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Environmental Reservoirs of Phage-encoded Exotoxin Genes

A Dissertation submitted in partial satisfaction of the

Requirements for the degree Doctor of Philosophy

in

Biology

by

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Professor Forest L. Rohwer

2011

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Chair

University of California, San Diego

San Diego State University

2011



## **DEDICATION**

To my parents, Braulio and Graciela Casas—without your sacrifices and dedication to your children, none of this would have been possible. I am eternally grateful.

## TABLE OF CONTENTS

SIGNATURE PAGE.....	IV
DEDICATION.....	IV
TABLE OF CONTENTS .....	V
LIST OF FIGURES .....	VI
LIST OF TABLES .....	VII
ACKNOWLEDGEMENTS .....	VIII
VITA.....	X
ABSTRACT OF THE DISSERTATION .....	XII
INTRODUCTION.....	1
<b>CHAPTER 1: WIDESPREAD DISTRIBUTION OF PHAGE-ENCODED EXOTOXIN GENES IN TERRESTRIAL AND AQUATIC ENVIRONMENTS ...</b>	<b>14</b>
<b>CHAPTER 2: DISTRIBUTION OF PHAGE-ENCODED EXOTOXIN GENES IN COASTAL WATERS FOLLOWING RAIN EVENT .....</b>	<b>25</b>
<b>CHAPTER 3: IDENTIFICATION OF PHAGE-ENCODED <i>SEA</i> GENE IN AN ATYPICAL BACTERIAL HOST CULTIVATED FROM THE ENVIRONMENT .....</b>	<b>47</b>
<b>CHAPTER 4: IDENTIFICATION OF PHAGE-ENCODED SHIGA TOXIN GENE IN AN ATYPICAL BACTERIAL HOST CULTIVATED FROM CANINE FECES .....</b>	<b>59</b>
CONCLUSIONS .....	77
APPENDICES.....	83
REFERENCES CITED .....	95

## LIST OF FIGURES

Figure I.1. Free phage pool of exotoxin genes.....	6
Figure 1.1 Map of sampling sites in San Diego County, California .....	16
Figure 1.2 Southern dot blot of <i>stx</i> in environmental samples .....	19
Figure 2.1. Estimated gene copies by QPCR for exotoxin-positive sediment samples....	37
Figure 2.2. Estimated gene copies by QPCR for exotoxin-positive water samples. ....	40
Figure 2.3. Map of sampling locations in San Diego County, California. ....	44
Figure 3.1 Phylogenetic tree of <i>sea</i> positive isolate, its nearest relatives, and select outgroups.....	50
Figure 3.2 ClustalX2 alignment of top BLASTN hits of the <i>sea</i> gene from the ambient environmental isolate .....	51
Figure 3.3 ClustalX2 guide tree of the top BLASTN hits of the <i>sea</i> gene from the ambient environmental isolate .....	51
Figure 3.4 Free phage pool of exotoxin genes .....	52
Figure 4.1. Agarose gel picture of <i>stx</i> -specific PCR on canine fecal DNA and canine fecal isolates.....	65
Figure 4.2. Phylogenetic analysis of <i>stx</i> gene PCR product of total DNA .....	67
Figure 4.3. Phylogenetic analysis of 16S rDNA PCR product from canine fecal bacterial isolates.....	69
Figure 4.4. Map of sampling location and surrounding waterways.....	73

## LIST OF TABLES

Table I.1. Select human diseases mediated by phage- or plasmid-encoded .....	4
Table 1.1 Conditions for conventional PCRs used in this manuscript .....	17
Table 1.2 Percent positives in exotoxin-specific PCR assays.....	18
Table 1.3 Abundances of exotoxin genes as determined using quantitative real-time PCR.....	19
Table 1.4 Occurrence of exotoxin genes in free phage fraction .....	20
Table 2.1. Exotoxin genes detected in sediment and water samples by standard and Quantitative PCR (QPCR). .....	31

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The Appendix, in full, is a reprint of the material as it appears in Methods in Enzymology 2007. Casas, V. and Rohwer, F., Elsevier Academic Press, 2007. I was the primary investigator and author of this paper.

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## PUBLICATIONS

- Casas, V., J. Magbanua, G. Sobrepeña, S.T. Kelley, and S.R. Maloy (2010) "Reservoir of Bacterial Exotoxin Genes in the Environment," *International Journal of Microbiology*, vol. 2010, Article ID 754368, 10 pages, 2010. doi:10.1155/2010/754368
- Casas, V, J. Magbanua, G. Sobrepena, and S.R. Maloy. (2008) "Reservoirs of Bacterial Toxins." *Nature Precedings* : hdl:10101/npre.2008.2065.1.
- Casas, V. and Forest Rohwer. (2007) "Phage Metagenomics." *Methods in Enzymology: Advanced Bacterial Genetics*. 421: 259-268.

Casas, V., J. Miyake, H. Balsley, J. Roark, S. Telles, S. Leeds, I. Zurita, M. Breitbart, D. Bartlett, F. Azam, and F. Rohwer (2006). "Widespread occurrence of phage-encoded exotoxin genes in terrestrial and aquatic environments in Southern California." *FEMS Microbiology Letters*. 261: 141-149.

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Studies in Molecular Biology:  
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Studies in Bacteriophage Biology and Ecology:  
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Studies in Genomics  
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## **ABSTRACT OF THE DISSERTATION**

Environmental Reservoirs of Phage-encoded Exotoxin Genes

by

Veronica Casas

Doctor of Philosophy in Biology

University of California, San Diego, 2011

San Diego State University, 2011

Professor Stanley R. Maloy, Chair

Exotoxins are virulence factors produced by some bacteria and are sometimes encoded by mobile genetic elements like bacteriophage (phage). Phage are viruses of bacteria that carry genetic information and through horizontal gene transfer this genetic information is sometimes transferred to the bacteria they infect. When virulence genes are transferred to an avirulent bacterium, this bacterium can be transduced to virulence and gains the capability to cause disease. Through this process between the phage and bacterium, a whole host of novel human pathogens may emerge. This has significant implications in the area of human health.

Using exotoxin-specific polymerase chain reaction (PCR), quantitative real-time PCR (QPCR), and Southern dot blot assays, over 500 environmental samples were screened for the presence of four phage-encoded exotoxin genes: cholera toxin (*ctx*), diphtheria toxin (*dtx*), *Staphylococcus* enterotoxin A (*sea*), and shiga toxin (*stx*). A minimum of one gene was found in ~15% of the samples screened. These genes were found in the phage fraction of some of the positive samples; suggesting these genes are

mobile within the environment. The gene most frequently observed in the environmental samples was *stx*. The samples that were positive for an exotoxin gene were spread throughout environmental type and there was no correlation with presence of exotoxin genes and sample type. Identification of some bacterial environmental isolates from samples positive for an exotoxin gene, showed that the bacterium carrying the exotoxin gene was not the known pathogen and was indeed an alternative host for the gene(s). This was shown for environmental isolates carrying the *sea* and *stx* genes. The data generated from this dissertation indicate that exotoxin genes are widespread in the environment and that these genes can be carried by alternative hosts.

## **INTRODUCTION**

### **BACTERIOPHAGE**

Bacteriophage (phage) are the most abundant biological entities on the planet, exceeding the number of prokaryotes by an order of magnitude. There are approximately  $10^6$  phage per ml in the world's oceans and lakes and  $10^9$  phage per gram of sediment and top soil [1-3]. Phage are the major predators of bacteria and are believed to influence the types and population density of bacteria in an environment. By killing bacteria, phage modulate global biogeochemical cycles and play a role in maintaining microbial diversity by selective killing [4-7].

In addition to controlling bacterial populations by lysis of infected bacteria, phage can also alter the physiology of bacteria. Many temperate phage carry genes that, when expressed after lysogeny, alter the phenotype of the bacterial host. One of the most common examples of such lysogenic conversion is immunity to superinfection by other phage. Lysogenic conversion can also result in expanded metabolic capabilities including resistance to antibiotics and reactive oxygen compounds [8, 9]. For phage that encode exotoxins, lysogenic conversion can change avirulent bacteria into human pathogens [10-12].

### **EXOTOXINS AND HUMAN DISEASE**

Exotoxins are products that are secreted from bacteria and are toxic to eukaryotic cells [13]. Certain exotoxins are among the most deadly substances known: for example, approximately 1 mg of botulinum or tetanus exotoxin is fatal to an adult human and a single A chain of diphtheria toxin can kill a eukaryotic

cell [14]. In many cases, exotoxins are responsible for most or all aspects of their associated diseases [15, 16].

There are at least 200 million reported incidences and 2 million deaths annually from the exotoxin-associated diseases listed in Table I.1. These values are clearly underestimates, because in many cases reliable statistics are not available [17]. In addition, exotoxins are critical virulence factors in at least six of the select agents considered to be potential bioterrorism threats [18]. For example:

- *Bacillus anthracis* (Anthrax) and *Clostridium botulinum* (Botulism) are Category A agents.
- *Shigella* and *Escherichia coli* EHEC strains (Shiga toxin), and *Vibrio cholerae* (Cholera) are Category B agents.

A number of newly emerging diseases are caused by exotoxins, of which some examples include:

- Diphtheria is increasing dramatically due to the growing lack of proper immunization. A recent epidemic of diphtheria in the former Soviet Union has caused over 5,000 deaths since 1990 [19].
- Highly virulent, toxigenic Streptococci have been increasing since the late 1980s, resulting in a variety of serious clinical symptoms including toxic shock and necrotizing fasciitis [20].
- *Clostridium difficile* infections are an increasing cause of morbidity and mortality in infants and those with inflamed bowel diseases [21, 22]
- Community acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) induced necrotizing pneumonia in young patients [23].

Recently there has been a significant increase in the incidences of food borne illnesses caused by toxin-producing pathogens such as *E. coli* [24-37]. These outbreaks were mostly attributed to food contaminated in some way from food originating from our complex agricultural system. This was either through the fruits and vegetables supplied by our farms or by the animal products supplied by the poultry, cattle, or dairy industries. Our food comes from our environment. As such, the pathogens that cause our diseases may also come from our environment. We hypothesize that this is a result of the virulence genes maintained in the environment by mobile genetic elements such as phage.

### **GENETIC EXCHANGE**

Many exotoxin genes are carried on mobile genetic elements such as phage or plasmids (Table I.1). Exotoxin genes may move between different plasmids and phage as selfish DNA (increasing its potential microbial host range). Movement of these elements between bacteria allows exotoxin genes to move freely through the environment. Despite this, epidemiology and ecological studies of the infectious diseases associated with these exotoxin genes have largely neglected the potential role mobile genetic elements play in the evolution of these diseases. In particular, phage may facilitate the rapid transmission of exotoxin genes between different bacterial hosts. Transfer of exotoxin genes to new hosts may facilitate the evolution of novel human diseases. In the Tampa

**Table I.1. Select human diseases mediated by phage- or plasmid-encoded**

Disease	Organism	Encoding Element	Comments
Anthrax	<i>Bacillus anthracis</i>	Plasmid pXO1 carries all three toxin genes [38, 39].	Three toxin proteins - edema factor, protective antigen, and lethal factor - [40]. Category A Select Agent [18].
Botulism	<i>Clostridium botulinum</i>	Serotype C1 & D are carried by phage [41, 42]; Type E can be moved by helper phage [43]; Type G carried on a plasmid [44].	At least 7 closely related neurotoxin serotypes - A, B, C <sub>1</sub> , D, E, F, and G - produced by various <i>C. botulinum</i> strains [45]. Type E neurotoxin also reported in <i>C. butyricum</i> [43]. Category A Select Agent [18].
Cholera	<i>Vibrio cholerae</i>	Cholera toxin carried on a phage that can also replicate as a plasmid [46, 47].	Genetic arrangement of <i>ctx</i> -phi similar to a compound transposon [48]. The <i>ctxAB</i> locus shown to move by generalized transduction [49]. Category A Select Agent [18].
Diphtheria	<i>Corynebacterium diphtheriae</i>	Two related phage, b and w, carry exotoxin gene [50, 51].	First phage-encoded exotoxin to be characterized [50].
Diarrhea-genic <i>E. coli</i>	<i>Escherichia coli</i> (EHEC strains)	Shiga toxin-1 ( <i>stx-1</i> ) carried on phages 933J and H19B [52, 53]; Shiga toxin-2 ( <i>stx-2</i> ) carried on phage 933W [53-55].	The <i>stx-1</i> and <i>stx-2</i> genes are associated with <i>E. coli</i> phage. A <i>stx-2</i> related gene has been reported in <i>Enterobacter cloacae</i> [56]. Category B Select Agents [18].
Tetanus	<i>Clostridium tetani</i>	Plasmid [57].	Neurotoxin that prevents the release of glycine.
Toxic shock	<i>Staphylococcus aureus</i>	Moved as part of pathogenicity island by phage phi 13 and 80-phi [58].	Toxic shock syndrome toxin 1 (TSST-1) is a superantigen [59].
Staph Food Poisoning	<i>Staphylococcus aureus</i>	<i>sea</i> phage [60, 61]; SED plasmid [62]; SEE a defective phage .	Staphylococcal enterotoxins (SE) belong to 5 serological groups. Staphylococcal enterotoxin B is considered a Category B Select Agent [18].
Scalded Skin Syndrome	<i>Staphylococcus aureus</i>	ETA - Phage [63]. ETB - Plasmid [64-66].	Exfoliative Toxins (ET) A and B serologically distinct, but evolutionarily related. ET have superantigen [67, 68] and serine protease activity [69-72]. Superantigen activity not necessary for exfoliative activity [72].
Scarlet fever	<i>Streptococcus pyogenes</i>	Phage [73-77].	Disease symptoms associated with Group A Streptococci are varied.

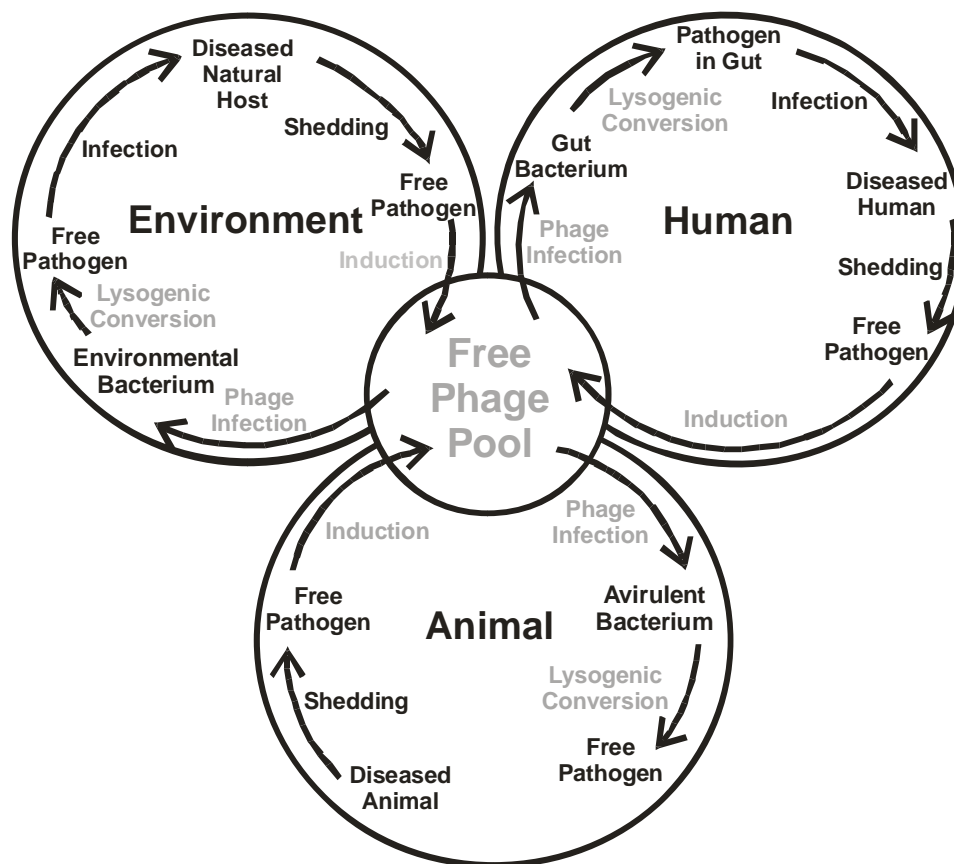
Bay Estuary, it has been estimated that  $10^{14}$  phage transduction events occur each year [78]. Using this estimate and extrapolating for all the World's Oceans, 20 million billion transduction events occur per second [79]. At such a high rate of transfer, if even only a

very small percentage of the genes being transferred are exotoxin genes, the potential for evolution of novel human pathogens is high indeed.

These exotoxin-encoding virulence genes may be maintained in the environment independent of the microbial host typically involved in the human disease. If these genes are located within free phage or in alternative microbial hosts, they could serve as environmental reservoirs for new disease outbreaks. If this hypothesis is correct, important environmental reservoirs of potential human pathogens have previously been ignored. I hypothesize that at least three different routes exist by which a reservoir of mobile exotoxin genes might be maintained and lead to human diseases (Figure 1.1).

### **GENETIC EXCHANGE IN THE ENVIRONMENT**

Creation of novel human pathogens via transduction of exotoxin genes to previously avirulent and innocuous bacteria may occur in the natural environment. Important to this proposition is the fact that phage can survive outside their microbial hosts for extended periods of time and are often more resistant to environmental factors than their bacterial hosts [80, 81]. Phage of *E. coli* encoding the *stx-2* toxin survive chlorination and heat treatments 100-1000x better than their host bacteria [81]. Coliphage and RNA phage are more resistant



**Figure I.1. Free phage pool of exotoxin genes.** Proposed scenarios for how exotoxin-encoding phage might be maintained in the environment and produce human pathogens through genetic exchange between the free phage pool and the natural, human and animal environments. Light grey writing indicates phage/host interaction and potential for horizontal gene transfer.

to sunlight inactivation than are their bacterial hosts [82]. Phage have also been shown to escape inactivation by sewage/water treatment processes more readily than their bacterial hosts [83-87]. By being more impervious to environmental conditions and stress, phage can maintain exotoxin genes in different natural environments, and thereby increase the potential for exchange.

In this scenario, a reservoir of mobile exotoxin genes might be maintained in the environment. The phage could infect an avirulent environmental bacterium, transduce the exotoxin gene they are carrying to that bacterium, and thereby create a novel human



pathogen. There are several lines of evidence that this process does occur in nature. One compelling example is provided by the phage *ctx-phi* of *Vibrio cholerae*. The *ctx-phi* carries the cholera toxin gene and is required for pathogenicity of *V. cholerae*. *V. cholerae* strains that lack cholera toxin are a common inhabitant of aquatic environments around the world and such strains have also been isolated from marine environments in the San Diego area [88-90]. Environmental non-*ctx V. cholerae* isolates can be transduced to virulence by *ctx-phi* *in vitro* and *in vivo* [91, 92]. Aquatic environmental non-*ctx V. cholerae* O139 strains were isolated, propagated, and subjected to infection by *ctx-phi* *in vitro* and *in vivo*. These non-*ctx V. cholerae* O139 isolates were efficiently transduced to virulence by *ctx-phi*, integrated into the bacterial chromosome, and produced viable and infectious *ctx-phi* upon induction (either naturally by sunlight, or experimentally by Mitomycin C) [91, 92]. Furthermore, environmental conditions associated with estuarine and marine environments (e.g., higher salinity and lower temperature) greatly stabilized *ctx-phi* phage particles, and these particles were induced upon exposure of toxigenic strains of *V. cholerae* to direct sunlight [92, 93].

Another compelling example that supports the proposition of avirulent environmental bacteria being transduced to virulence by exotoxin-encoding phage is provided by *E. coli* O157:H7 and one of its associated phages, *stx-2* phage. Avirulent strains of *E. coli* that could acquire *stx*-toxin genes are commonly found in soil [94]. *Stx-2* phage have also been found in sewage water that has undergone treatment to remove bacterial and viral pathogens [86, 95-97]. In a study examining the potential of defective *stx-2* prophages contained within the chromosome of *E. coli* O157:H7 strains to disseminate the *stx-2* gene, Asadulghani *et al.* [98] showed that the defective *stx-2* phage

were inducible and could even be transferred to other *E. coli* strains. An examination of *E. coli* strains isolated from municipal raw sewage and animal wastewater showed a high incidence of the *stx-2* gene in non-O157:H7 *E. coli* strains, suggesting a transfer of this gene to novel *E. coli* strains with the potential to become pathogenic [99]. Also, co-incubation of shiga toxin-encoding phage with phage-sensitive nonpathogenic *E. coli* strains results in production of shiga toxin [100].

### **GENETIC EXCHANGE IN HUMAN MICROBIOTA**

A second scenario by which a reservoir of phage-encoded exotoxin genes may be maintained and lead to disease, is that of transduction of a bacterium already present as part of our normal human microbiota, by a phage carrying an exotoxin gene. Humans, like all multicellular organisms, have an extensive associated microbiota (a.k.a. “normal flora”) [101-107]. Free phage from an environmental reservoir or a lysogen could be ingested and subsequently convert an avirulent member of this normal microbial flora into a pathogen. Several examples indicate that this process occurs in nature. Avirulent strains of pathogens are commonly found in humans (e.g., *Corynebacterium diphtheriae*, *E. coli*, etc.). Phage conversion of avirulent *C. diphtheriae* strains has been reported to occur within the upper respiratory tract [108], and phage conversion by *stx-1* phage and *ctx-phi* has been shown to occur in the gastrointestinal tract [93]. *V. cholerae ctx-phi* lysogens have also produced infectious virions within the gastrointestinal tract [109]. This facilitates the evolution of novel human pathogens because *V. cholerae* O139 strain can induce production of phage *ctx-phi* and cause transfer of *ctx* to classical standard strains 569B and O395 [110]. New virulent *V. cholerae* serotypes may have evolved via

phage-mediated transduction of avirulent strains within the host gastrointestinal tract. Furthermore, it has become quite evident that the evolution of the pathogenicity of *V. cholerae* strains involves cooperative interactions between multiple filamentous phages, including ctx-phi, VGJ-phi, VEJ-phi, KSF-I-phi, and TLC-Kn-phi1 [111-114].

