic strain of the El Tor biotype by changes in the genes determining the serogroup antigen. O139 strains of the propagation study and their derivatives were analyzed for their ribotype pattern, and it was observed that these two strains possess the same ribotype as one of the existing ribotype of the O139 strains (Figure 3.9.2). This supports the mechanism for the emergence of epidemic 0139 strains from nontoxigenic progenitors in the environment. Thus, lysogenic conversion may constitute an alternative mechanism for the emergence of epidemic 0139 strains from nontoxigenic progenitors in the environment.

Emergence of novel strains of toxigenic V. cholerae: Speculation on the role of CTXΦ as a component of the Ecosystem

Present study demonstrated that naturally occurring strains of toxigenic *V. cholerae* O1 and O139 are inducible lysogens of CTXΦ that can be induced *in vitro* by both chemical inducer mitomycin C and natural inducer sunlight. It seems possible that in the natural ecological settings, unidentified environmental factors induce lysogenic CTXΦ in toxigenic *V. cholerae* resulting in release of extracellular CTXΦ particles in the aquatic environment. The cell free phage particles participate in the emergence of novel toxigenic strains of *V. cholerae* through interactions with nontoxigenic strains, which exists in the environment and the human population, which consume the environmental waters. CTXΦ uses TCP as its receptor, and hence the phage can only infect *V. cholerae* cells expressing TCP. The TCP genes, which are part of a greater genetic element, referred to as the TCP pathogenicity island, appears to be the initial genetic factor required for the origination of epidemic strains. Analysis of the structure of the pathogenicity island suggests that this could be of phage origin and it has been speculated that the pathogenicity island can be transferred by transducing phages (56, 60). Genes responsible for the production of TCP are carried mostly by *V. cholerae* O1 or O139. Other serotypes of *V. cholerae* usually do not carry genes for TCP. It is therefore obvious

that the CTX element is also found mostly in the O1 and O139 vibrios while most non-O1 vibrios are usually nontoxigenic. This further supports the assumption that in the natural settings the CTXΦ probably plays an important role in the origination of new toxigenic strains of *V. cholerae*. Since *V. cholerae* strains, which are, TCP positive but CTX negative are not frequently isolated on environmental sampling, it is possible that such strains are normally present in very low numbers in the environment, but following conversion by CTXΦ to toxigenicity, the strains are enriched in the gastrointestinal environment, and later become detectable as new strains of toxigenic *V. cholerae* (Figure 4.1). Subsequent increase in the concentration of toxigenic *V. cholerae* in the aquatic environment may lead to epidemic outbreaks of cholera.

The study also demonstrated that CTXΦ infects recipient *V. cholerae* more efficiently in the intestinal environment where virulence factors such as TCP are adequately expressed (112). While the conversion of nontoxigenic *V. cholerae* is favored within the gastrointestinal tract of the mammalian host, the natural selection and persistence of the novel toxigenic strains may involve both intestinal and environment factors. Intestinal alkaline environment is the favorable condition for the growth and multiplication of this acid sensitive organism. Moreover the present study demonstrated that alkaline rather than acidic condition is favorable for the toxigenic conversion of the nontoxigenic progenitor strains by CTXΦ (Figure 3.5). Natural selection and persistence of the novel toxigenic strains may also involve the immune status of the host population (36), and antigenic properties of the new pathogenic strain.

Precise environmental signals such as optimum temperature, sunlight, osmotic conditions etc. probably control the induction of CTXΦ lysogens (Figure 4.1), and this may also account for the observed seasonal outbreaks of cholera in endemic regions. However in order to test this hypothesis, it is necessary to carry out further studies with environmental water samples in a cholera endemic region for the presence of cell-free CTXΦ particles during epidemic and interepidemic seasons and their ability to infect and