## **GENETIC EXCHANGE IN ANIMAL MICROBIOTA**

Infection of non-human organisms might maintain a reservoir of exotoxin genes that can lead to disease in humans through their interactions with animals; either with livestock or with wild animals. In both of the previously described scenarios only human-to-human routes are considered. The fact that exotoxins are encoded by mobile elements strongly suggests that these genes may move to other bacterial species and eventually infect non-human hosts.

The cellular targets of most exotoxins are conserved among all eukaryotes, therefore it is likely that the exotoxins will have similar effects even in unrelated hosts. There are several lines of evidence for an environmental reservoir of phage encoded exotoxin genes. Shiga toxin producing *E. coli* [115] have been found in sheep, pigs, cattle, houseflies, seagulls, and deer [116]. The *Staphylococcus aureus* exotoxins SEA, SEB, and SEC have been shown to be produced when *S. aureus* infects shrimp [117], pig, horses, cats, goats, cattle, and dogs [118]. In cows, bovine variant exfoliative toxin A (ETA) phage induced from *S. aureus* isolates of cows with mastitis were able to lysogenize ETA-negative *S. aureus* isolates and produced ETA [119]. The common animal husbandry practice of administering antibiotics as growth promoters has been

shown to induce stx phages *in vivo*. This induction may increase amounts of stx phage and subsequently lead to the spread of STEC and development of new pathogens [120].

### **BROAD BACTERIOPHAGE HOST RANGE**

Imperative to the model proposed above, is the ability of phage to infect alternative hosts. The classic textbook description of phage-bacteria interactions implies that phage infection is limited to a specific host. According to this dogma, phage host-range is limited by several requirements: (i) the host must have a specific receptor for the phage, (ii) phage gene expression must occur in the infected host, and (iii) replication, assembly, and release of the phage must occur in the new host. Although there are known examples where phage host-range is limited at one of these steps, phage can use a variety of tricks to overcome these barriers. For instance, Phage T2 can acquire mutations that alter its tail fiber and allow it to infect *E. coli* O157:H7 [121]. Phage Mu has an inversion mechanism that modulates its tail fibers to allow infection of hosts with distinct cell surface receptors [122]. Phage P22 expresses an anti-repressor that can inactivate endogenous lysogens which may interfere with phage gene expression and replication [123, 124]. *Bordetella* phage carry diversity generating retroelements that allow the phage to infect bacteria with different cell surface receptors and physiology [125]. Many phage encode anti-restriction systems that allow the phage to reproduce in hosts with different restriction-modification systems [126]. Recombination between phage yields novel types of chimeric phage that have novel features [127], and such recombination can occur by recombination with endogenous prophage even if the invading phage cannot

replicate in the infected bacterial host. These mechanisms allow phage to infect a wider variety of bacterial hosts.

Infection of alternative hosts clearly occurs in nature. Several examples of alternative hosts for phage which carry exotoxins are known: ctx-phi can infect both *V. cholerae* and *V. mimicus* [128], stx-2 phage can infect *E. coli* and *Enterobacter cloacae* [129], and Botulinum toxin E phage can infect *Clostridium botulinum* and *C. butyricum* [43]. Viral populations from lake water, marine sediments, and soil are able to replicate when incubated with marine microbes [130]. Detection of phage T7-like DNA polymerase genes in marine, freshwater, sediment, terrestrial, extreme, and metazoan-associated environments demonstrated that phage can move the genes they carry between these different environments [131]. Additionally, analyses of uncultured environmental phage libraries have revealed an abundance of mobile elements and genes involved in the mobilization of DNA [132-134]. Finally, widespread distribution of a T7-like DNA polymerase in different environmental ecosystems suggests that phage with quite broad host ranges must exist in nature [131]. Taken together, these results suggest that phage are capable of infecting different hosts from different environments, providing a major mechanism for the spread of genes between bacteria. Thus, phage may promote promiscuous horizontal gene transfer in nature.

## **FREQUENCY OF GENETIC EXCHANGE**

Though estimates of 20 million billion transduction events per second in the World's Oceans have been suggested [79], exactly how often phage infect and recombine with hosts in nature is not completely understood. The sequencing of many bacterial

genomes has helped provide insight into this question. Genome sequencing led to the realization that bacterial genomes contain a substantial amount of phage DNA [11, 135]. For example, the genome sequence of *Salmonella enterica* sv. Enteritidis revealed at least six prophages, corresponding to approximately 5% of the bacterial genome [136]. Some of this integrated phage DNA has been mutated and is no longer capable of yielding viable phage, but some of the integrated prophages can be induced to yield viable phage. In addition to encoding exotoxins, the gene products expressed from the phage can affect the bacterial host in many other ways, including changes in cell surface antigens and expression of other virulence factors [11].

Are the phage remnants of events that happened on an evolutionary time scale, or do these events happen in “real time?” The identification of phage in bacterial genomes coupled with the realization that there is such a large pool of phage in the environment suggests that phage mediated genetic exchange is rampant. However, there have been surprisingly few studies that document genetic exchange between phage and infected host cells in nature, and the frequency of such events has been estimated, but not exactly known [8, 10, 137-142]. Genetic exchange of *ctx* and *stx* in nature occur at low but detectable frequencies [47, 95, 143-147]. Such gene exchange may be enhanced by environmental conditions including presence of environmental contaminants and antibiotics [148, 149]. To fully understand how phage, and the genes they carry, play a role in the epidemiology of disease, it is important to determine the absolute frequency of genetic exchange between different phage and between phage and host. Adding to this understanding will be how environmental conditions modulate these events. The

expected frequency will depend upon the number of phage and host cells, and may be influenced by the presence of other bacteria as well.

A closer examination of the mechanism of horizontal gene transfer reveals that such events might disseminate a particular gene at the genetic level, and not necessarily to disseminate a particular pathogen at the organismal level [150-153]. Known as the “selfish gene,” this theory proposes that horizontal gene transfer counteracts the loss of gene clusters via genetic drift within microorganisms [153]. The genes perpetuate themselves in the microbial population by promiscuous genetic exchange, thereby increasing their frequency in the gene pool, and in turn increasing the fitness of the organism that carries it (phage or bacteria). A computational analysis of the “selfish gene” theory indicated that a high horizontal gene transfer rate promotes evolution of gene clusters [154]. The theory helps explain the mosaicism of bacterial and phage genomes that has been discovered through genomic analyses [155-160]. Hence, the phage and bacteria may solely be vessels or vectors for dissemination exotoxin genes.

Many human diseases are caused by pathogens that produce exotoxins, and exotoxin genes are carried phage. We hypothesize that phage may facilitate the movement of exotoxin genes between microbial hosts, thereby converting avirulent bacteria into pathogens. Transfer of these exotoxin genes to new hosts in the environment may be an important mechanism for evolution of new strains of pathogens and emerging infectious diseases. The goal of this dissertation research was to determine where exotoxin genes are found in the environment and which bacteria are carrying the exotoxin gene. The results presented herein will hopefully provide valuable insights into the role of microbial ecology in the evolution of new infectious diseases.

**CHAPTER 1: WIDESPREAD DISTRIBUTION OF PHAGE-ENCODED  
EXOTOXIN GENES IN TERRESTRIAL AND AQUATIC ENVIRONMENTS**





## Widespread occurrence of phage-encoded exotoxin genes in terrestrial and aquatic environments in Southern California

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### Keywords

phage; cholera toxin; diphtheria toxin; shiga  
toxin; *Staphylococcus enterotoxin A*; horizontal  
gene transfer.

### Abstract

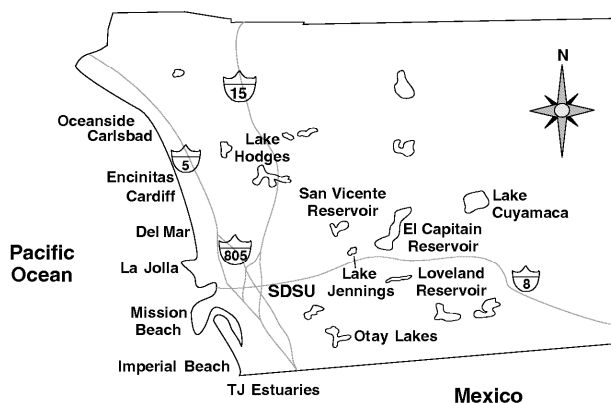
Many human diseases are caused by pathogens that produce exotoxins. The genes that encode these exotoxins are frequently encoded by mobile DNA elements such as plasmids or phage. Mobile DNA elements can move exotoxin genes among microbial hosts, converting avirulent bacteria into pathogens. Phage and bacteria from water, soil, and sediment environments represent a potential reservoir of phage- and plasmid-encoded exotoxin genes. The genes encoding exotoxins that are the causes of cholera, diphtheria, enterohemorrhagic diarrhea, and *Staphylococcus aureus* food poisoning were found in soil, sediment, and water samples by standard PCR assays from locations where the human diseases are uncommon or nonexistent. On average, at least one of the target exotoxin genes was detected in ~15% of the more than 300 environmental samples tested. The results of standard PCR assays were confirmed by quantitative PCR (QPCR) and Southern dot blot analyses. Agreement between the results of the standard PCR and QPCR ranged from 63% to 84%; and the agreement between standard PCR and Southern dot blots ranged from 50% to 66%. Both the cholera and shiga exotoxin genes were also found in the free phage DNA fraction. The results indicate that phage-encoded exotoxin genes are widespread and mobile in terrestrial and aquatic environments.

### Introduction

Exotoxins of bacteria are secreted polypeptides involved in pathogenesis and are among the most deadly substances known. Exotoxins are responsible for many or all aspects of their associated diseases and, although other pathogenic factors might be involved, the host bacterium is often relatively innocuous without the exotoxin gene (Pittman, 1984; Davis & Waldor, 2002). Most exotoxin genes are carried on plasmids or phage (Freeman, 1951; Eklund & Poysky, 1974; Betley & Mekalanos, 1985; Calderwood *et al.*, 1987; Waldor & Mekalanos, 1996; Okinaka *et al.*, 1999a, b) and epidemiological and ecological studies of infectious disease have ignored the potential role of phage and plasmids in the evolution of pathogens.

There are *c.* 10 million phage per mL in the World's oceans and lakes and one billion phage per gram of sediment and top soil (Bergh *et al.*, 1989; Ogunseitan *et al.*, 1990; Maranger & Bird, 1996; Danovaro & Serresi, 2000; Hewson *et al.*, 2001). Phage capable of infecting more than one

bacterium may influence the emergence of infectious diseases by facilitating horizontal gene transfer (HGT) of virulence genes to avirulent hosts. Phage infection of more than one host occurs in nature – examples of which include *ctxφ* infecting both *Vibrio cholerae* and *Vibrio mimicus* (Faruque *et al.*, 1999) and *stx-2* phage infecting *Escherichia coli* and *Enterobacter cloacae* (Mehrotra *et al.*, 2002). Also, examination of phage-encapsulated 16S rRNA genes isolated from activated sludge demonstrated the presence of generalized transducing phages in *Aeromonas*, *Acinetobacter*, and *Acrobacter* species that had not previously been observed to contain phage (Sander & Schmieger, 2001). Viral populations from lake water, marine sediments, and soil have been shown to replicate when incubated with marine microorganisms (Sano *et al.*, 2004). Breitbart *et al.* (2003) showed that phage T7-like DNA polymerase genes are present in multiple biomes tested. Additionally, analyses of uncultured environmental phage libraries have revealed an abundance of mobile elements and genes involved in the mobilization of DNA (Breitbart *et al.*, 2002, 2003, 2004).



**Fig. 1.** Map of sampling sites in San Diego County, California. A general map of locations sampled in this study. Water and sediment samples were gathered from beaches along the San Diego coastline as far north as Oceanside and as far south as Imperial Beach. Lake and reservoir samples (water and sediment) were gathered from various locations around San Diego, a few of these locations are indicated here. Soil samples were gathered from all around San Diego County – from urban and suburban areas in the western side of the county, to rural and campground areas in the eastern side of the county. Numerous samples were gathered from San Diego State University (SDSU) campus. Refer to Table S1 for information on when samples were collected, who processed the samples, and in which laboratory they were processed. Map is not drawn to scale.

Taken together, these results suggest that phage are capable of infecting different hosts in the environment, providing a major mechanism for the spread of genes between bacteria.

Plasmids are also very common in the environment and are important in the exchange of genetic information between microorganisms. Between 17% and 60% of marine bacteria isolates contain plasmids 5–400 kb in length (Sobecky, 1999). Jiang & Paul (1998) estimated that  $1.3 \times 10^{14}$  transduction events occur per year in the Tampa Bay Estuary in Florida. Transfer of marked plasmids in mixed bacterial communities occurred at a frequency between  $1.58 \times 10^{-8}$  and  $3.7 \times 10^{-8}$  (Jiang & Paul, 1998). These results suggest that plasmids are a major conduit for DNA mobilization between different environments and as such serve as a potential mechanism for the spread of exotoxin genes.

Here we screened water, soil, and sediment samples for shiga (*stx*), cholera (*ctx*), *Staphylococcus* enterotoxin A (*sea*), and diphtheria (*dtx*) exotoxin genes. The genes for *ctx* and *stx* were found in the three environments tested, while *sea* and *dtx* were not detectable in the water samples tested. At least one specific exotoxin gene was amplified by PCR, on average, from 15% of the samples tested. The results were confirmed through the use of secondary and tertiary tests in independent labs with different operators. The cholera and shiga exotoxin genes were also found in the phage from these environments. Together, these results show that the phage-encoded exotoxin genes are common in the environ-

ment and that they may be moving around through phage–host interactions.

## Materials and methods

### Environmental sample collection and handling

Soil, sediment, and water samples were collected from San Diego County, CA (Fig. 1). Environmental samples were collected between May 2001 and June 2004. Water and sediment sampling included estuarine, freshwater, and marine sites. Small water samples were collected in 1 L high-density polyethylene (HDPE) bottles and large water samples were collected in 38 L polystyrene sampling bags. Sediment samples were collected in new 1 L disposable plastic containers, usually just underneath a corresponding water sample. Soil samples were collected in new 3.8 L resealable plastic freezer bags. All samples were returned to the laboratory and stored at 4 °C within 2 h of collection. Whenever possible DNA was isolated from environmental samples within 48–72 h of collection.

To prevent contamination from PCR products, physically separate laboratories were used for DNA isolation from environmental samples (template-negative) and for PCR amplification of exotoxin genes (template-positive). Pipettors, plasticware, chemicals, etc. from each laboratory were kept entirely separate and PCR-amplified DNA products were never brought into the template-negative laboratory.

Exotoxin genes in the environment

**Table 1.** Conditions for conventional PCRs used in this manuscript

Exotoxin gene	MgCl <sub>2</sub> (mM)	No. of PCR cycles	Annealing temperature (°C)	Known bacterial host	Primer designations and sequences	Reference
<i>dtx</i>	2.5	35	56.0	<i>Corynebacterium diphtheriae</i>	DT15F: GTTTCGCTCAATCTTAATAGGG DT1643R: ACCTTGGTGTGATCTACTGTTT	Efstratiou <i>et al.</i> (1998)
<i>stx</i>	2.0	45	50.0	<i>Escherichia coli</i>	STXF: GAGCGAAATAATTTATATGTG STXR: TGATGATGGCAATTCAGTAT	Makino <i>et al.</i> (2000)
<i>ctx</i>	2.5	45	67.4	<i>Vibrio cholerae</i>	CTXA73F: CGGGCAGATTCTAGACCTCCTG CTX636R: CGATGATCTGGAGCATTCCCAC	Fields <i>et al.</i> (1992)
<i>sea</i>	1.5	45	63.1	<i>Staphylococcus aureus</i>	SEA126F: GCAGGGAACAGCTTTAGGC SEA624R: GTTCTGTAGAAGTATGAAACACG	This study

Listed in the table are the final concentrations of magnesium chloride used for each exotoxin-specific PCRs (50 µL final volume) as well as the various thermocycling conditions. The sequences of the forward and reverse primer for each PCR assay and their reference are also included. The *sea* primers were designed from *Staphylococcus aureus* enterotoxin A gene sequence (GenBank accession number 349129).

*dtx*, diphtheria; *stx*, shiga; *ctx*, cholera; *sea*, *Staphylococcus* enterotoxin A.

Aerosol-resistant pipette tips were always used and nondisposable were routinely UV sterilized. PCR reactions were assembled in the template-negative laboratory, covered, and transported to the template-positive laboratory for amplification. To ensure reproducibility between operators and laboratory locations, four different operators isolated environmental DNA and setup the PCR assays in two different template-negative laboratories. (see Table S1).

#### Isolation of total community DNA from environmental samples

Total DNA was extracted from 1 g of 228 soil samples and 67 sediment samples using the Ultra-Clean Soil DNA Kit (MoBio, Carlsbad, CA). Forty-six 1 L water samples were split into two 500 mL aliquots and precipitated overnight with 10% polyethylene glycol at 4 °C (Liu *et al.*, 2002). The polyethylene glycol (PEG) precipitated sample was centrifuged in a Sorvall RC5C (Asheville, NC) at 8300 g using a GS-3 rotor at 4 °C and the pellets from each aliquot were combined. DNA was extracted from the pellet using conventional cetyltrimethyl-ammonium bromide (CTAB) DNA extraction methods (Sambrook *et al.*, 1989).

#### Isolation of separate phage and prokaryotic DNAs from environmental samples

Phage and prokaryotic DNA was isolated from soil and sediment samples by a previously described method (Breitbart *et al.*, 2002, 2003). Phage DNA was isolated from small volume water samples (≤ 1 L) by PEG precipitation followed by filtration (0.2 µm) and CTAB DNA extraction (Sambrook *et al.*, 1989). For large volume water samples

(> 1 L), phage DNA was isolated by tangential flow filtration (TFF), cesium chloride gradient centrifugation, and a formamide/CTAB extraction as described previously (Breitbart *et al.*, 2002, 2003).

#### PCR detection of exotoxin genes

PCR assays were developed for *dtx*, *stx*, *ctx*, and *sea* exotoxin genes. Table 1 indicates the PCR conditions for each of the exotoxin gene PCR assays. The forward and reverse primers used in each assay and their corresponding references are also listed in Table 1. The *sea* primers were designed from the *Staphylococcus aureus* enterotoxin A gene sequence (34912-9) and a BLASTN search against the nonredundant GenBank database was performed to ensure their specificity.

Positive control DNA for the PCR assays was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA) from *V. cholerae* El Tor N16961, *Corynebacterium diphtheriae*, *Escherichia coli*, and *S. aureus* containing the exotoxin genes of interest (Heidelberg *et al.*, 2000). The DNA was quantified on a DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA) and positive control DNA standard curves (1–10<sup>5</sup> genomic targets) were made for use in each assay.

#### Quantitative real-time PCR (QPCR) for *dtx*, *ctx*, and *sea* exotoxin genes

QPCR assays were developed for *dtx*, *ctx*, and *sea* exotoxin genes. Primer and probe sequences were designed using the Beacon Designer (Premier Biosoft International, Palo Alto, CA) and Primer3 (Rozen & Skaletsky, 1998). A BLASTN search against the nonredundant GenBank database was performed to ensure specificity of the QPCR primers and

**Table 2.** Percent positives in exotoxin-specific PCR assays

Environment	Exotoxin gene			
	<i>stx</i>	<i>sea</i>	<i>dtx</i>	<i>ctx</i>
Soil	29% <i>n</i> = 229	11% <i>n</i> = 229	5% <i>n</i> = 146	3% <i>n</i> = 146
Sediment	15% <i>n</i> = 66	14% <i>n</i> = 66	11% <i>n</i> = 61	31% <i>n</i> = 61
Water	18% <i>n</i> = 44	0% <i>n</i> = 40	0% <i>n</i> = 44	49% <i>n</i> = 41
Total % positive	25	10	6	18

DNA isolated from soil, sediment, and water samples were tested for each exotoxin gene by PCR.

Results of the four different experiments are shown as percent positives of samples tested. A positive result indicates a sample that produced a visible band of the correct target size on a 1% agarose gel. Using a standard curve, the detection limit for each assay were:  $1 \times 10^4$  gene copies for *dtx* and *sea*,  $1 \times 10^3$  gene copies for *ctx*, and 100 gene copies for *stx*. *n* = total numbers of samples tested combined from different time points, different operators, and different laboratories in which the PCR was performed.

*dtx*, diphtheria; *stx*, shiga; *ctx*, cholera; *sea*, *Staphylococcus enterotoxin A*.

probe. The primers for the *dtx* QPCR were DTXrprcF (5'-CGGTTGCAGAGCTATAGACG-3') and DTXrprcR (5'-CGAGCTGCTTCTGTGAAATG-3'). The primers for the *ctx* QPCR were CTX564F (5'-GCATAGAGCTTGGAGGGAAGA-3') and CTX654R (5'-ATCGCAAGTATTACTCATCGA-3'). The primers for the *sea* QPCR were SEArpcrF (5'-ACAGATCATTCTGGTATAACGAT-3') and SEArpcrR (5'-TACACCACCATACATACAAGCTG-3'). The *ctx* probe, CTX (5'-CCGTGGATTTCATCATGCACCGCCG-3'), was labeled with a fluorescein (FAM) dye on the 5' end and a black hole quencher 1 (BHQ1) on the 3' end. The samples tested by quantitative PCR had also been previously tested by exotoxin-specific PCR as outlined above. Real-time amplification of each exotoxin gene consisted of one cycle of 4 min at 95 °C and then 45 cycles of (10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C). For the *ctx* QPCR, each 20 µL reaction contained final concentrations of  $1 \times$  Bio-Rad iQ Supermix (Hercules, CA – 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM dNTPs, 25 U mL<sup>-1</sup> iTaq DNA polymerase, 3 mM MgCl<sub>2</sub>) which did not contain SYBR Green (Molecular Probes Inc., Eugene, OR), 3 µg µL<sup>-1</sup> bovine serum albumin (BSA), 200 nM of each primer, 50 nM of the *ctx* probe and 1 µL of sample DNA. For the *dtx* and *sea* assays, the same conditions apply except that the Bio-Rad iQ Supermix contained SYBR Green. The assays were performed in a Bio-Rad iCycler (Hercules, CA) QPCR machine. For testing, samples were run undiluted and at either a 1/50 or 1/500 dilution, in duplicate or triplicate. Data generated on estimated number of gene copies is an average of all replicates.

#### Southern dot blot hybridizations to detect exotoxin genes

DNA from the total community, bacterial community, and phage community of samples were fixed onto Hybond-N

nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) using standard procedures (Sambrook *et al.*, 1989) and hybridized with an exotoxin-gene-specific probe. The  $\gamma$ -[<sup>32</sup>P]ATP-labeled DNA probes were made using the ethanol-precipitated and concentrated PCR product resulting from amplification of 1 µL containing  $1 \times 10^5$  copies of the positive control (Fig. 2).

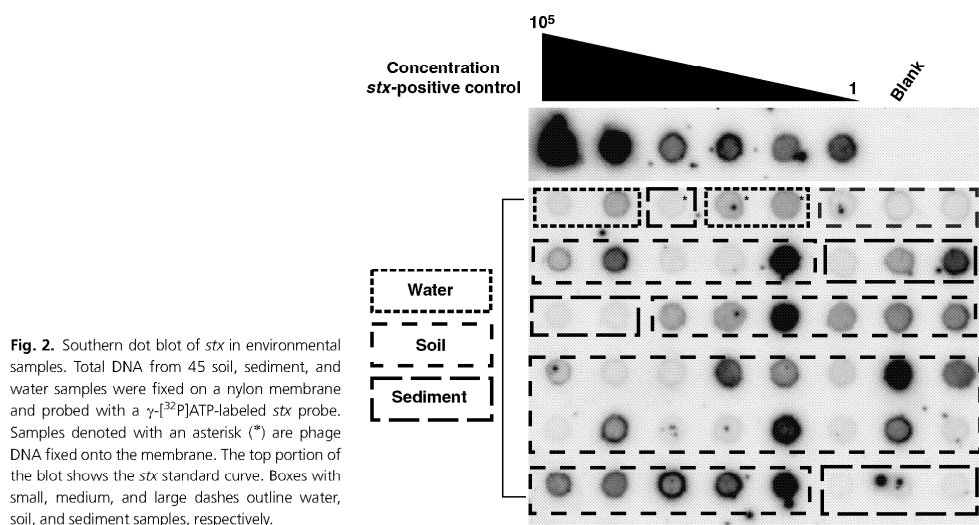
## Results and discussion

### Phage-encoded exotoxin genes are widespread in water, sediment, and soil samples from San Diego, CA and surrounding areas

Exotoxin-specific PCR assays were developed for *ctx*, *dtx*, *sea*, and *stx* exotoxin genes. Table 2 shows the compilation of the PCR results for the four exotoxin genes tested. The results represent over 300 sample locations, spanning 4 years (2001–2004), four different assay operators, and two different laboratory settings. The results showed that at least one of the four exotoxin genes was found in each environment tested. The *sea* exotoxin gene was found in soil and sediment samples, but was absent from the 38 water samples tested. The *stx* exotoxin gene, with the most sensitive PCR, was the most commonly observed gene (25%). The *stx* gene was found mostly in soil samples. Thirty-two sample locations tested positive for > 1 exotoxin gene. Of these locations, 26 contained two exotoxin genes and six contained three exotoxin genes. Some PCR positives were also cloned and sequenced to confirm the target gene. A BLASTN (Altschul *et al.*, 1990) comparison of these sequences showed that the target gene was amplified and was > 98% identical to the sequences in GenBank (DQ491342, DQ491343, DQ491344; see Fig. S1).

To show that standard PCR assay results from samples collected and screened between 2001 and 2003 were

## Exotoxin genes in the environment



**Fig. 2.** Southern dot blot of *stx* in environmental samples. Total DNA from 45 soil, sediment, and water samples were fixed on a nylon membrane and probed with a  $\gamma$ -[ $^{32}$ P]ATP-labeled *stx* probe. Samples denoted with an asterisk (\*) are phage DNA fixed onto the membrane. The top portion of the blot shows the *stx* standard curve. Boxes with small, medium, and large dashes outline water, soil, and sediment samples, respectively.

**Table 3.** Abundances of exotoxin genes as determined using quantitative real-time PCR

Environment	Location	Average no. <i>dtx</i> gene copies	Standard deviation
Soil	Perkins Elementary	211	57 (6)
	Valhalla High School	83	90 (5)
	Cuyamaca College	239	222 (4)
Sediment	Otay Mesa Reservoir (freshwater)	181	233 (4)
	Imperial Beach (seawater)	625	488 (5)
Water	National City Marina (seawater)	279	204 (4)
	Cardiff (seawater)	124	178 (4)
	Torrey Pines (seawater)	961	751 (4)
Environment	Location	Average no. <i>sea</i> gene copies	Standard deviation
Soil	El Monte Park	114	32 (4)
Sediment	Imperial Beach (seawater)	31	14 (4)
	Scripps Ranch (freshwater)	1936	161 (5)
	Lake Hodges (freshwater)	529	55 (5)
	Tijuana Estuary (estuary)	557	786 (5)
Environment	Location	Average no. <i>ctx</i> gene copies	Standard deviation
Sediment	Lindo Lake (freshwater)	602	165 (6)
	San Diego River (freshwater)	1800	484 (6)
	Ocean Beach (seawater)	1514	482 (7)

The DNA from a total of 90 soil, sediment, and water samples collected in San Diego, CA was tested for *dtx*, *sea*, and *ctx* using real-time PCR. For soil and sediment samples, DNA was isolated from 1 g of material and numbers shown represent gene copies per gram. For water samples, DNA was isolated from 1 L and numbers shown represent gene copies per liter. The number of replicates is shown in parentheses. *dtx*, diphtheria; *stx*, shiga; *ctx*, cholera; *sea*, *Staphylococcus enterotoxin A*.

reproducible and not a result of contamination by PCR products, 130 'new' soil and sediment samples were collected between April and June 2004. A different operator, in

a laboratory housed in an entirely separate building, processed these additional samples. These results are included in the compilation of data in Table 2. Of these 130 'new'

**Table 4.** Occurrence of exotoxin genes in free phage fraction

Environment	Location	Exotoxin gene PCR results		
		<i>stx</i>	<i>ctx</i>	<i>sea</i>
Soil	SDSU: Hepner Hall*	(-)	(-)	Negative
	SDSU: Mediterranean Garden*	(-)	(-)	Negative
	Balboa Park 2*	Positive	(-)	(-)
	Balboa Park 3*	(-)	(-)	Negative
	Balboa Park 6*	Positive	(-)	(-)
	Oxford Community Park*	Negative	(-)	Negative
	Fred H. Rohr Park*	Negative	(-)	Negative
	SDSU: Aztec Student Center*	Negative	(-)	Negative
	Lindo Lake Park*	Positive	(-)	(-)
	Water	Lake Hodges (freshwater) <sup>†</sup>	Positive	Positive
Mission Beach (seawater) <sup>†</sup>		Negative	Negative	(-)
Torrey Pines (seawater) <sup>†</sup>		Positive	Positive	(-)
Salton Sea (hypersaline) <sup>†</sup>		Negative	(-)	(-)
Scripps pier May 2001 (seawater) <sup>†</sup>		Positive	Positive	(-)
Sediment		Scripps pier June 2001 (seawater) <sup>†</sup>	Negative	Positive
	Mission Beach (seawater) <sup>†</sup>	Positive	Positive	(-)
	Cuyamaca Lake (freshwater)*	Negative	(-)	(-)
	Lindo Lake (freshwater)*	Negative	(-)	(-)

Phage DNA was isolated from 15 samples that had tested positive for *stx*, *ctx*, or *sea* by the exotoxin-specific PCR (Table 2). Phage DNA was screened for the presence of the corresponding gene by exotoxin-specific PCR. The table includes results from samples tested in 2001 and in 2004. A positive result indicates the presence of a correctly sized band on a 1% agarose gel. The detection limit for each assay is as follows:  $1 \times 10^4$  gene copies for *ctx* and *sea*,  $1 \times 10^3$  gene copies for *stx*, and 100 gene copies for *stx*.

\*Samples processed by PEG precipitation followed by CTAB DNA extraction

<sup>†</sup>Samples processed by TFF followed by CsCl centrifugation and formamide/CTAB DNA extraction.

SDSU, San Diego State University; (-), not tested; PEG, polyethylene glycol; CTAB, cetyltrimethyl-ammonium bromide; TFF, tangential flow filtration; *ctx*, diphtheria; *stx*, shiga; *ctx*, cholera; *sea*, *Staphylococcus enterotoxin A*.

samples, 26 tested positive for *stx* and six were positive for the *sea* gene. Three soil samples were positive for both *stx* and *sea*. Two of the *stx* positives were from sediments and the remaining *stx* and *sea* positives were from soil samples. Testing of these 'new' samples in a different laboratory by a different operator showed that the standard PCR assays are robust and that the results are reproducible.

The data indicates that phage-encoded exotoxin genes are relatively common in the environment. On average, at least one of four exotoxin genes was found in 15% of the samples screened. This presents the first step in the process of gathering the information necessary to determine how the presence of these exotoxin genes in the environment may facilitate the evolution of novel pathogens.

#### QPCR agrees with standard PCR results and shows varying amounts of *ctx*, *stx*, and *sea* genes in environmental samples

QPCR assays were developed for *ctx*, *stx*, and *sea* exotoxin genes to confirm results from conventional PCR and to assess the approximate concentration of these genes in the environmental samples. During the development of these assays it was determined that PCR inhibitors were present in

DNA isolated from some of the environmental samples. Addition of BSA and dilution of the environmental sample by a factor of 50 or 500 helped to relieve this inhibition while BSA had no adverse effects on the reaction (see Fig. S2).

Thirty of each soil, sediment, and water samples (90 samples total) collected in July of 2003 were tested by QPCR (Table 3). Most samples that were positive for an exotoxin gene by this method were from soil and sediment environments. Analysis of the melt-curve of the QPCR products confirmed that there was only one species of product. Each sample was tested a minimum of four times and the estimated number of gene copies present per gram or liter of material represents an average of these replicates. Estimated gene copies in an environmental sample were extrapolated from the standard curve run with each QPCR assay (see Fig. S3). In these positive samples, the *ctx* QPCR detected a range of 83–961 gene copies per gram soil or per liter water, the *sea* QPCR detected a range of 31–1936 gene copies per gram of soil or sediment, and the *stx* QPCR detected 602–1800 gene copies per gram sediment. Only *ctx* had positives in the water samples tested. The *ctx* gene had eight of 90 positives (9%) with varying amounts of the gene present in each sample. There were five *sea* positives (6%) in the 90 samples screened, one that was from a soil sample and

the other four from sediment samples. The *ctx* QPCR detected the fewest positives with only three positives of 90 (3.33%) – all in sediment samples. There was no apparent correlation between the quantity of exotoxin gene and sample type.

The QPCR analyses confirmed the results obtained by standard exotoxin-specific PCR. From these results it was estimated that the concentration of phage-encoded exotoxin genes in the environment ranged between 31 and 1936 gene copies per gram or liter of material tested. The caveat here, however, is that these figures are only estimates because the standard deviation between replicates varied substantially in some samples. As shown in the Fig. S2, the QPCR can be inhibited by environmental sample DNA. While this inhibition was relieved by dilution and addition of BSA, the nature of the environmental samples and the sensitivity of the QPCR make this variation inevitable. Nonetheless, the results from standard PCR and QPCR did agree in the 90 samples tested by both methods. The results of the standard PCR and QPCR assays for *ctx*, *ctx*, and *sea* agreed 63%, 80%, and 84%, respectively.

#### **Southern dot blot analyses agree with standard PCR assays**

Some environmental samples were also analyzed by Southern dot blot to corroborate the results from standard PCR assays (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). An example of an *stx* Southern dot blot is shown in Fig. 2. Together, the results from the *ctx*, *sea*, and *stx* blots showed the presence of exotoxin genes in soil, sediment, and water samples. As shown in Fig. 2, *stx* was also found in the phage fraction of two water samples. The agreement between the results of standard PCR and Southern dot blot analyses was 64%, 50% and 66% for the *ctx*, *sea*, and *stx* assays, respectively. Using Southern dot blot analysis, at least one exotoxin gene was detected in 29% of the samples screened. These Southern dot blot assays also confirmed that phage-encoded exotoxin genes were widespread in the environment.

#### **Exotoxin genes were found in phage DNA isolated from environmental samples**

Phage DNA was purified from a set of environmental samples that had tested positive for *stx*, *ctx*, and/or *sea* (Table 4). The *stx* gene was found in seven of the 15 samples tested (46.7%). Three of the *stx* positives were from soil samples and the remaining three were from water samples. The *ctx* gene was detected in five of the six samples screened (83%) – four in water samples and one in a sediment sample. Water samples from Lake Hodges, Torrey Pines, and Scripps Pier (May 2001) tested positive for both *stx* and

*ctx*. The Mission Beach sediment sample also tested positive for both *stx* and *ctx*. Another water sample from Scripps Pier obtained in June 2001 tested positive for *ctx*. The *sea* gene was not detectable in the six soil samples tested. This is not surprising however, because the *sea* PCR assay is not as sensitive as the *stx* and *ctx* assays (detection limit =  $10^4$  gene copies). Finding these genes in the free phage fraction suggests that they are mobile within the environment, and as such could be serving to facilitate the development of novel human pathogens.

#### **Phage-encoded exotoxin genes are present where the corresponding human disease is not**

These phage-encoded exotoxin genes are found in environments where the corresponding human disease is not prevalent. In San Diego only one case of cholera was reported between 1995 and 2004 (HHSA, 2005) and while statistic on incidences of diphtheria in San Diego County were not available, in the entire United States only 0.001 diphtheria cases per 100 000 people have been reported since 1980 (Centers for Disease Control and Prevention, USA). Why are these exotoxin genes present without the disease? We speculate that the exotoxin genes are present within microorganisms that are not currently human pathogens, but may be in the process of evolving into human pathogens or may be pathogens of other organisms. Alternatively, the exotoxin genes could be performing a different function not at all related to virulence.

The sensitive and specific methods developed for detecting phage-encoded exotoxin genes have shown them to be widespread in the environments tested. These exotoxin genes were found in the phage from the terrestrial and aquatic environments sampled. We hypothesize that a reservoir of phage-encoded exotoxin genes is maintained in the environment in alternative microbial hosts not typically associated with the human disease. The fact that these genes are mobile within phage means they could be facilitating the evolution and emergence of novel human pathogens through HGT. Our future studies will focus on identifying these environmental reservoirs, the alternative microbial hosts carrying the exotoxin genes, and elucidating the rate at which HGT events could be happening in nature.

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Exotoxin genes in the environment

### Supplementary material

The following supplementary material is available online:

**Fig. S1.** ClustalX sequence alignment of *stx* PCR products from water, soil, and sediment samples with target *stx* 2A gene.

**Fig. S2.** Effects of environmental DNA on QPCR.

**Fig. S3.** Example standard curve for *sea* QPCR.

**Table S1.** Details of sample collection and processing.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

Chapter 1, in full, is a reprint of the material as it appears in FEMS Microbiology Letters 2006. Casas, V., Miyake, J., Balsley, H., et al., Blackwell Publishing, 2006. I was the primary investigator and author of this paper.

**CHAPTER 2: DISTRIBUTION OF PHAGE-ENCODED EXOTOXIN GENES IN  
COASTAL WATERS FOLLOWING RAIN EVENT**

## INTRODUCTION

Pollution of coastal waters from storm runoff is a common problem in San Diego County. Following a storm event, it is commonplace for San Diego beachgoers to be advised not to enter the water for up to three days due to contamination by microorganisms that may cause human disease. These potential disease microorganisms are the bacteria and viruses that are harbored by human feces—such as fecal coliform indicator bacteria (FIB) and enteric viruses. The diseases associated with swimming or coming into contact with polluted waters are often due to infection by enteric viruses as well as FIB [161-171]. Due to difficulties in cultivating the enteric viruses, however, it is more common to cultivate and enumerate FIB to determine the water quality of potentially polluted waters [172-176]. The problem with relying on this type of monitoring is that the relationship between presence of FIB and presence of pathogenic enteric viruses has not been clearly established [162, 163, 177]. This lack of a clear relationship between FIB and the pathogenic enteric viruses present in polluted waters after a storm event necessitates a more direct approach to determining contamination of coastal waters [178-185]. Molecular assays testing for virulence genes may offer an alternative approach to assessing water quality without the need for cultivation of bacterial or viral pathogens.

Depending on the season, San Diego County monitors its coastal waters rigorously and regularly for FIB. The Ocean and Bay Recreational Water Program (OBRWP) under the San Diego County Department of Environmental Health (DEH) is responsible for monitoring the water quality of San Diego's recreational waterways [186, 187]. From the period of April 1 through October 31, California generally requires

weekly water-quality testing at beaches that have more than 50,000 visitors a year and are located next to storm drains that flow during the summer [188]. San Diego County monitors 55 beach sites and reviews the results of tests conducted by wastewater agencies at 41 other spots. From the period of November 1 through March 31, San Diego County typically takes weekly samples at 13 or 14 sites and reviews information from the wastewater agencies during this period [186, 188]. The data gathered from the monitoring is used by the OBRWP to determine if local waterways may be used by the public without risk of illness. If the waters sampled exceed specific FIB levels, the local DEH official will issue a water contact advisory or complete beach closure until additional testing reveals bacterial levels have been reduced to meet state standards. From 2000 to 2006 the DEH issued an average of 42 general advisory days due to urban runoff following a rain event [187]. During that same period, sewage spills caused complete beach closures on an average of 40 days per year [187].

Beach closures due to sewage spills are unfortunately a common occurrence at the southern beaches of San Diego County due to the untreated raw sewage that flows from the Tijuana River into the Pacific Ocean [189]. The OBRWP uses the term Beach Mile Days (BMDs) to represent the number of miles and days a beach is closed for exceeding state standards (BMD = miles closed x days closed) [187]. From 2000 to 2006, the southern San Diego County beaches, Coronado Beach and Imperial Beach, had average BMD values of 60 and 66, respectively. During that same period, the northern beaches of San Diego County rarely exceeded ten BMDs [187]. The raw sewage effluent from the Tijuana River is cited as the major reason for pollution and subsequent beach closures in the beaches of southern San Diego County. The Tijuana River contributed to more than

70% of beach closures in six of the seven years from 2000 to 2006 [187]. Contamination of these waters by human feces from the raw sewage is a major public health concern because although monitoring for FIB is performed, there is a lack of a clear direct relationship between FIB and the viruses of human fecal origin that generally cause waterborne illness [177, 181, 184]. A study quantifying the levels of fecal indicator organisms, both bacterial and viral, near the US/Mexico border during the wet and dry seasons sought to determine if there was a direct correlation between FIB and the enteroviruses present in human feces [181]. The results from this study indicated a direct positive relationship between levels of FIB and enteroviruses, as well as a direct relationship between levels of these organisms and Tijuana River flow rate and precipitation levels [181]. Altogether, these results suggested that during the wet season the sewage flowing into the Pacific Ocean from the Tijuana River has a large impact on the water quality of San Diego County's southern beaches.

To investigate the extent of the impact of the Tijuana River on the water quality of San Diego County beaches, we screened water and sediment samples collected from the Tijuana River Estuary, Imperial Beach, Ocean Beach, and the beach near Scripps Institute of Oceanography for the presence of bacterial virus-encoded exotoxin genes. Exotoxin-specific PCR was used to screen for cholera toxin (*ctx*), diphtheria toxin (*dtx*), staphylococcus enterotoxin a (*sea*), and shiga toxin (*stx*). We hypothesized that bacterial viruses are indicators of total viruses (bacterial and eukaryotic) present in the environment and are therefore better indicators of contamination and potential for human disease outbreaks.

The samples were collected for one week in the rainy season during and for one week following a storm event. We utilized this sampling strategy to determine if a temporal concentration gradient of exotoxin gene(s) existed, with exotoxin gene(s) being detected on the days immediately following the storm event and tapering off the rest of the week. The four locations sampled were chosen based on their proximity to a potential contamination source, either a river mouth outlet or storm drain outlet. The locations were distributed from southern San Diego County to northern San Diego County to determine if a concentration gradient of exotoxin gene(s) existed—emanating from the highly polluted Tijuana River Estuary and Imperial Beach areas to the beaches of northern San Diego County.

## **RESULTS**

### **Standard exotoxin PCR and QPCR assays detect *ctx*, *dtx*, and *stx* genes in sediment samples**

Imperial Beach, Ocean Beach, and Scripps Institute of Oceanography (SIO) near-shore sediment samples were collected for eight days and each sample was screened for the presence of the genes for cholera toxin (*ctx*), diphtheria toxin (*dtx*), *Staphylococcus* enterotoxin A (*sea*), and shiga toxin (*stx*) by standard PCR and quantitative real-time PCR (QPCR). The combined standard PCR and QPCR results are listed in Table 2.1. These assays detected the presence of at least one of the four exotoxin genes in at least one location on each day of sampling (Table 2.1).