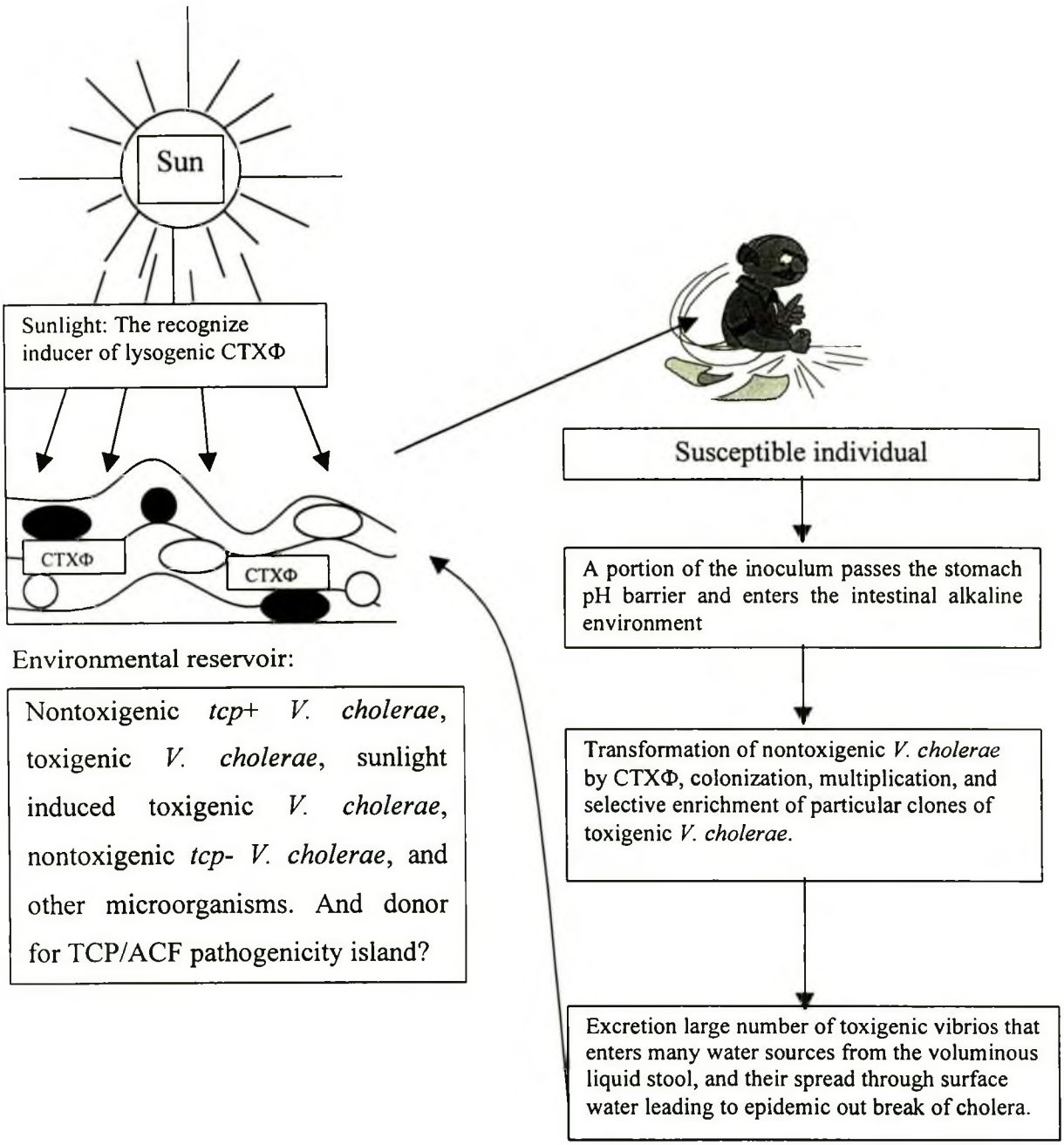


Figure 4.1: A model for the emergence of new strains of *V cholerae*: Possible role of CTXΦ, environmental factors, and the human host in the ecology of toxigenic *V. cholerae*.

lysogenize non-toxigenic strains of *V. cholerae* in animal models and laboratory microcosms, and characterize toxigenic and non-toxigenic *V. cholerae* strains isolated from the environment and cholera patients by genetic fingerprinting. Studies are also required to confirm the origin of the TCP pathogenicity island and its role in the ecology and evolution of epidemic *V. cholerae*. Our efforts are at present directed towards understanding the transmission of the CTX phage in the aquatic environment and its possible relationship with the emergence of seasonal epidemics in areas where cholera is endemic.

Natural selection of toxigenic V. cholerae:

V. cholerae offers genetic system to study the relationship between pathogenesis and the natural selection of pathogens so as to ensure their continued existence. It appears that acquisition of pathogenic gene clusters by *V. cholerae*, which is normally marine or brackish water species, has allowed the bacteria to become adapted to the human intestinal environment. Although the donor for the TCP pathogenicity island has not yet been identified, the TCP island seems to have a bacteriophage origin, and it has been speculated that the TCP island can be transferred by transducing phages (55,60). On the other hand the donor for the CTX genetic element has been confirmed to be a filamentous bacteriophage, which has been characterized to some details (112, 113). It has been postulated that the acquisition of the CTX element by *V. cholerae* provides a survival advantage to the bacterium, and leads to enrichment of toxigenic *V. cholerae* in the intestinal environment. Thus the CTX phage confers increased evolutionary fitness to its host and hence to its own nucleic acids. With growing immunity in the host population against certain toxigenic clones of *V. cholerae*, new toxigenic clones emerge and replace existing clones by a process of natural selection. Thus a continual emergence of new strains of toxigenic *V. cholerae* and their selective enrichment during cholera outbreaks constitute an essential component of the ecosystem for the survival and evolution of *V. cholerae* and the genetic elements that mediate the transfer of virulence genes. Studies have demonstrated that potential epidemic strains can originate from nonpathogenic

progenitor strains and thus account for the observed clonal diversity among epidemic *V. cholerae* strains. However, many aspects regarding the epidemiology and the ecology of the pathogen remain unknown, particularly the mechanism controlling the seasonal pattern of epidemics in areas of endemic infection.

Chapter 5

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