At the Imperial Beach location, at least one exotoxin gene was detected everyday except day three where no exotoxin genes were detected by either standard PCR or QPCR (Table 2.1). The *stx* gene was detected each day except day three. The *dtx* and *stx*

genes were detected together at this location on day five. The *ctx* and *sea* genes were not detected by any assay at the Imperial Beach location.

At least one exotoxin gene was detected on each day except day five at the Ocean Beach location (Table 2.1). Only the *ctx* gene was detected on day two. Only the *stx* gene was detected on day four. The *ctx* and *stx* genes were detected together on days one, three, six, seven, and eight. The *dtx* and *sea* genes were not detected by any assay at the Ocean Beach location.

At least one exotoxin gene was detected on each day except day two at the beach near SIO (Table 2.1). The *ctx* gene was detected on days five and six. The *dtx* gene was detected on days one, six, and eight. The *stx* gene was detected on days three through eight. There were two exotoxin genes detected on days five and eight (*ctx* and *stx*; *dtx* and *stx*, respectively). The *ctx*, *dtx*, and *stx* genes were all detected on day six. The *sea* gene was not detected by any assay at the beach near SIO.

The *stx* gene was the gene detected most frequently in the sediment samples by either standard PCR or QPCR, followed by the *ctx* and *dtx* genes. The *sea* gene was not detected by either assay in any of the sediment samples from any location. The *stx* and *ctx* gene also were most frequently found together in the same sample. All four exotoxin genes were never detected together in any sediment sample by either standard PCR or QPCR. The results for all negative controls were always negative.



**Table 2.1. Exotoxin genes detected in sediment and water samples by standard and Quantitative PCR (QPCR).** Each sediment and water sample gathered was screened by each exotoxin-specific PCR (standard and QPCR). The exotoxin gene(s) detected in each sample is/are indicated in the table. *ctx* = cholera toxin gene; *dtx* = diphtheria toxin gene; *sea* = staphylococcus enterotoxin A gene; *stx* = shiga toxin gene; SIO = Scripps Institute of Oceanography; NS = not sampled; — = no exotoxin genes detected.

Day	Sample Location	Sediment	Water
1	Tijuana River Estuary	NS	<i>stx</i>
	Imperial Beach	<i>stx</i>	<i>stx</i>
	Ocean Beach	<i>ctx, stx</i>	<i>stx</i>
	SIO	<i>dtx</i>	<i>stx</i>
2	Tijuana River Estuary	NS	<i>ctx, sea, stx</i>
	Imperial Beach	<i>stx</i>	<i>stx</i>
	Ocean Beach	<i>ctx</i>	<i>stx</i>
	SIO	—	<i>ctx, stx</i>
3	Tijuana River Estuary	NS	<i>sea, stx</i>
	Imperial Beach	—	<i>sea</i>
	Ocean Beach	<i>ctx, stx</i>	<i>sea, stx</i>
	SIO	<i>stx</i>	<i>ctx, stx</i>
4	Tijuana River Estuary	NS	<i>stx</i>
	Imperial Beach	<i>ctx</i>	<i>dtx, sea, stx</i>
	Ocean Beach	<i>stx</i>	<i>dtx, stx</i>
	SIO	<i>stx</i>	<i>dtx</i>
5	Tijuana River Estuary	NS	<i>ctx, sea, stx</i>
	Imperial Beach	<i>dtx, stx</i>	<i>ctx, sea, stx</i>
	Ocean Beach	—	<i>stx</i>
	SIO	<i>ctx, stx</i>	<i>sea</i>
6	Tijuana River Estuary	NS	<i>stx</i>
	Imperial Beach	<i>ctx, stx</i>	<i>sea, stx</i>
	Ocean Beach	<i>ctx, stx</i>	<i>dtx, sea, stx</i>
	SIO	<i>ctx, dtx, stx</i>	<i>stx</i>
7	Tijuana River Estuary	NS	<i>stx</i>
	Imperial Beach	<i>stx</i>	<i>dtx, stx</i>
	Ocean Beach	<i>ctx, stx</i>	<i>stx</i>
	SIO	<i>stx</i>	—
8	Tijuana River Estuary	NS	<i>ctx, sea, stx</i>
	Imperial Beach	<i>stx</i>	—
	Ocean Beach	<i>ctx, stx</i>	<i>ctx, stx</i>
	SIO	<i>dtx, stx</i>	<i>stx</i>

### **Standard exotoxin PCR and QPCR assays detect *ctx*, *dtx*, *sea*, and *stx* genes in water samples**

Tijuana River Estuary, Imperial Beach, Ocean Beach and SIO near-shore water samples were collected for eight days and each sample was screened for the presence of the *ctx*, *dtx*, *sea*, and *stx* genes by standard PCR and QPCR. The combined results from these assays detected the presence of at least one of the four exotoxin genes in at least one location on each day of sampling (Table 2.1).

In the water samples collected from the Tijuana River Estuary, at least one exotoxin gene was detected each day by either standard PCR or QPCR (Table 2.1). The *ctx* gene was detected on days two, five, and eight. The *sea* gene was detected on days two, three, five and eight. The *stx* gene was detected on each day. There were two exotoxin genes (*sea* and *stx*) detected on day three. There were three exotoxin genes (*ctx/sea/stx*) detected on days two, five, and eight. The *dtx* gene was not detected by either standard PCR or QPCR at the Tijuana River Estuary.

At least one exotoxin gene was detected by either standard PCR or QPCR in the Imperial Beach water samples from days one through seven (Table 2.1). The *ctx* gene was detected on day five. The *dtx* gene was detected on days four and seven. The *sea* gene was detected on days three through six. The *stx* gene was detected on days one, two, and four through seven. There were two exotoxin genes detected on days six and seven (*sea* and *stx*; *dtx* and *stx*, respectively). There were three exotoxin genes detected on days four and five (*dtx/sea/stx*; and *ctx/sea/stx*, respectively). All four exotoxin genes were detected by either standard PCR or QPCR in the water samples from Imperial Beach.

In the water samples from Ocean Beach, at least one exotoxin gene was detected by either standard PCR or QPCR on all eight sampling days (Table 2.1). The *ctx* gene

was detected on day eight. The *dtx* gene was detected on days four and six. The *sea* gene was detected on days three and six. The *stx* gene was detected on days one through eight. There were two exotoxin genes detected on days three, four, five, and eight (see Table 2.1). There *dtx*, *sea*, and *stx* genes were detected together on day six. All four exotoxin genes were detected by either standard PCR or QPCR in the Ocean Beach samples.

At least one exotoxin gene was detected by either standard PCR or QPCR in the samples collected from SIO on each day except day seven (Table 2.1). The *ctx* gene was detected on days two and three. The *dtx* gene was detected on day four. The *sea* gene was detected on day five. The *stx* gene was detected on days one, two, three, six, and eight. The *ctx* and *stx* genes were detected together on days two and three. All four exotoxin genes were detected by either standard PCR or QPCR in the SIO water samples.

The *stx* gene was the gene detected most frequently in the water samples by either standard PCR or QPCR, followed by the *sea*, *ctx*, and *dtx* genes, respectively. The *sea* and *stx* genes were more frequently detected together in the same sample. The *ctx* and *stx* genes were also frequently detected together. The *dtx* and *stx* genes were also detected together. The *ctx*, *sea*, and *stx* genes were detected together four times from the Tijuana River Estuary and Imperial Beach samples on days two, five, and eight. On day five, the three genes were detected in both the Tijuana River Estuary and Imperial Beach water samples. The results for all negative controls were always negative.

### **QPCR results on estimated exotoxin gene copies present in sediment samples indicate mostly low level exotoxin genes throughout sampling week**

QPCR assays were utilized to estimate the number of exotoxin gene copies in the sediment samples collected from Imperial Beach, Ocean Beach, and SIO. A standard

curve of positive control DNA was run for each exotoxin gene QPCR and was used to estimate the number of exotoxin gene copies in the samples collected. Negative controls were also run in each QPCR assay and the results were always negative. The *ctx*, *dtx*, and *stx* genes were detected by QPCR in the sediment samples. The *sea* gene was not detected by QPCR in the sediment samples.

QPCR assays detected the *ctx*, *dtx*, and *stx* genes in sediment samples collected from Imperial Beach (Figure 2.1). The *ctx* gene was detected on day four at 5.41 copies ml<sup>-1</sup>. The *dtx* gene was detected on day five at 1.63 copies g<sup>-1</sup>. The *stx* gene was detected on days five and eight at 0.29 and 0.22 copies g<sup>-1</sup>, respectively.

QPCR assays detected the *ctx* and *stx* genes in sediment samples collected from Ocean Beach (Figure 2.1). The *ctx* gene was detected on days two and three at 5.84 and 0.72 copies ml<sup>-1</sup>, respectively. The *stx* gene was detected on days one, two, and eight at 1.61, 1.91, 2.23 copies g<sup>-1</sup>, respectively.

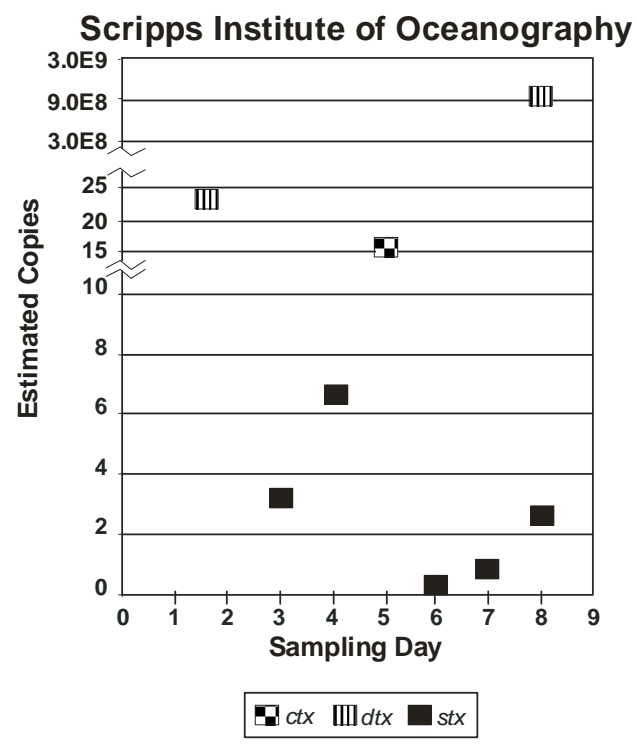
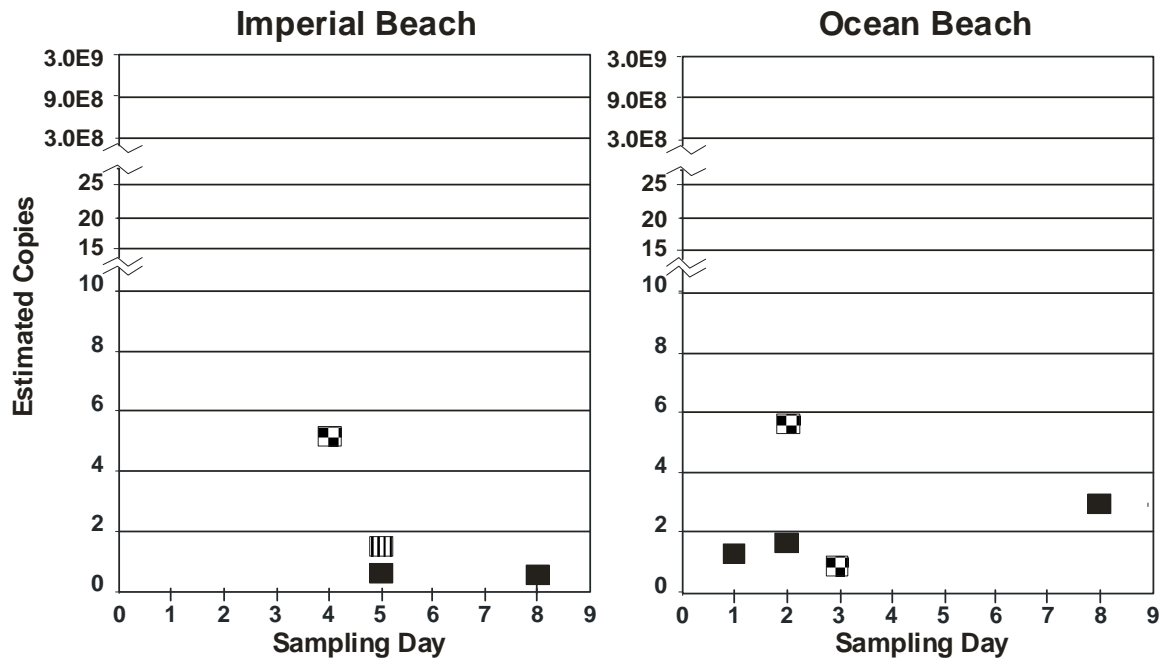
QPCR assays detected the *ctx*, *dtx*, and *stx* genes in sediment samples collected from the beach near SIO (Figure 2.1). The *ctx* gene was detected on day five at 16.13 copies g<sup>-1</sup>. The *dtx* gene was detected on days one and eight at 24.0 and 9.14E+08 copies g<sup>-1</sup>, respectively. The *stx* gene was detected on days three, four, six, seven, and eight at 2.23, 6.50, 0.19, 0.78, and 2.46 copies g<sup>-1</sup>, respectively.

### **QPCR results on estimated exotoxin gene copies present in water samples indicate mostly low level exotoxin genes throughout sampling week**

QPCR assays were utilized to estimate the number of exotoxin gene copies in the water samples collected from Imperial Beach, Ocean Beach, and SIO. A standard curve

of positive control DNA was run for each exotoxin gene QPCR and was used to estimate the number of exotoxin gene copies in the samples collected. Negative controls were also run in each QPCR assay and the results were always negative. The *ctx*, *sea*, and *stx* genes were detected by

**Figure 2.1. Estimated gene copies by QPCR for exotoxin-positive sediment samples.** Sediment samples were collected at Imperial Beach, Ocean Beach, and Scripps Institute of Oceanography and screened for exotoxin genes by QPCR. A standard curve was run in each assay to generate the estimated exotoxin gene copies ml<sup>-1</sup> in each sample where exotoxin genes were detected. The estimated gene copy levels for each location across the week sampled are depicted in the figure. The sea gene was not detected by QPCR in any of the sediment samples at any location on any day. ctx = cholera toxin gene; dtx = diphtheria toxin gene; stx = shiga toxin gene.



QPCR in the sediment samples. The *dtx* gene was not detected by QPCR in the water samples (Figure 2.2).

QPCR assays detected the *ctx* and *stx* genes in the water samples collected from the Tijuana River Estuary (Figure 2.2). The *ctx* gene was detected on day two at 2.73 copies ml<sup>-1</sup>. The *stx* gene was detected on days one, two, three, four, and seven at 4.78, 7.17, 1.43, 5.22E+04, 0.01, and 2.85 copies ml<sup>-1</sup>, respectively.

QPCR assays detected the *sea* and *stx* genes in the water samples collected from Imperial Beach (Figure 2.2). The *sea* gene was detected on day five at 97.5 copies ml<sup>-1</sup>. The *stx* gene was detected on days one through eight at 8.38, 1.06, 2.17, 6.79, 5.99, 70.6, 2.99, and 3.04 copies ml<sup>-1</sup>, respectively.

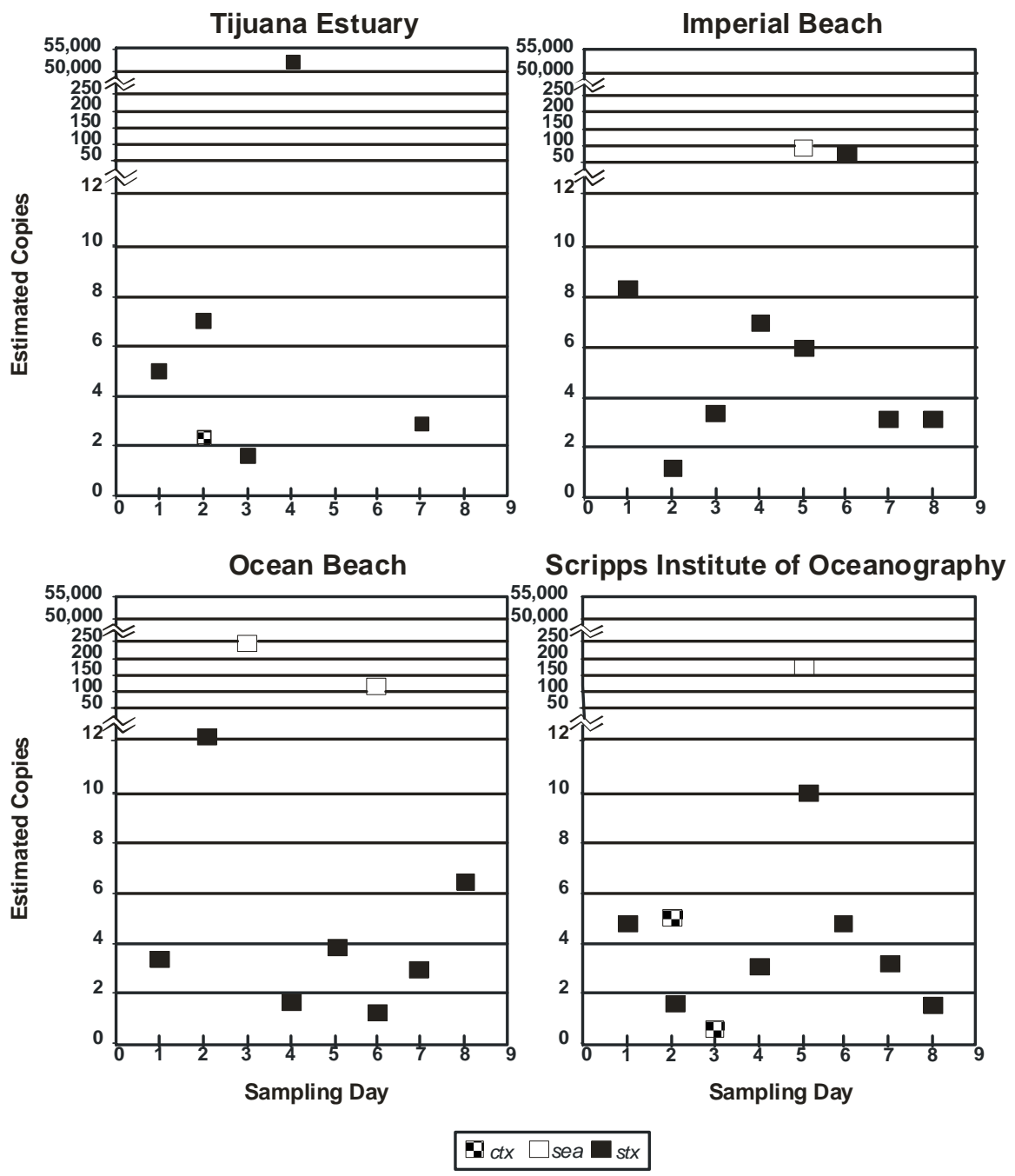
QPCR assays detected the *sea* and *stx* genes in the water samples collected from Ocean Beach (Figure 2.2). The *sea* gene was detected on days three and six at 239 and 103 copies ml<sup>-1</sup>, respectively. The *stx* gene was detected on days one, two, four, five, six, seven, and eight at 2.31, 11.9, 1.61, 3.78, 1.39, 2.83, 6.36 copies ml<sup>-1</sup>, respectively.

QPCR assays detected the *ctx*, *sea*, and *stx* genes in the water samples collected from the beach near SIO (Figure 2.2). The *ctx* gene was detected on days two and three at 4.90 and 0.57 copies ml<sup>-1</sup>, respectively. The *sea* gene was detected on day five at 181 copies ml<sup>-1</sup>. The *stx* gene was detected on days one, two, four, five, six, seven, and eight at 4.68, 1.53, 2.97, 10.1, 4.82, 2.12, and 1.58 copies ml<sup>-1</sup>, respectively.



**Figure 2.2. Estimated gene copies by QPCR for exotoxin-positive water samples.**

Water samples were collected at Tijuana River Estuary, Imperial Beach, Ocean Beach, and Scripps Institute of Oceanography and screened for exotoxin genes by QPCR. A standard curve was run in each assay to generate the estimated exotoxin gene copies ml<sup>-1</sup> in each sample where exotoxin genes were detected. The estimated gene copy levels for each location across the week sampled are depicted in the figure. The dtx gene was not detected by QPCR in any of the water samples at any location on any day. ctx = cholera toxin; sea = Staphylococcus enterotoxin A; stx = shiga toxin gene.



## DISCUSSION

Pollution of local recreational waterways in San Diego County is a major health concern. Routine monitoring of levels of fecal indicator bacteria (FIB) is performed for San Diego County beaches using traditional microbiological methods and often finds high levels of FIB following rain events. These methods test for the presence and concentration of bacterial pathogens that are transmitted by fecal contamination such as *Escherichia coli*. Levels of FIB are monitored because it is thought that there is a direct correlation between presence of high levels of FIB and presence of high levels of human viral pathogens, such as enteroviruses and hepatitis A virus (HAV). Whether this correlation truly exists and is applicable across all environmental situations is currently being debated. What is certain is that the standard cell culture assays for viral pathogens are difficult to perform on viruses from environmental samples. An alternative approach would be to screen for the presence and concentration of the virulence genes carried by bacterial viruses as a proxy for the presence and concentration of total eukaryotic and bacterial viruses.

This study focused on the screening of water and sediment samples from the local San Diego recreational waterways for the presence of bacterial virulence genes that are encoded by bacterial viruses. Using molecular assays we screened water and sediment samples for the genes that encode the cholera toxin (*ctx*), diphtheria toxin (*dtx*), staphylococcus enterotoxin A (*sea*), and shiga toxin (*stx*). The samples were gathered at the onset and for one week following a storm event. The purpose was to determine if detection of bacterial virulence genes encoded by bacterial viruses could serve as an alternative method for determining water quality of recreational waterways.

The standard PCR and QPCR assays we have developed for detection of virulence genes encoded by bacterial viruses were able to detect exotoxin genes in the sediment and water samples collected after the storm event. The results from the standard PCR and QPCR assays generally agreed with each other. The *ctx*, *dtx*, *sea*, and *stx* genes were all detected in the water samples by either standard PCR or QPCR, but the *sea* gene was not detected by either assay in the sediment samples. The *stx* gene was the gene most frequently detected by any assay in the sediment and water samples. This is not surprising because if fecal contamination is present, the *stx* gene is harbored in microorganisms commonly associated with human feces such as *E. coli*. This suggests using the molecular assays to screen sediment and water samples for the *stx* gene may be a suitable alternative for determining presence of fecal contamination.

We also wanted to determine if there was a prevalence of bacterial virus-encoded exotoxin genes at the onset of the rain event and immediately following the event, but tapering off throughout the week. The QPCR assays were utilized not only to determine presence of an exotoxin gene, but also to estimate its concentration within the sediment or water sample. The results from these assays indicated a mostly low level (<12 copies ml<sup>-1</sup>) presence of exotoxin genes in the sediment and water samples throughout the week, with a few instances of high exotoxin gene levels. It should be noted that the efficiency of our DNA extraction methods have not been determined, therefore the copy levels generated by the QPCR assays serve as estimations of exotoxin gene copies in the original sample. The QPCR assays did not detect a discernable concentration gradient of exotoxin genes in sediment and water samples following the rain event.

We were also interested in determining if there was a correlation between presence and/or concentration of bacterial virus-encoded exotoxin genes and proximity of sampling location to the Tijuana River outlet. The Tijuana River flows from Mexico along the U.S./Mexico border and outlets into the Pacific Ocean just south of Imperial Beach in the southern portion of San Diego County (Figure 2.3). Raw sewage often flows from the Tijuana River into the Tijuana Estuary and Pacific Ocean due to the lack of a sewage treatment facility in Tijuana [189-192]. The study by Gersberg et al. [181] showed increased presence of fecal indicator organisms in the vicinity of the Tijuana River outlet. We wanted to investigate this phenomenon further using molecular assays we developed to detect the virulence genes that are encoded and carried by bacterial viruses.

Exotoxin genes were detected in the sediment and water samples by either standard PCR or QPCR. Exotoxin genes were detected more frequently in the water samples than in the sediment samples. This may be due to inhibition of the PCR assays by compounds present in trace amounts in the DNA extracted from the sediment samples and that this inhibition could not be overcome by dilution of the DNA.



**Figure 2.3. Map of sampling locations in San Diego County, California.** Four locations along the San Diego County, California coastline were sampled. The four sites sampled were the Tijuana River Estuary, Imperial Beach, Ocean Beach, and Scripps Institute of Oceanography and are indicated by a black star on the map. Water and sediment samples were gathered from each location, with the exception of the Tijuana River Estuary where only water samples were gathered due to protection of wildlife on the reserve.

The standard PCR and QPCR results did not indicate a correlation between presence and/or concentration of exotoxin genes in the sediment and water samples from the four sampling locations and proximity of those locations to the Tijuana River outlet.

Our methodologies were not able to determine the extent of the impact of the Tijuana River on the water quality of San Diego County beaches.

Bacterial virus-encoded exotoxin genes were detected in sediment and water samples collected during the onset and for one week following a storm event in San Diego County. Neither a spatial nor temporal pattern in the presence or concentration of exotoxin genes could be discerned in the sediment and water samples collected. It is possible that collecting and screening a higher number of samples from each location on each sampling day may have been able to generate more data from which a pattern could have been distinguished. Nonetheless, the methodologies utilized were able to detect exotoxin genes encoded by bacterial viruses in the water and sediment samples collected. With further refinement of these molecular assays, specifically the efficiency of the QPCR assays, they could serve as an alternative approach to determining presence of disease-causing organisms of human fecal origin in sediment and water samples gathered from recreational waterways.

## **MATERIALS AND METHODS**

### **Water and sediment sampling strategy**

Water and sediment samples were gathered from southern to northern coastal waters in San Diego County for one week during the wet season (March 21, 2007-March 28, 2007). The sampling sites were the Tijuana River Estuary, Imperial Beach, Ocean Beach (where the San Diego River and other waterways empty into the Pacific Ocean), and the beach near Scripps Institute of Oceanography (SIO; sampled at the water's edge where a storm drain empties into the Pacific Ocean). A map indicating the locations

sampled is depicted in Figure 2.3. Sediment samples were not collected from the Tijuana River Estuary due to nesting of protected bird species. All samples were collected on the day of and for one week after a rain event that produced 0.10-0.25 inches of precipitation. For collecting sediment samples, new disposable plastic containers were used for gathering up to 750 ml of each sample. For water samples, clean and sanitized 1.0 liter Nalgene® containers were used to collect 1.0 liter of each sample. Samples were gathered, kept on ice, and returned to the lab for processing within 2 hours. A total of eight water and sediment samples were gathered from each location (with the exception of the Tijuana River Estuary where only water was sampled).

#### **DNA extraction, exotoxin-specific standard PCR, and QPCR of water and sediment samples**

Methods for extracting DNA and performing the exotoxin-specific standard and real time quantitative PCRs (QPCR) on environmental samples are as described previously [193, 194]. Each sample was screened by standard PCR and QPCR. The QPCR assays were utilized to corroborate standard PCR assay results and also to estimate exotoxin gene copy levels by extrapolation from a standard curve. To reduce possibilities for PCR contamination, processing of environmental samples to set up for standard PCR and QPCR assays was performed in a separate laboratory in a separate building from where PCR amplifications were performed. All PCR amplification products were contained to the amplification laboratory. All reagents and equipment used were also kept separate. A negative control was also included in all assays. For the QPCR, samples were tested in triplicate and dilutions of the total DNA were tested, where necessary, to overcome assay inhibition from the undiluted DNA.



**CHAPTER 3: IDENTIFICATION OF PHAGE-ENCODED *sea* GENE IN AN ATYPICAL BACTERIAL HOST CULTIVATED FROM THE ENVIRONMENT**

## Research Article

# Reservoir of Bacterial Exotoxin Genes in the Environment

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Many bacteria produce secreted virulence factors called exotoxins. Exotoxins are often encoded by mobile genetic elements, including bacteriophage (phage). Phage can transfer genetic information to the bacteria they infect. When a phage transfers virulence genes to an avirulent bacterium, the bacterium can acquire the ability to cause disease. It is important to understand the role played by the phage that carry these genes in the evolution of pathogens. This is the first report of an environmental reservoir of a bacterial exotoxin gene in an atypical host. Screening bacterial isolates from the environment via PCR identified an isolate with a DNA sequence >95% identical to the *Staphylococcus aureus* enterotoxin A gene (*sea*). 16S DNA sequence comparisons and growth studies identified the environmental isolate as a psychrophilic *Pseudomonas* spp. The results indicate that the *sea* gene is present in an alternative bacterial host, providing the first evidence for an environmental pool of exotoxin genes in bacteria.

## 1. Introduction

Exotoxins are secreted polypeptides produced by certain bacterial pathogens. Many exotoxin genes are carried on mobile genetic elements, including bacterial viruses (bacteriophage or phage). These virulence genes are responsible for many of the symptoms associated with the human disease [1–3]. As highly mobile genetic elements, phage can readily move between different environments, and are generally more resistant to environmental stress than their bacterial counterparts [4–11]. As a result, phage may survive in the environment in reservoirs not yet characterized. Living in these environmental reservoirs, the phage can influence evolution of the bacteria within these environments in several different manners. Horizontal gene transfer between the phage and the bacterium can result in the rapid evolution of new pathogens and may have serious implications in public health [12].

When a phage infects a bacterium, two alternative possibilities may result. One possibility is that the phage can replicate itself using phage and host factors, resulting in lysis of the bacterial host and release of new phage (the lytic lifestyle). Alternatively, the phage can integrate into the bacterial genome and the bacterial host can utilize certain

genes the phage carries in its genome for its own benefit (the lysogenic lifestyle) [13]. If a phage encodes virulence genes, such as exotoxin genes, the phage could facilitate the transfer of these genes to nontoxic bacterial hosts, thereby increasing the exotoxin gene pool.

The toxins of *Vibrio cholera* (cholera toxin), *Escherichia coli* (shiga toxin), *Corynebacterium diphtheria* (diphtheria toxin), and *Staphylococcus aureus* (enterotoxin A) are encoded by phage [14–17]. The *Staphylococcus aureus* enterotoxin A (*sea*) gene carried by virulent strains of *S. aureus* is encoded by multiple phages, including  $\phi$ 11,  $\phi$ 12,  $\phi$ 13, 80 $\alpha$ , and 42D [18–21]. Other toxins are carried by phages isolated from *S. aureus* strains from animals, food, and the environment [20, 22–31]. Given that multiple *S. aureus* toxins are encoded by phage, it is possible that multiple transduction events over time have resulted in the generation of the current virulent *S. aureus* strains. An environmental reservoir of toxin genes would provide novel virulence genes, and the genetic exchange between phage and novel bacterial hosts, could provide the mechanism for evolution of novel human pathogens.

To explore this hypothesis, we cultured environmental bacteria and screened them for a phage-encoded exotoxin gene. In this study, we describe the isolation of bacteria from

environmental ambient air and the screening of the isolates for the phage-encoded *sea* gene using an exotoxin-specific colony PCR assay. One isolate was confirmed positive for the *sea* gene, and the sequence of the *sea* gene was determined. Using 16S rDNA PCR sequencing and comparison to the nonredundant GenBank nucleotide database, we determined that the environmental isolate was a novel host for the *sea* exotoxin. This is the first report of an alternative bacterial host from the environment that carries a phage-encoded exotoxin gene that is commonly associated with a different bacterial host.

## 2. Results

**2.1. Cultivation and Exotoxin-Specific (*sea*) PCR Screening of Environmental Isolates.** Bacterial isolates were cultivated from the ambient environment by exposing Luria Bertani (LB) plates to air and then incubated at room temperature for 48–72 hr. Eighty nine isolates were subcultured into sterile 96-well plates containing LB with 15% glycerol, and grown with aeration for 48–72 hr. Using colony PCR specific for the *Staphylococcus enterotoxin A (sea)* gene, each of the cultivated environmental isolates was screened for the presence of the *sea* gene. Of 89 isolates screened, one putative *sea* positive isolate was identified. The isolate was single colony purified, and the *sea* PCR was repeated on this purified isolate to confirm that the isolate (SEAB3C070426 lab-designated identification) was positive for the *sea* gene. The resulting *sea*-specific PCR product was gel-purified and sequenced.

**2.2. Characterization of Environmental Isolate.** The cultivated and purified environmental isolate “SEAB3C070426” was microbiologically characterized by Gram staining and microscopic evaluation. The environmental isolate was identified as a Gram negative rod. Furthermore, the purified isolate’s growth characteristics were evaluated against a *S. aureus* known to carry the *sea* exotoxin gene (*S. aureus* Food Research Institute 913 strain). The environmental isolate did not grow on *S. aureus* enrichment media at 35°C [32], but grew on LB at room temperature after 48–72 hr. In contrast, the *S. aureus* FRI913 control grew on both the enrichment media and LB at both temperatures.

To molecularly identify the cultivated environmental isolate carrying the *sea* sequence, 16S rDNA colony PCR was performed and the resulting PCR product was sequenced. The resulting 16S rDNA sequence was imported into the ARB bacterial 16S rDNA database to identify its nearest relatives for downstream phylogenetic analyses [33]. The 16S rDNA PCR product sequence grouped with *Pseudomonas* spp. using the ARB alignment. The nearest relatives identified by ARB were exported and used to generate a phylogenetic tree. The phylogenetic analyses of the ambient air isolate, its nearest relatives, and select outgroups (including *S. aureus*) were performed using the PAUP\* program (Figure 1) [33]. The consensus tree generated from Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor Joining (NJ) analyses grouped the ambient

air isolate with *Pseudomonas* spp., not with *S. aureus*. The ML, MP, and NJ bootstrap values for the main branches of the consensus tree separating the ambient air isolate from *S. aureus* and grouping it with *Pseudomonas* spp. were  $\geq 94$ . The GenBank Accession number of environmental isolate “SEAB3C070426” is FJ979636.

**2.3. Characterization of the *sea* PCR Product.** Sequencing of the *sea* PCR product obtained from the environmental isolate generated a 280 bp DNA sequence. The nucleotide composition of the amplified sequence was analyzed using BioEdit [34]. The sequence of the amplified PCR product contained a G + C content of 30% over 92 predicted amino acids. A ClustalW [35] alignment of the translated partial sequence of the *sea*-related gene with known *sea* genes is presented in Figure S1 in Supplemental Material available online at doi:10.1155/2010/754368. A BLASTN alignment of the sequence against the nonredundant GenBank database confirmed that the amplified PCR product shared 95–96% nucleotide sequence identity with known *sea* genes. Most notably, the amplified PCR product was 96% similar to a known *S. aureus* phage,  $\phi$ NM3. A multiple alignment of the partial sequence of the *sea*-related gene and guide tree of the top BLASTN hits were produced using ClustalX2 [36] (Figures 2 and 3). BLASTN alignment of the *sea*-related gene against annotated *S. aureus* genomes from The SEED database was also performed [37]. This alignment indicated the *sea*-related gene was related to other *S. aureus* phage-associated enterotoxin genes (supplementary Figure S2). The *sea*-related gene was uploaded to the GenBank database and the Accession number is HQ698309.

## 3. Discussion

Transfer of exotoxin genes to new, as yet uncharacterized, bacterial hosts may facilitate the evolution of novel human pathogens. Many of the exotoxins produced by phage-encoded genes target key eukaryotic cellular processes such as protein synthesis [38–43]. These phage encode the genes responsible for many of the symptoms associated with the human disease. Until recently, most epidemiological and ecological studies of infectious diseases have focused on the presence and activity of the bacteria per se, neglecting the potentially significant role of the phage that carry the exotoxin genes and their role in transmitting these virulence traits [44–47].

Sequence analysis of several environmental metagenomes has shown that phage carrying exotoxin genes are common in the environment, however 16S rDNA analysis of metagenomes from the same environmental samples did not identify the cognate bacterial phage host [48]. A potential explanation of this finding is that phage in the environment may propagate in alternate bacterial hosts rather than those commonly associated with the human disease. The classic textbook description of phage-bacteria interactions implies that phage infection is limited to a specific host. However, as the ecology and physiology of phages has been further investigated, it is clear that some phage can infect multiple

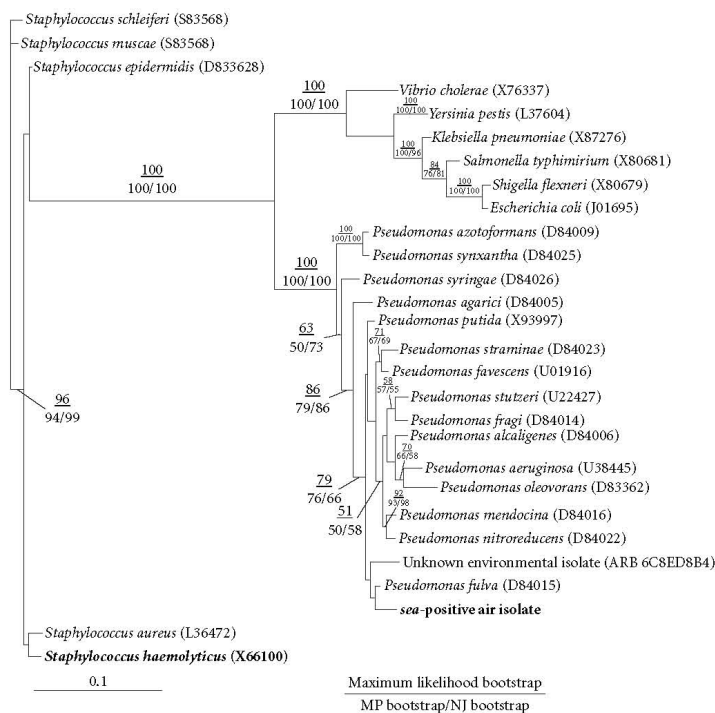


FIGURE 1: Phylogenetic tree of *sea*-positive isolate, its nearest relatives, and select outgroups. PAUP\* was used to generate a consensus phylogenetic tree. Maximum Likelihood (ML) analysis was performed using 100 replicates with 10 random addition sequences searches per replicate. Maximum Parsimony (MP) analysis was performed using searches on 100 bootstrap replicated datasets with 10 search replicates. The Neighbor-joining (NJ) bootstrap analysis was performed with 1000 replicates. The (ML bootstrap)/(MP bootstrap/NJ bootstrap) values are as indicated. The *sea*-positive isolate is highlighted in bold and GenBank Accession numbers of each organism are in parentheses.

bacterial hosts [23, 49–73]. Examples of phage that carry exotoxin genes and can infect alternative hosts include *ctxϕ* (infects both *Vibrio cholerae* and *Vibrio mimicus* [74]), *stx-2* phage (infects *E. coli* and *Enterobacter cloacae* [75]), and Botulinum toxin E phage (infects *Clostridium botulinum* and *Clostridium butyricum* [76]).

The mechanisms phage have developed to allow infection of a range of hosts are varied. Phage T2 and phage Mu alter their tail fibers to allow infection of alternative hosts [77, 78]. *Bordetella* phage carry diversity generating retroelements that allow the phage to infect bacteria with different cell surface receptors and physiology [79]. Some phage can inactivate or recombine with endogenous lysogens to alter their host range [45, 80, 81]. Inter- and intraspecies examples of phage showing a larger infection range than previously believed have been demonstrated through experimentation with lab strains [52, 62, 65, 67, 71, 72, 82–87].

Infection of alternative hosts in nature has also been demonstrated. Phage isolated from natural marine environments have been shown to subtly influence the composition of the bacterial community of those same environments [88]. Conversely, phage populations from various natural environments such as soil, lake water, and marine sediments have been shown to replicate when incubated with microbes from a different marine environment [6]. Comparison of the distribution of phage types in particular environments with the types of bacteria found in that same environment suggests that phage with quite broad host ranges must exist in nature [89]. Additionally, analyses of uncultured environmental phage libraries have revealed an abundance of mobile elements and genes involved in the mobilization of DNA [90–92]. Altogether these results suggest that phage are capable of infecting different hosts in the environment, providing a major mechanism for the spread of genes



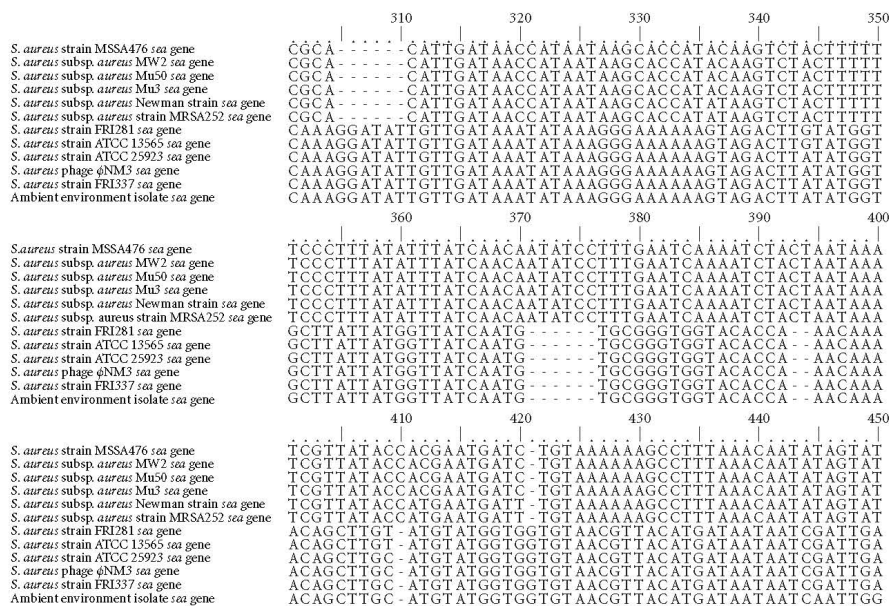


FIGURE 2: ClustalX2 alignment of top BLASTN hits of the *sea* gene from the ambient environmental isolate. The *sea* PCR product amplified from the cultured ambient air isolate was verified against the GenBank nonredundant database. The FASTA files of the top hits were downloaded and aligned using ClustalX2. The Accession number for the *sea*-related gene is HQ698309.

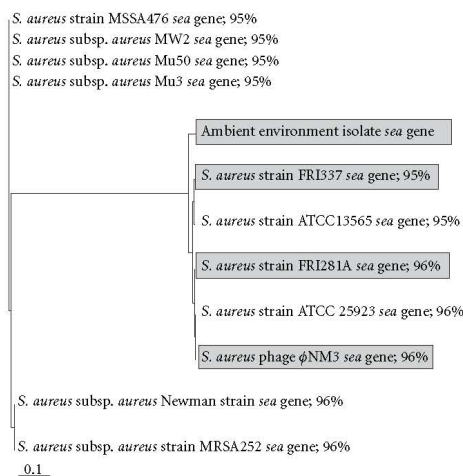


FIGURE 3: ClustalX2 guide tree of the top BLASTN hits of the *sea* gene from the ambient environmental isolate. The ambient air isolate is highlighted by a gray box along with *S. aureus* strains FRI337 and FRI281A (Food Research Institute) because the positive control used for *sea* exotoxin PCR comes from *S. aureus* strain FRI913. Percent identities are indicated beside the organism name as listed in GenBank. The GenBank Accession number for the *sea*-related gene is HQ698309.

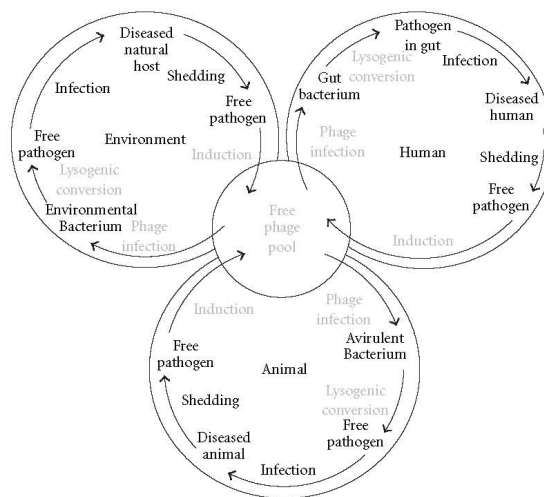


FIGURE 4: Free phage pool of exotoxin genes. Proposed scenarios for how exotoxin-encoding phage might be maintained in the environment and produce human pathogens through genetic exchange between the free phage pool and the natural, human, and animal environments. Light grey writing indicates phage/host interaction and potential for horizontal gene transfer.

between bacteria. Thus, phage may promote promiscuous horizontal gene transfer in nature.

This report provides the first direct evidence that alternative microbial hosts can carry exotoxin genes. The environmental isolate cultivated from outdoor ambient air was confirmed to carry the *sea* exotoxin gene by repeated exotoxin-specific PCR, and by sequencing and alignment of the *sea*-specific PCR product. The *sea*-specific PCR product was 95-96% identical at the nucleotide level to known *sea* genes in the GenBank nonredundant database.

When characterized microbiologically, the cultivated environmental isolate did not share the same characteristics as the control *S. aureus* FR1913 strain known to carry the *sea* gene. It did not grow on *Staphylococcus* enrichment media, nor did it share the same Gram staining properties as the *S. aureus* FR1913 strain (the environmental isolate was a Gram negative rod versus *Staphylococcus* which are Gram positive cocci). These results suggested that the *sea* gene was present in an alternative environmental host.

Further support for this conclusion was provided by sequencing of the 16S rDNA from the cultivated environmental isolate. Alignment of the 16S rDNA sequence from this isolate with the ARB database indicated the isolate was a *Pseudomonas* spp. and did not group with *S. aureus*. Moreover, phylogenetic analyses using PAUP\* indicated that the isolate was related to *Pseudomonas* spp., not *S. aureus*. The ML, MP, and NJ bootstrap values of the main branches of the consensus tree that group the isolate with *Pseudomonas* spp. were  $\geq 94$ , indicating high probability that these branches are robust. This provides robust statistical evidence that the

isolate is a *Pseudomonas* spp. Further evidence supporting this identification of the ambient air isolate was its relatively high G + C content (52.36%) like many *Pseudomonas* spp., as compared to the low G + C content of *S. aureus* (32.8%).

We were unable to induce a *sea* positive phage by treatment from the environmental isolate via mitomycin C treatment [81]. There are at least two possible explanations why the environmental isolate was carrying the *sea*-related gene, but did not produce phage. First, generalized transduction could have transferred a region of bacterial chromosomal DNA containing the *sea* gene into the *Pseudomonas* spp. without integration of the phage itself [93]. Second, it is possible that the exotoxin was transferred via a phage, but the phage genes were subsequently mutated due to selection against harmful phage genes in the lysogen [94-102].

Based on the examples from the literature and the data generated from this study, we propose the following model for transfer of phage-encoded exotoxin genes to a novel bacterium leading to the creation of a new human pathogen. A "free phage pool" (Figure 4) can potentially lead to new disease outbreaks in three ways: (i) transduction of exotoxin genes to an environmental bacterium that subsequently infects a human; (ii) transduction of exotoxin genes from the environmental reservoir to a bacterium in the normal human microbiota; (iii) transduction of a bacterium associated with a nonhuman animal, with subsequent infection of a human. In all three scenarios, once the phage is propagated within the alternate bacterial hosts it can lyse the host and re-enter the "free-phage pool" ready to transduce the exotoxin genes to other bacteria. In this manner, the genes are maintained in

the environment independent of the bacterial host typically involved in the human disease.

The evidence that there is an environmental reservoir of exotoxin genes in bacteria that are not normally associated with human disease, suggests the possibility that novel diseases may evolve through horizontal transfer of virulence genes via transduction to new microbial hosts (Figure 4).

#### 4. Materials and Methods

**4.1. Sampling of Indoor and Outdoor Air.** To collect bacterial air isolates, Luria Bertani (LB) agar plates containing 50 mg of cyclohexamide to prevent growth of fungi, were exposed to ambient air. These plates were then incubated at room temperature for 48–72 hr. All isolates that grew on the LB plates conditions were then subcultured into sterile 96-well plates containing LB and 15% glycerol. These subcultured isolates were grown, with aeration, at room temperature for 48–72 hr then stored at 4°C until screened by PCR.

**4.2. PCR Assays and Sequencing.** A colony PCR assay was used to initially screen for the *Staphylococcus enterotoxin A (sea)* gene. The *sea*-specific PCR primers amplified a 498 bp partial sequence within the coding region of the *sea* gene. The *sea* primers were (forward primer) 5'-GCAGGGAACAGCTTTAGGC-3' and (reverse primer) 5'-GTTCTGTAGAAGTATGAAACACG-3'. To identify the bacterial isolate carrying the *sea* gene, a 16S rDNA PCR assay was used and the resulting PCR product sequenced. The primers used for the 16S rDNA PCR were (forward) 5'-AGAGTTTGATCMTGGCTCAG-3' and (reverse) 5'-TACGGYTACCTTGTTACGACTT-3'. Five microliters of the subcultured isolates suspension was used as a template in the *sea*-specific PCR and 16S rDNA PCR. The PCR thermocycling conditions for the *sea*-specific PCR and 16S rDNA PCR were as described previously [103]. To control against possible PCR contamination of the environmental sample DNAs, all PCRs were performed in a completely separate room and different building with different air handling systems. The PCR products were run on a 1% agarose gel at 150 V. Sequencing of the *sea*-specific PCR and 16S rDNA PCR products was performed at the SDSU Microchemical Core Facility. To confirm the *sea* PCR product was the target gene, a BLASTN alignment of the PCR product was performed against the nonredundant GenBank database [104]. The top 12 hits were then aligned and a tree generated using ClustalX2 [36].

**4.3. Microbiological Characterization of Environmental Isolate.** The air isolate carrying the *sea*-like gene was characterized by plating onto Staph 110 media [105], a media that enriches for *Staphylococcus aureus*, and LB medium. The plates were incubated at room temperature and at 35°C. A Gram stain was also performed on the isolate identified as *sea*-positive by colony PCR.

**4.4. Molecular Characterization of Environmental Isolate.** Phylogenetic analyses were performed on the 16S rDNA

PCR product sequence to molecularly identify the ambient air isolate. An alignment with the ARB 16S rDNA database was used to identify the nearest relatives to the ambient air isolate for use in creating a phylogenetic tree in PAUP\* [33]. Maximum Likelihood (ML) was implemented in PAUP\* to build the highest likelihood tree under an HKY85 model of sequence evolution with estimated nucleotide frequencies, shape parameter, and number of invariant sites. The heuristic search approach to find the best ML tree included 100 random addition sequence searches using TBR branch swapping. The ML bootstrap involved 100 replicates with 10 random addition sequences searches per replicate. The best Maximum Parsimony (MP) tree was found through a random addition sequence heuristic search strategy with 100 replicates. The maximum number of trees kept during each search was capped at 1000. MP bootstrap analyses were performed using searches on 100 bootstrap replicated datasets using the same heuristic search strategy except with 10, rather than 100, search replicates. The Neighbor-joining (NJ) bootstrap analysis was performed with 1000 replicates.

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**CHAPTER 4: IDENTIFICATION OF PHAGE-ENCODED SHIGA TOXIN GENE  
IN AN ATYPICAL BACTERIAL HOST CULTIVATED FROM CANINE FECES**

## INTRODUCTION

Each year millions of people flock to the beaches of San Diego County for fun in the sun, sand, and surf. Monitoring water quality at these beaches is therefore extremely important to limit the possibility of illness from contact with these recreational waterways. Through San Diego County's Department of Health (DEH), the Ocean and Bay Recreational Water Program (OBRWP) is responsible for monitoring the water quality of San Diego's recreational waterways [187]. Most beaches in San Diego County are relatively clean, with little to no advisories or beach closures, but there are also areas prone to advisories and/or beach closures because of known sources of pollution [186, 187]. Posted warning signs, a daily water quality report hotline, and an internet podcast are some of the ways the OBRWP informs the public about general advisories or beach closures they have issued as a result of elevated bacterial levels in the water [187]. Typical sources of bacterial contamination at San Diego County beaches include urban runoff from storm drains and rivers, animal waste, human activities, and sewage.

Contamination from human and animal feces is of particular concern because infectious disease agents like bacteria, viruses, and protozoa are shed in the feces of infected individuals [195]. *Vibrio cholera* (cholera), *Salmonella* spp. (typhoid fever, gastroenteritis), and *Shigella* spp. (shigellosis) are bacteria found in feces and cause gastrointestinal disease [195]. Some pathogenic viruses that can be transmitted through use of recreational waterways include enteroviruses, hepatitis A viruses (HAV), polioviruses, coxsackie viruses, echoviruses, rotaviruses, and Norwalk viruses [177, 181, 184, 196-198]. They cause a broad range of disease, not necessarily gut related, from hepatitis to polio to acute viral gastroenteritis [177, 198]. *Cryptosporidium*, *Giardia*, and

*Entamoeba* are common protozoan pathogens that cause cryptosporidiosis, giardiasis, and amoebic dysentery, respectively [184, 195, 198-202].

Though these organisms are common sources of waterborne diseases, their presence in these waters is not easily determined. As a result, presence of fecal indicator organisms has been accepted as the factor in determining water quality. Ideally, fecal indicator organisms would be good predictors of fecal contamination, and therefore good predictors of the potential for human illness. These fecal indicator organisms would be present whenever the pathogens were present, would survive in the environment as long as the pathogen was present, and would be easily detectable and cultivable from environmental samples [195]. Unfortunately, the relationship between presence of fecal indicator organisms, bacterial and viral pathogens, and actual fecal contamination is poorly understood. This leaves public health agencies at a disadvantage in determining the potential risk to the public's health when using recreational waterways.

The various San Diego County water quality monitoring laboratories currently use culturing assays (membrane filtration, multiple-tube fermentation, Colilert 18®, Enterolert®) to assess presence of fecal indicator organisms—namely coliforms [187, 203]. These assays take advantage of metabolic and enzymatic properties common to coliform bacteria [195]. Many studies have been performed to evaluate if fecal indicator organisms serve as a good proxy for monitoring water quality. Comparing cultivation tests for coliforms to molecular tests for other pathogenic bacteria and viruses, the studies showed mixed results—mostly indicating that relationships depend upon the water type, exposed population, and weather conditions (see [204] for a review) [163, 181, 204-207].

It is important to be able to have a broader view of the bacterial and viral community present in recreational waters because of their potential to cause human disease. This environment may provide selection for new virulence traits that could be missed by current methods. One mechanism for acquiring new virulence traits is through horizontal gene transfer (HGT). Bacteriophage are common mediators of this genetic exchange and they often carry genes that code for virulence factors. Since the discovery that phage beta from *Corynebacteria diphtheria* carried the gene for diphtheria toxin and was responsible for the virulence traits of *C. diphtheria*, many more bacteriophage-encoded virulence genes have been discovered [50, 51]. Some of these include the shiga toxin (*stx*) gene of *Escherichia coli* O157:H7 species, the cholera toxin gene (*ctx*) carried by *ctx-phi* of *V. cholera*, and the *Staphylococcus* enterotoxin A (*sea*) gene of *Staphylococcus aureus* [47, 208, 209]. Considering the high concentrations of bacteriophage and bacteria in the ocean, transduction frequencies in the World's oceans has been estimated to be as high as 20 million billion transduction events per second [79]. It is therefore important to understand the interactions between bacteria and bacteriophage in recreational waters and the potential for evolution of novel pathogens within these environments, to be able to better understand the risk to the public's health when using these waters.

## RESULTS

### **Shiga toxin (*stx*) gene detected in total and bacterial fraction DNA and bacterial isolate cultivated from canine feces**

Twenty canine fecal samples were gathered from the shoreline at the Ocean Beach Dog Beach in San Diego, California. Total and bacterial fraction DNA was

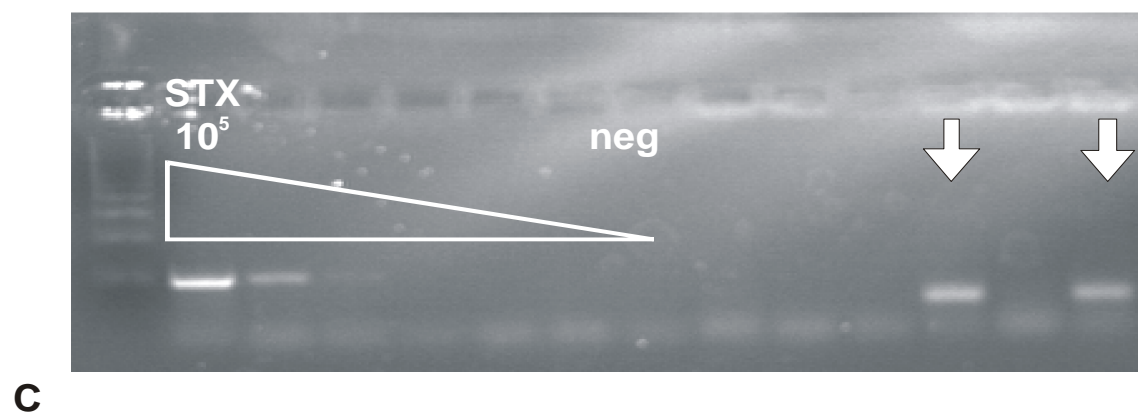
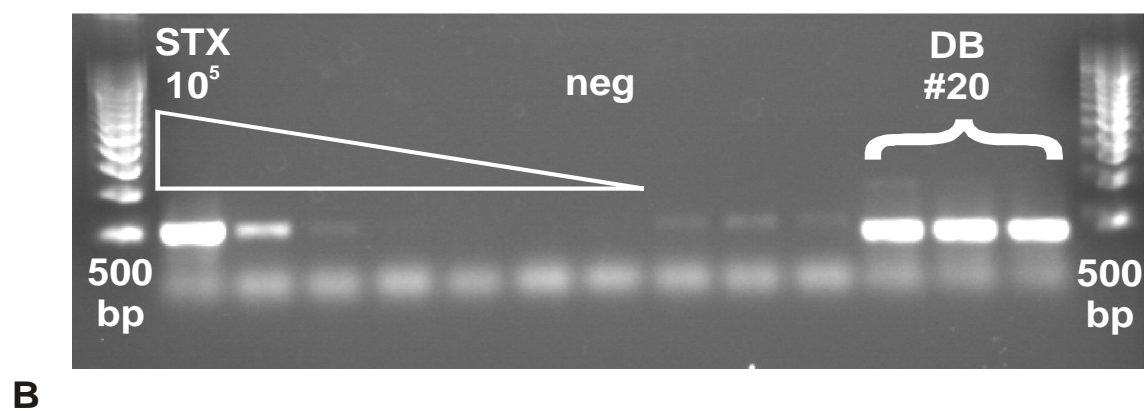
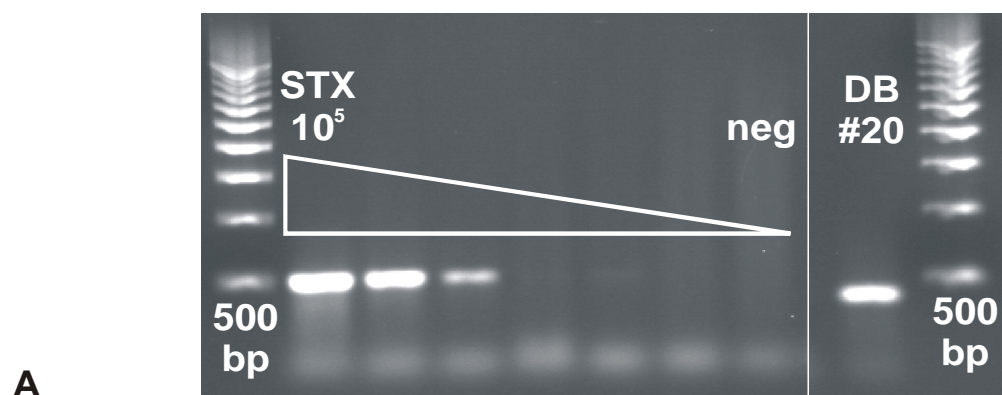


extracted from the fecal samples and screened for the *stx* gene by *stx*-specific PCR. The *stx* gene was detected in both the total and bacterial fraction DNA (Figure 4.1A and 4.1B, respectively). Bacterial isolates were cultivated from the canine fecal sample where the *stx* gene was detected. Eighty nine bacterial isolates were screened for the *stx* gene and it was detected in five of these isolates (Figure 4.1C). No PCR products were ever detected in the negative controls.

### **Phylogenetic analyses of *stx* PCR product**

The *stx* PCR products generated from the three FastGroup II representative canine fecal bacterial isolate groups were purified and sequenced (designated group 1-3, see 16S rDNA sequence results below for description). A BLASTN alignment of the sequences to the GenBank non-redundant nucleotide database was performed and they were confirmed to be the *stx* gene. A phylogenetic analysis of the *stx* sequences was also performed [210, 211].

**Figure 4.1. Agarose gel picture of *stx*-specific PCR on canine fecal DNA and canine fecal isolates.** A) Initial *stx*-specific PCR screening of DNA extracted from canine fecal samples; B) *stx*-specific PCR on bacterial DNA extracted from canine fecal samples (n=3, 10ml and 20ml loaded into wells); and C) *stx*-specific PCR showing two of the five positive results from the cultured bacterial isolates. White arrows indicate the *stx*-positive isolates. White triangle is *stx* standard curve from 10E5 copies ml<sup>-1</sup> to one copy ml<sup>-1</sup>, neg = negative control, DB#20 = Dog Beach fecal sample #20, 500 bp = 500 bp DNA ladder. Negative controls consistently had no detectable PCR products.

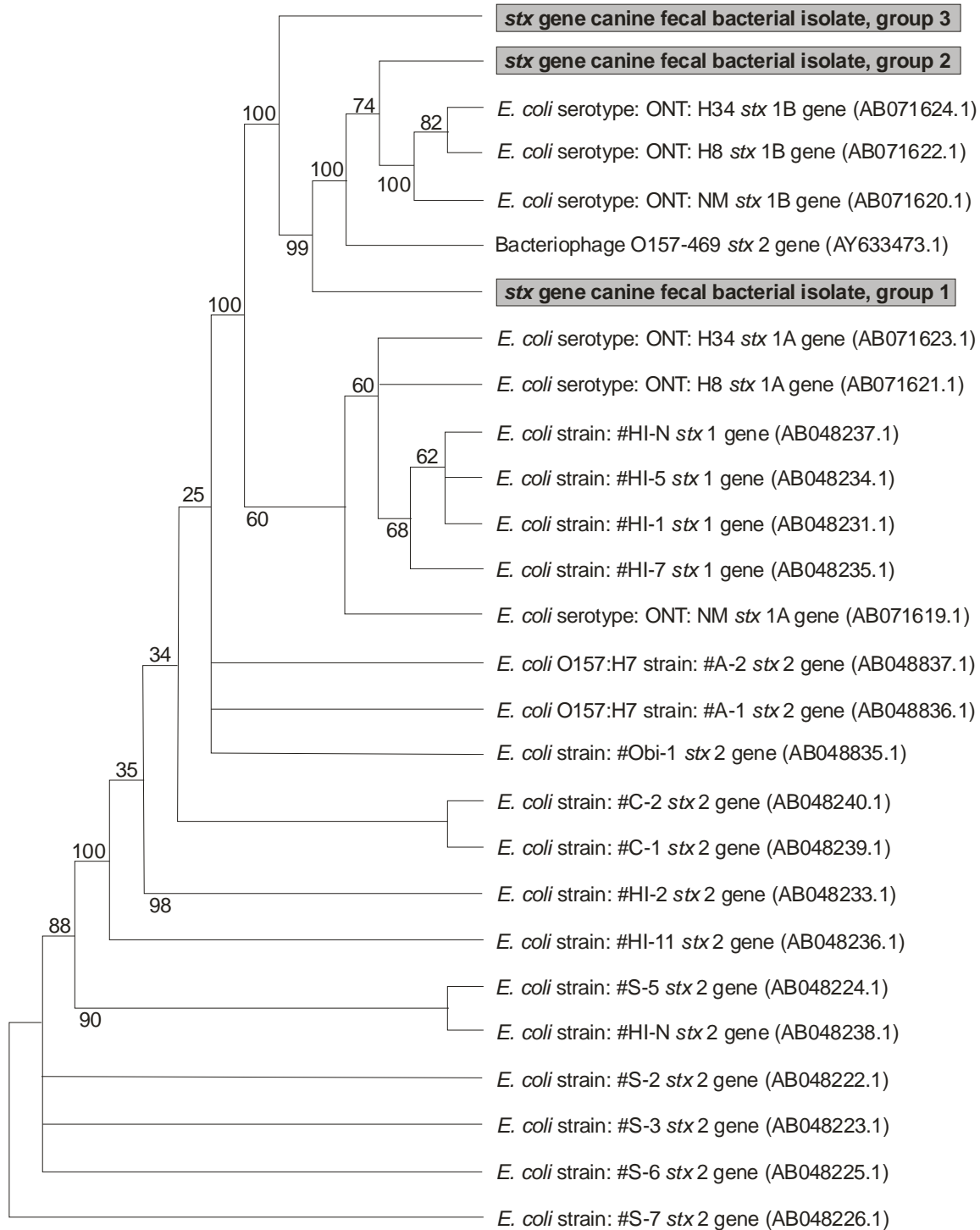


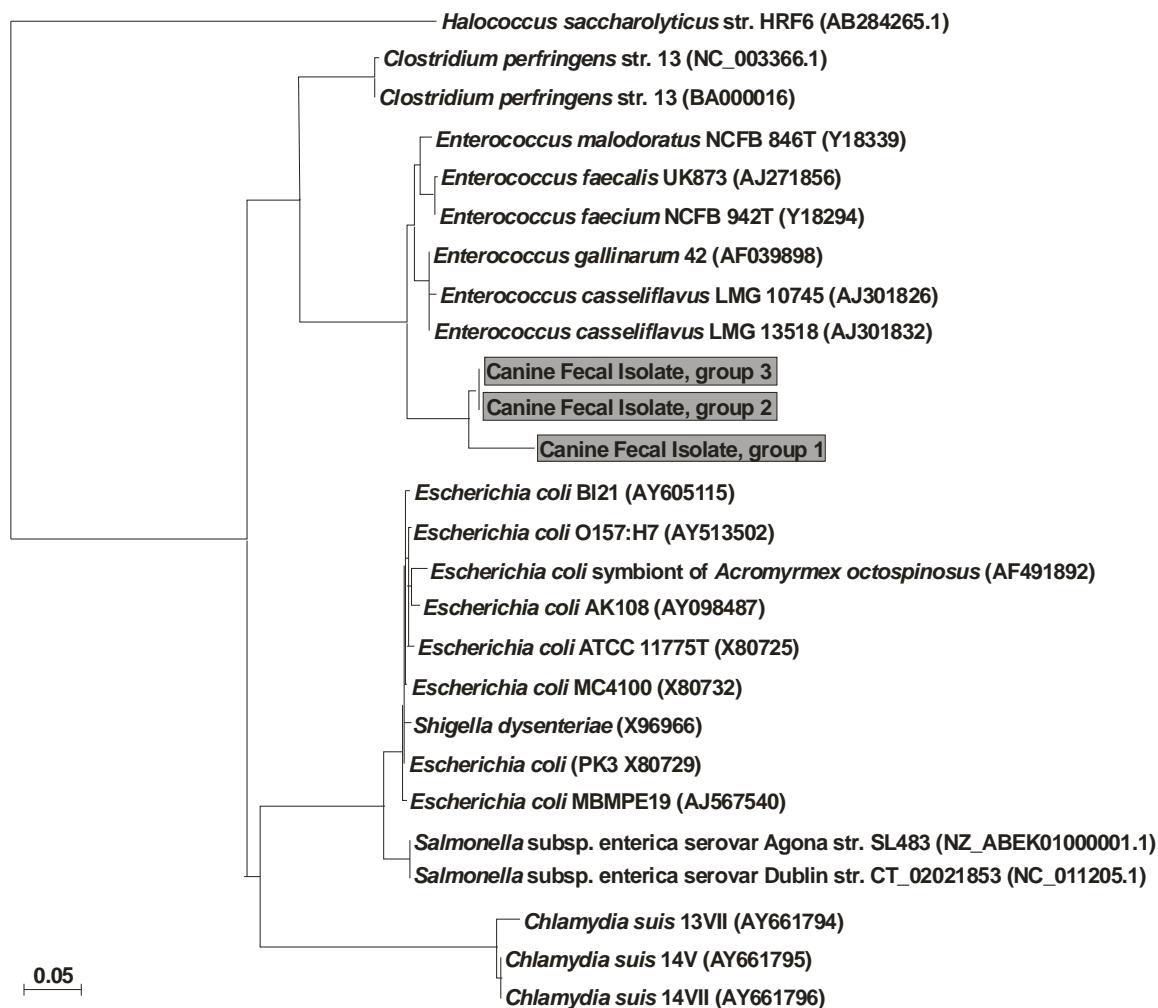
The three representative *stx* gene sequences were grouped with known *E. coli* O157:H7 *stx* gene sequences (Figure 4.2). The *stx* gene from groups 2 and 3 clustered together. The bootstrap values for these groupings were greater than 74.

**Canine fecal bacterial isolates carrying *stx* gene identified as *Enterococcus* spp.**

The *stx* gene was detected in five of the 89 bacterial isolates screened by *stx*-specific PCR (designated G516S.STX, H616S.STX, H716S.STX, H816S.STX, H916S.STX). A 16S rDNA PCR was performed on the five bacterial isolates. The resulting PCR product was purified and sequenced. The five 16S rDNA sequences were de-replicated using FastGroup II [212] and three distinct groups were identified (group 1= G516S.STX; group 2= H716S.STX, H816S.STX; group 3= H616S.STX, H916S.STX). Representative sequences from these groups were utilized to molecularly identify the canine fecal bacterial isolates. First, the 16S rDNA sequences were identified taxonomically by using the Ribosomal Database Project (RDP) Classifier [213]. The RDP Classifier identified the three groups as belonging to the genus *Enterococcus*. To confirm this classification, a phylogenetic tree was generated (Figure 4.3). These phylogenetic analyses confirmed that the canine fecal isolates belonged to the genus *Enterococcus*.

**Figure 4.2. Phylogenetic analysis of *stx* gene PCR product of total DNA.** The DNAPARS DNA parsimony program was used to generate the phylogenetic trees of the representative *stx* PCR product sequences [214]. The bootstrap method with 1000 replicates, 2822 steps, at 1278 sites was performed. The consensus tree is shown. The three canine fecal bacterial isolate groups are indicated by the gray boxes. GenBank accession numbers are indicated in parentheses.





**Figure 4.3. Phylogenetic analysis of 16S rDNA PCR product from canine fecal bacterial isolates.** The DNAPARS DNA parsimony program was used to generate the phylogenetic trees of the three representative 16S rDNA PCR product sequences [214]. The bootstrap method with 1000 replicates, 989 steps, at 580 sites was performed. The consensus tree is shown. The three canine fecal bacterial isolate groups are indicated by the gray boxes. GenBank accession numbers are indicated in parentheses.

## DISCUSSION

The beaches of San Diego, California are a popular destination for residents and tourists alike. Some of these beaches are designated as canine-friendly where people and their canine pets are allowed to enjoy the recreational waters together. One of these beaches is the Ocean Beach Dog Beach. This location is unique because not only is it a

dog beach, but it is also an outlet into the Pacific Ocean for the San Diego River and the Mission Bay recreational waterways. As a result, this area is impacted by pollution and often closed for exceeding bacterial standards levels [186, 187]. Important for water quality monitoring agencies is keeping the possibility of spread of infectious disease from bacteria and viruses relatively low. A paramount concern is pollution by human and animal waste because it carries disease-causing bacteria and viruses. With the close interaction of humans and animals at locations like Dog Beach, a potential reservoir for novel infectious disease pathogens may exist.

Exactly what kind of impact does the close interaction of humans and canines have on this environment and the microorganisms inhabiting it? Bacteria and their viruses (bacteriophage) are abundant in aquatic environments [1, 3, 215-217] and genetic exchange between these microorganisms occurs at a high frequency [79]. Some bacteriophage carry exotoxin genes that, when integrated into the bacterial chromosome, can transduce an avirulent bacterium to virulence. The influx of human- and animal-associated microorganisms to this environment may be providing a selective niche for the evolution of novel pathogens.

Many enteropathogenic microorganisms are found in animal feces and examples of the bacterial pathogens include *Salmonella* spp., *Shigella* spp., *Vibrio cholera*, and *Escherichia coli*. Certain strains of *Shigella* spp. and *E. coli* cause gastrointestinal illnesses and can carry the bacteriophage-encoded shiga toxin (*stx*) gene [218]. We hypothesized that bacteria cultivated from canine fecal samples collected from the Ocean Beach Dog Beach may contain the *stx* gene normally found in the fecal *Shigella* spp. or *E. coli*.



Canine fecal samples were collected from the shoreline of the Ocean Beach Dog Beach in San Diego, California and screened for the *stx* gene. The *stx* gene was detected in one of the 20 fecal samples. Bacterial isolates were cultivated from this fecal sample and the *stx* gene was detected in five of these isolates. Phylogenetic analyses of the 16S rDNA sequences from these isolates determined these isolates belonged to the *Enterococcus* genus and not the typical *E. coli* or *Shigella* spp. host. These results suggest a unique horizontal gene transfer event has occurred. One possibility for this event is that bacteriophage carrying the *stx* gene has a broad host range that allows it to infect *Enterococcus* spp. as well as *E. coli* and *Shigella* spp., or a genetic exchange has occurred between the *stx*-carrying-bacteriophage and a bacteriophage that infects *Enterococcus* spp.

Our results suggest that canine feces may be a reservoir for the bacteriophage-encoded *stx* gene and that this gene can be transferred to new bacterial hosts. This has implications in the development of new infectious diseases. A further, in depth assessment of the genomics of the bacterial and bacteriophage communities present in these recreational waterways and the potential fecal sources of contamination would shed more light on the extent to which horizontal gene transfer is occurring in these environments. Our study presents a first step in examining the reservoir of bacteriophage-encoded virulence genes present in animal waste and the potential impact exchange of these genes between atypical hosts may be having on the evolution of novel human pathogens.

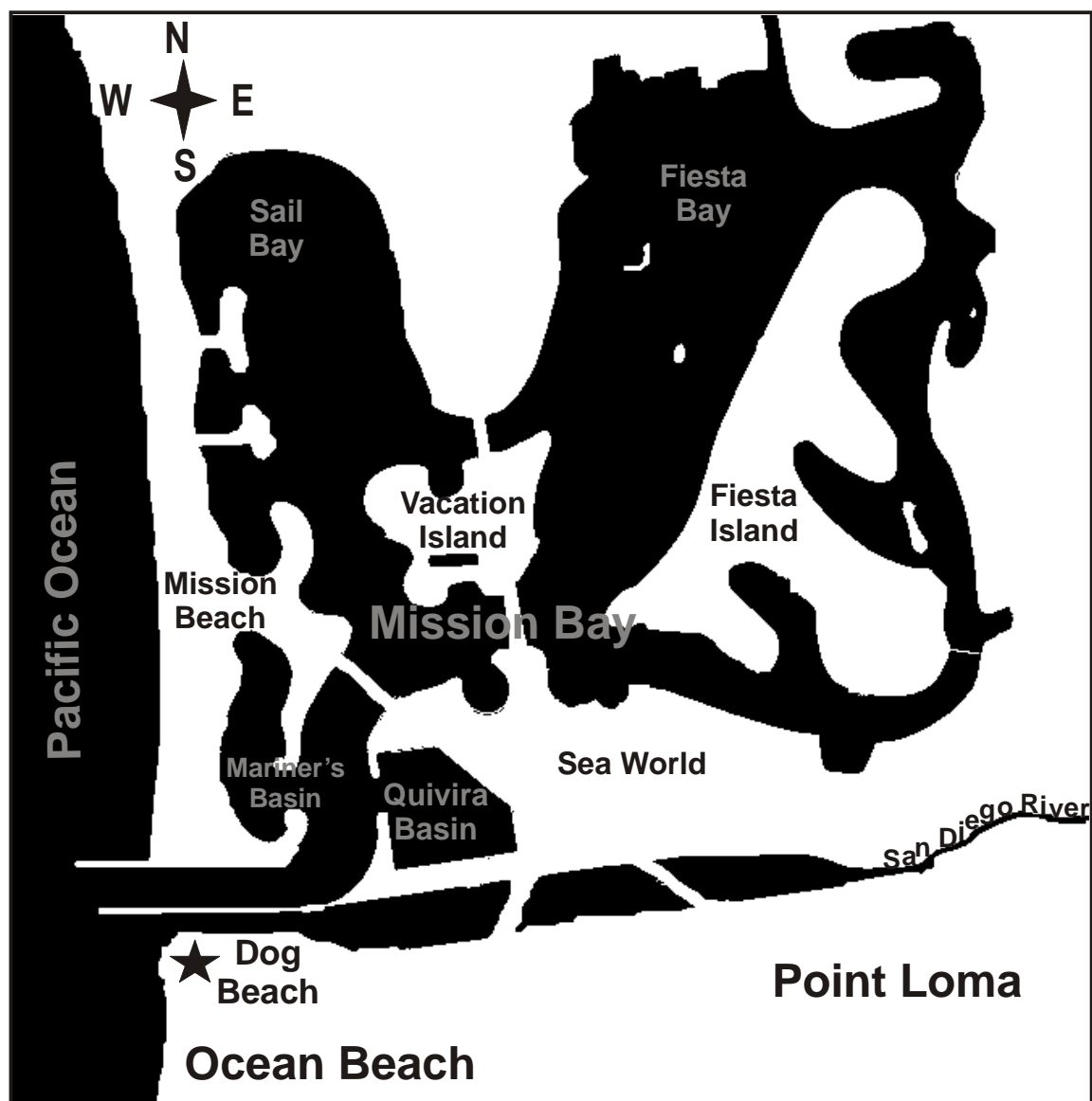
## **MATERIALS AND METHODS**

### **Canine feces sampling strategy**

A total of 20 canine fecal samples were gathered at the water's edge at the Ocean Beach Dog Beach in San Diego, California (Figure 4.4). The fecal samples were identified visually in the sand and gathered carefully with gloved hands using new disposable sandwich bags. The samples varied in size, color, consistency, and dampness. Samples were gathered and immediately returned to the lab for processing.

### **Extraction of DNA from canine fecal samples**

Total DNA was extracted from 1.0 g canine fecal samples using the MoBio UltraClean Soil DNA kit (Carlsbad, CA) maximum yield protocol. A previously described method was modified and used to extract bacterial fraction DNA from the canine fecal samples [193, 194]. Briefly, fecal samples were suspended in 1:1 (w/v) 1X storage media (SM) buffer in a 50 ml conical vial and mixed overnight on an orbital shaker set at 100 rpm [194]. The samples were then centrifuged at 11,000 x g to pellet the biosolids. The supernatant was then filtered through a 0.2um Sterivex® filter to capture the bacteria. The filter was then aseptically removed from its housing and DNA was extracted from the bacteria trapped on the filter using the MoBio UltraClean Soil DNA kit (Carlsbad, CA) maximum yield protocol.



**Figure 4.4. Map of sampling location and surrounding waterways.** The Dog Beach area of Ocean Beach in San Diego, CA and surrounding waterways are depicted in this map. Dog Beach is one of a few beaches in San Diego where people are allowed to bring their canine companions. The San Diego River and popular Mission Bay recreational waterways empty into the Pacific Ocean at this location. The black star indicates the Dog Beach area where fecal samples were gathered.

#### **Exotoxin and 16S rDNA PCRs and sequencing of PCR products**

Exotoxin-specific PCR was performed on the total and bacterial fraction DNA of the canine fecal samples. The primers and PCR conditions used in this study are as

described previously [193]. Exotoxin-specific and 16S rDNA colony PCRs were performed on 89 canine fecal bacterial isolates, as described previously [193]. Five microliters of the bacterial isolate suspension cultivated from the canine feces was used as template in the PCRs. To control against contamination by PCR products, amplification and all subsequent downstream procedures were carried out in an entirely separate laboratory in a separate building from where DNA extraction and PCR assay set up procedures were performed. Separate and dedicated equipment and reagents were maintained in each laboratory. Also a negative control was included in each PCR assay performed.

The *stx* and 16S rDNA PCR products from the canine fecal bacterial isolates were gel purified using the MoBio UltraClean GelSpin kit (Carlsbad, CA) to prepare for sequencing. Sequencing of the *stx* and 16S rDNA PCR products was performed by the SDSU MicroChemical Core facility using the ABI Prism® 3100 capillary electrophoresis DNA sequencer.

### **Cultivation of bacterial isolates from canine fecal samples**

Canine fecal samples were re-suspended 1:1 (w/v) in 1X SM buffer in a 50 ml conical vial. The samples were mixed overnight on an orbital shaker set at 100 rpm. The samples were then centrifuged at 11,000 x g to pellet the biosolids. One hundred microliters of serial 10-fold dilutions of the supernatant were plated onto Luria Bertani (LB) agar plates to allow for isolation of single colonies. All plates were incubated at room temperature for 2-3 days until colonies were visible. Isolates were then sub-cultured into 96-well plates containing 150 ul LB broth with 15% glycerol. These sub-cultured

isolates were then grown for another 2-3 days with aeration. Isolates were stored in the 96-well plates at 4°C until tested in the exotoxin-specific and 16S rDNA PCRs. Isolates were then placed at -80°C for permanent storage.

### **Bioinformatic analyses of *stx* and 16S rDNA gene sequences**

The *stx* PCR product sequences were identified by BLASTN alignment against the GenBank non-redundant nucleotide database [210, 211]. The 16S rDNA sequences were de-replicated using FastGroup II [212]. The representative 16S rDNA FastGroup II sequences were used for the subsequent phylogenetic analyses. The 16S rDNA sequences were identified taxonomically by using the Ribosomal Database Project (RDP) Classifier [213] and phylogenetic trees were also generated as a means of classifying the canine fecal bacterial isolates. The *stx* sequences were also grouped according the FastGroup II analyses and analyzed phylogenetically to visualize their relationship to other known *stx* sequences. The DNAPARS DNA parsimony program was used to generate the phylogenetic trees of both the 16S rDNA and *stx* PCR product sequences [214]. The bootstrap method with 1000 replicates, 989 steps, at 580 sites was performed for the 16S rDNA sequence analyses. For the *stx* gene sequence analyses, the bootstrap method with 1000 replicates, 2822 steps, at 1278 sites was performed. Consensus trees were used to represent the data and those groups at a relative frequency less than 10% were not shown.

Chapter 4, in full, has been submitted for publication of the material as it may appear in BMC Microbiology open access journal 2011. Casas, V., Sobrepeña, G., Rodriguez-Mueller, B., and Maloy, S.R., BioMed Central 2011. I was the primary investigator and author of this paper.

## CONCLUSIONS

Bacteriophage, or phage, are the most numerous biological entities in the world. They are widespread and have been found in many environments from natural, to animal, to human. Phage carry genetic material that they often transfer to the bacterial hosts they infect. Through the process of transduction, phage serve as mediators of genetic exchange between members of a microbial community present in the particular environment. It is through this genetic exchange that phage play a role in bacterial pathogenesis. The extent to which this is true is not fully understood.

Phage carry many types of genes that can be utilized to the benefit of the bacterial host. The types of genes transferred are varied and, when expressed, can alter the phenotype of the bacterium to which they are transferred. This is very important in terms of pathogenesis because when virulence genes are transferred, a previously avirulent bacterium can become virulent. A few examples of phage-encoded exotoxin genes that can confer virulence to the bacterial host are the genes for cholera toxin (*ctx*), diphtheria toxin (*dtx*), Staphylococcal enterotoxin A (*sea*), and shiga toxin (*stx*).

The goal of this dissertation research was to investigate the distribution and abundance of these phage-encoded exotoxin genes, the bacteria and phage that carry these genes, and the role the phage carrying the exotoxin genes may be playing in the evolution of novel human disease.

## DISTRIBUTION OF PHAGE-ENCODED EXOTOXIN GENES IN TERRESTRIAL AND AQUATIC ENVIRONMENTS

Molecular and microbiological assays were developed and used to investigate the distribution and abundance of phage-encoded exotoxin genes. A large number of samples

from different natural environments were screened using these assays. The results from this investigation determined that phage-encoded exotoxin genes were widespread in terrestrial and aquatic environments. The extent to which human activities a particular terrestrial or aquatic environment varied and the presence and/or abundance of phage-encoded exotoxin genes was not dependent upon impact of human activities. Exotoxin genes were found distributed in areas with a high amount of human activities as well as areas with a minimal amount of human activities. These genes were found in the bacterial DNA fraction as well as the phage DNA fraction. Together, these results suggest there is a reservoir of phage-encoded exotoxin genes, that the presence of exotoxin genes is not dependent on level of human activities, and that the genes are mobile within these environments.

#### **DISTRIBUTION AND ABUNDANCE OF PHAGE-ENCODED EXOTOXIN GENES FOLLOWING RAIN EVENT**

The distribution and abundance of phage-encoded exotoxin genes in coastal waters following a rain event were investigated. Many human diseases are caused by bacteria and viruses (both eukaryotic and their bacterial counterparts, bacteriophage) that enter the waterways following a rain event. Some of these bacteria and viruses may be carrying exotoxin genes that cause disease symptoms in humans. In southern San Diego, the coastal waters are highly impacted by raw sewage from the Tijuana River that flows, untreated, into the Tijuana River Estuary and Pacific Ocean. Water and sediment samples were gathered at four locations along the San Diego coastline for one week following a rain event to determine if pollution from the Tijuana River affected water quality.



Phage-encoded exotoxin genes were detected in the water and sediment samples throughout the sampling week. There was no observable increase or decrease in frequency of exotoxin genes detected throughout the week. There was also no observable increase or decrease in frequency of exotoxin genes detected according to location sampled. The raw sewage flowing from the Tijuana River into the Tijuana River Estuary and the Pacific Ocean at Imperial Beach did not lead to increased detection or concentration of exotoxin genes at these locations. The *stx* gene was the gene most detected. Estimation of exotoxin gene copy level by quantitative real-time PCR also showed no observable trend in exotoxin gene concentration according to time or location. The sums of these results suggest that phage-encoded exotoxin genes are present in coastal sediment and waters at some level and that weather patterns do not affect their distribution or concentration.

#### **IDENTIFICATION OF *sea* GENE IN ATYPICAL BACTERIAL HOST FROM THE ENVIRONMENT**

Screening of environmental samples determined that phage-encoded exotoxin genes were widespread. It was important to also determine if the bacteria carrying these exotoxin genes were the known host, or if they were being carried by alternative hosts. We examined bacterial isolates cultivated from environmental samples to investigate the hypothesis that these phage-encoded exotoxin genes could be carried by alternative hosts.

Bacterial isolates cultivated from outdoor ambient air were screened for the Staphylococcal enterotoxin A (*sea*) gene. The *sea* gene was detected in one of 89 cultivated isolates. The bacterial isolate carrying the *sea* gene was molecularly identified by its 16S rDNA sequence through BLASTN alignment with the GenBank database and

phylogenetic alignments using ARB and PAUP\*. The bacterial isolate was also characterized microbiologically by Gram stain, cell and colony morphology, and by selective growth conditions. These results indicated that the bacterial isolate from outdoor ambient air was a Pseudomonad and not the typical *Staphylococcus aureus* host known to carry the phage-encoded *sea* gene. This was the first report of a phage-encoded exotoxin gene being found in an atypical host.

### **IDENTIFICATION OF *stx* GENE IN ATYPICAL BACTERIAL HOST FROM CANINE FECES**

Bacterial isolates cultivated from canine feces were screened for the shiga toxin (*stx*) gene. Twenty fecal samples were collected from the Ocean Beach Dog Beach and their DNA extracted. The *stx* gene was detected in the DNA of one of the 20 canine fecal samples. Bacterial isolates were then cultivated from this canine fecal sample and screened for the *stx* gene. Of the 89 bacterial isolates screened, the *stx* gene was detected in five of the isolates. These isolates were molecularly identified by 16S rDNA sequencing and bioinformatics analyses. The sequences were de-replicated using FastGroup II into three distinct groups. Phylogenetic alignments using DNAPARS identified the canine fecal bacterial isolate groups as *Enterococcus* spp. This was the second report of a phage-encoded exotoxin gene being found in an atypical bacterial host.

### **FUTURE STUDIES**

A reservoir of phage-encoded exotoxin genes exists in the natural and animal environments sampled. The *sea* and *stx* genes were found in alternative bacterial hosts cultivated from these environmental samples. These phage-encoded exotoxin genes were also found in the DNA from the phage fraction of some of these samples. Altogether,

these data suggest that through the phage life cycle of infection, transduction, lysogeny, and lysis, these phage-encoded exotoxin genes are being transferred to alternative bacterial hosts. These novel hosts, upon incorporation of the phage-encoded exotoxin genes into their genome, can become virulent. Through this cycle of horizontal gene transfer, a mechanism of bacterial pathogenesis is created in the environment and has implications in the evolution of novel human pathogens.

The results presented here serve as groundwork for investigating the hypothesis that a reservoir of free phage exists and that horizontal gene transfer through this reservoir creates new virulent bacteria capable of causing disease in humans. We hypothesize that phage cycle between the free phage reservoir and three types of environments—natural, animal, and human (see Figure I.1 in the Introduction). The natural environment was thoroughly explored in these studies; and the animal environment was studied indirectly through sampling of canine feces, but further research is needed to determine if bacteria present in and on the human body also carry phage-encoded exotoxin genes.

The second part of the hypothesis posits that phage-encoded exotoxin genes are being transferred to bacterial hosts not typically associated with the gene and human disease. We found two examples of this in our studies, whereby the *sea* gene was carried by a *Pseudomonas* spp. and the *stx* gene was carried by *Enterococcus* spp. It is likely that more examples like this exist, but have yet to be explored. It is not important just to identify these novel exotoxin-carrying bacteria, however. It is also important to investigate exactly how these genes were acquired. How did a gene known to be carried by a certain phage that is known to infect a certain host, end up in an altogether different

bacterial host? Does the known phage carrying the exotoxin gene have a much broader host range than is currently known? Is the exotoxin gene present in a different phage that infects the different bacterial host? Did a high transducing phage acquire the exotoxin gene from the typical bacterial host and transfer it to the different bacterial host?

To help answer these questions it would be necessary to further examine the genomes of the novel bacteria carrying the phage-encoded exotoxin genes. Do bacterial or phage genes surround the exotoxin gene? It is also important to identify the phage carrying the exotoxin genes in the environments sampled. This would require consistently cultivating individual environmental phage at a concentration high enough from which to extract DNA, and then screening them for the exotoxin genes with the molecular assays. This would not be a simple endeavor, but a necessary one to understand both sides of the horizontal gene transfer process between the phage carrying exotoxin genes and their bacterial hosts.

Finally, to start to understand the potential role phage carrying exotoxin genes may be playing in the evolution of novel human pathogens, it would be important to investigate the rate or frequency at which horizontal gene transfer is happening between the free phage reservoir and the bacteria in the different environments. Model phage/host systems in which genetic transfer events can be traced and measured could be established, or existing systems could be modified. Microcosms of different environments and different phage/host interactions could be tested. The data generated could then be utilized to generate mathematical models that could predict the impact of these horizontal gene transfer events on the evolution of novel human pathogens.

## APPENDICES

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## [20] Phage Metagenomics

By VERONICA CASAS and FOREST ROHWER

### Abstract

The vast majority of novel DNA sequences deposited in the databases now comes from environmental phage DNA sequences. Methods are presented for the cloning and sequencing of phage DNA that might otherwise be lethal to bacterial host vectors or contain modified DNA bases that prevent standard cloning of such sequences. In addition, methods are presented for the isolation of viral particles directly from soil and sediment environmental samples or from large volumes of environmental water samples. The viral particles are then purified by cesium-chloride density centrifugation followed by DNA extraction. This purified viral metagenomic DNA is then used for cloning and sequencing.

### Introduction

Bacterial viruses (bacteriophage or simply phage) are the most abundant biological entities on the planet. There are approximately  $10^6$  phage per milliliter in the world's oceans and lakes and  $10^9$  phage per gram of sediment and topsoil (Bergh *et al.*, 1989; Danovaro and Serresi, 2000; Hewson *et al.*, 2001; Maranger and Bird, 1996; Ogunseitan *et al.*, 1990). Phage are the major predators of bacteria and are believed to influence the types and population density of bacteria in an environment. By killing bacteria, phage modulate global biogeochemical cycles, an example of which is the marine microbial

food web (Azam, 1988; Azam and Ammerman, 1984; Azam *et al.*, 1983). Phage have also been implicated in the maintenance of microbial diversity by selective killing (Bratbak *et al.*, 1992; Fuhrman, 1999; Thingstad *et al.*, 1993; Wommack and Colwell, 2000).

In addition to controlling bacterial populations by lysis of infected bacteria, phage can also alter the physiology of infected bacteria through horizontal gene transfer. Many temperate phage express gene products that alter the phenotype of the bacterial host through lysogenic conversion. One of the most common examples of lysogenic conversion is immunity to superinfection by other phage. Lysogenic conversion can also result in expanded metabolic capabilities including resistance to antibiotics and reactive oxygen compounds (Mlynarczyk *et al.*, 1997; Ochman *et al.*, 2000). For phage that carry exotoxin genes, lysogenic conversion can change avirulent bacteria into human pathogens (Banks *et al.*, 2002; Canchaya *et al.*, 2003, 2004).

Studies have also suggested that phage can readily move between different types of ecosystems and have the ability to infect bacteria from these ecosystems. In a study surveying the distribution of T7-like podophage, DNA polymerase genes these sequences were found to occur in marine, freshwater, sediment, terrestrial, extreme, and metazoan-associated ecosystems (Breitbart and Rohwer, 2004). This suggested that the phage have moved in relatively recent evolutionary time (Breitbart and Rohwer, 2004). Moreover, it has also been shown that phage from one type of ecosystem (e.g., soil, sediment, or freshwater) can grow on bacterial hosts isolated from a distinctly different ecosystem (e.g., marine environments [Sano *et al.*, 2004]). Together these results implied that phage from various ecosystem types are capable of infecting more than one type of bacterial host and, as a result, are capable of moving DNA between these ecosystem types.

Considering the impact that phage have on biogeochemical cycling, bacterial population densities and community structure, horizontal gene transfer, and bacterial virulence, it is surprising that more is not known about their diversity and biogeography. The standard methods used to study their bacterial counterparts cannot be directly applied to the study of phage diversity and biogeography. Phage DNA is lethal to bacterial cells and it contains modified nucleotide bases that are a barrier to standard cloning techniques (Wang *et al.*, 2000; Warren, 1980; Xu *et al.*, 2002). Development of novel cloning and sequencing techniques have overcome these obstacles and viral metagenomics is beginning to provide a better understanding of the ecology of viruses (Breitbart *et al.*, 2002; Margulies *et al.*, 2005; Rohwer *et al.*, 2001).

The term “viral metagenomics” can be defined as the culture-independent functional and sequence-based analysis of an assemblage of phage genomes in an environmental sample (Handelsman *et al.*, 1998; Riesenfeld *et al.*, 2004).

To date, there have been five dsDNA and two RNA viral metagenomic libraries published. The dsDNA viral metagenomic libraries included viruses from two near-shore marine water samples (Breitbart *et al.*, 2002), one marine sediment sample, one human fecal sample (Breitbart *et al.*, 2003), and one equine fecal sample (Cann *et al.*, 2005). The two RNA viral metagenomic libraries were derived from viruses isolated from coastal waters off of Canada (Culley *et al.*, 2006) and from human feces (Zhang *et al.*, 2006). What was overwhelming in these studies was that the vast majority of viral sequences showed no significant similarity (E-value >0.001) to sequences deposited in the GenBank nonredundant database (Edwards and Rohwer, 2005). The knowledge of the community structure and composition of uncultured microbes has grown dramatically via the use of metagenomics, and viral metagenomics is likely to provide similar insights into the number and types of phage in the environment.

### Procedures

The methods described here focus on the isolation, purification, and extraction of DNA from double-stranded DNA viruses. Appropriate modifications are needed for cloning and sequencing single-stranded DNA and RNA viruses (see Culley *et al.*, 2006; Zhang *et al.*, 2006). Random-primed reverse transcriptase and strand displacement DNA polymerases may be viable options for these types of viruses (Edwards and Rohwer, 2005).

#### *Protocol 1. Isolation of Viral Particles from Soil and Sediment Environmental Samples*

1. For soil samples:
  - a. In a sterile appropriately sized container, add equal amounts of 1× SM buffer and soil sample volumes (i.e., 50 ml buffer to 50 g soil).
  - b. Shake vigorously for a few minutes until soil is well suspended and to ensure that viral particles are released from the soil sample.
  - c. Continue to step 3.
2. For sediment samples (freshwater or salt water):
  - a. In a sterile appropriately sized container, add equal volumes of sediment sample and 0.2  $\mu\text{m}$  filtered, autoclaved water from the sample location.
  - b. Shake vigorously for a few minutes until sediment is well suspended and to ensure that viral particles are released from the sediment sample.
  - c. Continue to step 3.



3. Allow soil/sediment to settle to the bottom of the container at 4°. This may take a few hours, so it may be best to store at 4° overnight.
4. Using a sterile pipette, transfer the supernatant to an appropriately sized sterile container for centrifugation. Conical or centrifuge tubes work best for this step.
5. Spin for 15 min at the highest speed allowed for the container and centrifuge you are using (10,000g if allowable). This will pellet any soil/sediment debris remaining in your supernatant.
6. Pour supernatant into sterile 60-ml syringe with 0.2  $\mu\text{m}$  Sterivex (Millipore, Billerica, MA) filter attached to the tip.
7. Filter supernatant into an appropriately sized sterile conical or centrifuge tube.
8. To the filtrate add  $\sim$ 10 units or Kunitz units of DNase I per milliliter of filtrate. This amount of DNase I can be increased, or the treatment repeated, if all the free DNA is not removed from the filtrate.
9. Incubate at room temperature for 1 h.
10. Precipitate viral particles by adding 10% (w/v) solid polyethylene glycol (PEG) 8000. Make sure that the PEG is well dissolved. For best results, precipitate overnight at 4°.
11. Centrifuge sample with PEG for 15 to 30 min at 11,000g.
12. Decant the supernatant. The viral particles are in the pellet.
13. Invert the conical or centrifuge tube containing the pellet in a tilted position for 5 min to remove any residual liquid.
14. Add the desired/necessary volume of TE buffer (pH 7.6) to the viral pellet. Let stand for a few minutes at room temperature, and then resuspend the pellet with a wide-bore pipette. Do this gently, as the viruses can be sheared if pipetted too violently.
15. Transfer suspension to new appropriately sized container.
16. DNA can now be extracted from the pelleted viral particles using protocol 4. If contamination with exogenous DNA is a problem, the viral particles may first be further purified using protocol 3 and then the DNA extracted. (This protocol was adapted from Maniatis *et al.*, 1982; Sambrook *et al.*, 1989; and Sambrook and Russell, 2001.)

*Protocol 2. Isolation of Viral Particles from Large Water Environmental Samples Using Tangential-Flow Filtration*

1. Using a vacuum pump, first filter the sample through a 0.45  $\mu\text{m}$  GF/F filter (Whatman Inc.; Florham Park, NJ) to remove protists and large bacteria from the sample. Repeat this step to ensure that all

protists are removed (Wilcox and Fuhrman, 1994). This is the 0.45  $\mu\text{m}$  filtrate.

2. Set up the tangential flow-filter (TFF) system. The main components are the TFFs (Amersham Biosciences, Piscataway, NJ), peristaltic pump, tubing to connect the reservoirs to the filter and pump, and reservoirs to contain the sample as well as to capture the filtrate. The reservoir can be any container ranging from a beaker to a large trashcan.
3. Also needed are pressure gauges to monitor the pressure within the TFF system. It is important that the correct amount of pressure is maintained within the system such that the sample is forced through the TFF pores, while at the same time not exceeding 10 psi so that the viral particles do not burst.
4. Run the 0.45- $\mu\text{m}$  filtrate through a 0.2- $\mu\text{m}$  TFF. The 0.2- $\mu\text{m}$  TFF removes bacteria, but allows viruses to pass through.
5. Circulate the entire 0.45- $\mu\text{m}$  filtrate through the filter until it is concentrated down to a volume of  $\sim 1$  l. This is the 0.2- $\mu\text{m}$  filtrate.
6. Run the 0.2- $\mu\text{m}$  filtrate through a 100-kDa TFF. Viral particles will either be trapped on the filter or recycled back into the sample reservoir. The filter is run until there is very little volume left in the sample reservoir. At this point, the pressure on the retentate tube is released, allowing the sample to wash over the filter, freeing any attached viruses. The sample is then concentrated further, with the final step of removing the input tube that runs air through the filter and forces all remaining sample out of the filter and tubing. The viral concentrate is in the sample reservoir.
7. This method will concentrate volumes  $>10$  l down to  $\sim 1$  l, and volumes less than 10 l down to  $\sim 100$  ml.
8. Viral particles can now be further purified using protocol 3. (This protocol was based on Breitbart *et al.*, 2002, 2004a,b; Sano *et al.*, 2004; Wommack *et al.*, 1995).

*Protocol 3. Purification of Viral Particles by Cesium Chloride (CsCl) Density Centrifugation*

1. To the viral concentrate, add 0.2 g CsCl per milliliter of viral concentrate.
2. Make three CsCl solutions of 1.35-g/ml, 1.5-g/ml, and 1.7-g/ml densities. Make the CsCl gradient from the same solution in which the viral concentrate is diluted, and make sure that it has been filtered with a 0.02- $\mu\text{m}$  filter to remove any possible contaminating viral particles.

3. Set up the CsCl gradient in clear plastic centrifuge tubes that fit the rotor of the high-speed centrifuge being utilized (e.g., Beckman Ultra-Clear centrifuge tubes).
4. Layer the three CsCl solutions from greatest density to least density by slowly trickling the solution down the side of the centrifuge tube, being careful not to mix the gradients in the tube. The CsCl step gradient should take up ~60% of the centrifuge tube. The number of step gradients required to purify the entire viral concentrate is the final aqueous volume from step 1 divided by 40% of the volume of the centrifuge tube.
5. As you add each solution, mark the outside of the tube to denote the location of each fraction.
6. Carefully layer the appropriate volume of viral concentrate on top of the gradient (~40% of the capacity of the centrifuge tube).
7. Load an even number of step gradients into the centrifuge, making sure that opposite tubes are carefully balanced.
8. Centrifuge the gradients at 87,000g for 2 h at 4°.
9. Remove the gradients from the centrifuge and wipe the outside of each tube with ethanol to remove grease or oils. Apply a piece of clear tape to the outside of the tube level with the 1.35-g/ml and 1.5-g/ml densities.
10. Use a 21-gauge needle to pierce the tube, through the tape, just below the 1.5-g/ml density. Be careful to keep fingers away from the other side of the tube, just in case the needle punctures all the way through the tube.
11. Collect the 1.5-g/ml density and the interface between the 1.35-g/ml and 1.5-g/ml densities.
12. Extract DNA from the viral particles using protocol 4. (This protocol was adapted from Maniatis *et al.*, 1982; Sambrook and Russell, 2001; Sambrook *et al.*, 1989.)

*Protocol 4. Extraction of DNA from Viral Particles Using Formamide and CTAB/NaCl*

*Formamide Preparation*

1. To the viral concentrate, add the following:
  - a. 0.1 volume of 2 M Tris-Cl (pH 8.5)/0.2 M EDTA
  - b. 0.05 volume of 0.5 M EDTA
  - c. 1 volume of deionized formamide
  - d. 10  $\mu$ l glycogen (10 mg/ml)
2. Incubate at room temperature for 30 min.
3. Add 2 volumes of room temperature 100% ethanol.
4. Incubate overnight at -20°.

5. Centrifuge at high speed (e.g., 10,000g) for 20 min at 4°.
6. Decant supernatant.
7. Add 500  $\mu$ l of cold 70% ethanol to wash the pellet.
8. Centrifuge at high speed for 10 min.
9. Decant supernatant and repeat wash step.
10. Resuspend into 567  $\mu$ l TE buffer (pH 8.0). Be careful not to vortex or pipette too vigorously.
11. Continue with “CTAB/NaCl preparation” protocol below.

*CTAB/NaCl Preparation*

1. To 567  $\mu$ l of the resuspended viral pellet, add 30  $\mu$ l SDS (0.5% final concentration) and 3  $\mu$ l proteinase K (100  $\mu$ g/ml final concentration). Mix.
2. Incubate for 1 h at 37°.
3. Add 100  $\mu$ l of 5M NaCl to the resuspended viral pellet and mix thoroughly by inversion. Ensure that the final NaCl concentration is >0.5 M so that the nucleic acid does not precipitate.
4. Add 80  $\mu$ l CTAB/NaCl solution to the resuspended viral pellet and mix thoroughly by inversion.
5. Incubate for 10 min at 65°.
6. Add an equal volume of 24:1 chloroform/isoamyl alcohol and mix thoroughly by inversion.
7. Centrifuge for 5 min at high speed.
8. Transfer supernatant to a new tube, being careful not to transfer the debris in the interface. DNA is in the supernatant.
9. Add equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol to the supernatant and mix thoroughly by inversion.
10. Centrifuge for 5 min at high speed.
11. Transfer supernatant to a new tube.
12. Add 0.7 volume isopropanol to the supernatant fraction, and mix gently by rocking the tube parallel to the ground until a white DNA precipitate forms.
13. Centrifuge at high speed for 15 min at 4°.
14. Decant supernatant being careful not to discard the pellet.
15. Add 500  $\mu$ l 70%-ethanol to the pellet.
16. Centrifuge for 5 min at high speed. Decant ethanol.
17. Repeat steps 15 and 16.
18. Remove all residual ethanol with a pipette and let DNA pellet air dry, or dry pellet using a lyophilizer.
19. Resuspend pellet in 50  $\mu$ l sterile, DNase-, RNase-free water.
20. This purified viral metagenomic DNA can be used for cloning and sequencing. Many phage modify their DNA in ways that complicate

traditional cloning approaches. The phage DNA can be cloned and sequenced as described by Rohwer *et al.* (2001). Alternatively, a more economical solution is to take advantage of companies offering technologies that circumvent problems with cloning phage DNA. Two solutions are the PicoTiterPlate technology from 454 Life Sciences (Branford, CT), the Linker-Amplified-Shotgun-Libraries (LASLs) from Lucigen Corporation (Middleton, WI), or both.

#### *Reagents*

##### CTAB/NaCl (100 mL)

1. Add 4.1 g NaCl to 80 ml water.
2. Slowly add 10 g cetyltrimethyl ammonium bromide (CTAB) while stirring (heat to 65° if necessary).
3. Bring volume up to 100 ml.

##### 5× STORAGE MEDIA (SM) BUFFER (1 L)

1. Weigh out 29 g NaCl and 10 g MgSO<sub>4</sub> \* 7H<sub>2</sub>O.
2. Measure out 250 ml 1 M Tris-Cl at pH 7.5.
3. Add water to a final volume of 1 l. Make appropriate dilution for working stock of 1X. (This protocol was adapted from Ausubel *et al.*, 1995, 2002; Maniatis *et al.*, 1982; Sambrook and Russell, 2001; Sambrook *et al.*, 1989.)

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The Appendix, in full, is a reprint of the material as it appears in *Methods in Enzymology* 2007. Casas, V. and Rohwer, F., Elsevier Academic Press, 2007. I was the primary investigator and author of this paper.



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