

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

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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 *ctx*φ 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).

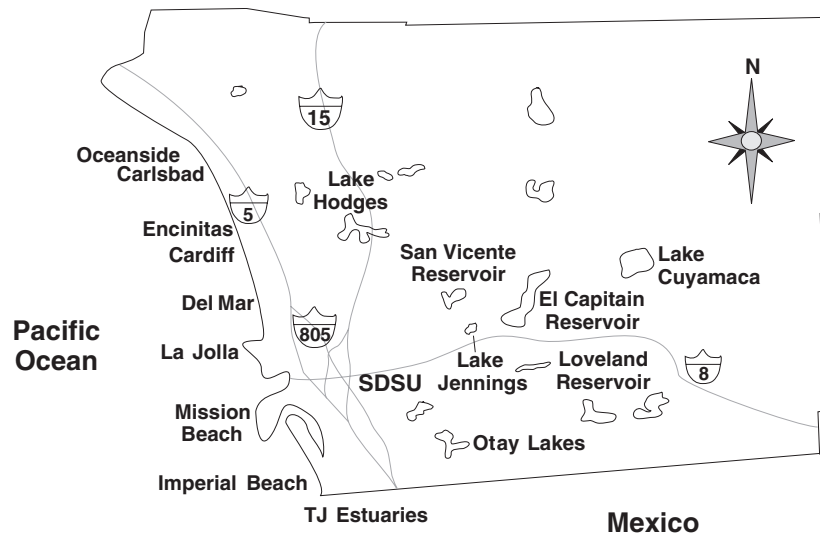


Fig. 1. Map of sampling sites in San Diego County, California. A general map of locations sampled in this study. Water and sediment samples were gathered from beaches along the San Diego coastline as far north as Oceanside and as far south as Imperial Beach. Lake and reservoir samples (water and sediment) 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 1.3×10^{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 1.58×10^{-8} and 3.7×10^{-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.

Table 1. Conditions for conventional PCRs used in this manuscript

Exotoxin gene	MgCl ₂ (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: GTTTGCGTCAATCTTAATAGGG	Efstratiou <i>et al.</i> (1998)
<i>stx</i>	2.0	45	50.0	<i>Escherichia coli</i>	DT1643R: ACCTTGGTGTGATCTACTGTTT 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: CGATGATCTTGGAGCATTCCAC	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 (1–10⁵ 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: 1×10^4 gene copies for *dtx* and *sea*, 1×10^3 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'-GCGTTGACAGCTATAGACG-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'-ACAGATCATTCGTGGTATAACGAT-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⁻¹ iTaq DNA polymerase, 3 mM MgCl₂) which did not contain SYBR Green (Molecular Probes Inc., Eugene, OR), 3 µg µL⁻¹ 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 γ -[³²P]ATP-labeled DNA probes were made using the ethanol-precipitated and concentrated PCR product resulting from amplification of 1 µL containing 1×10^5 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

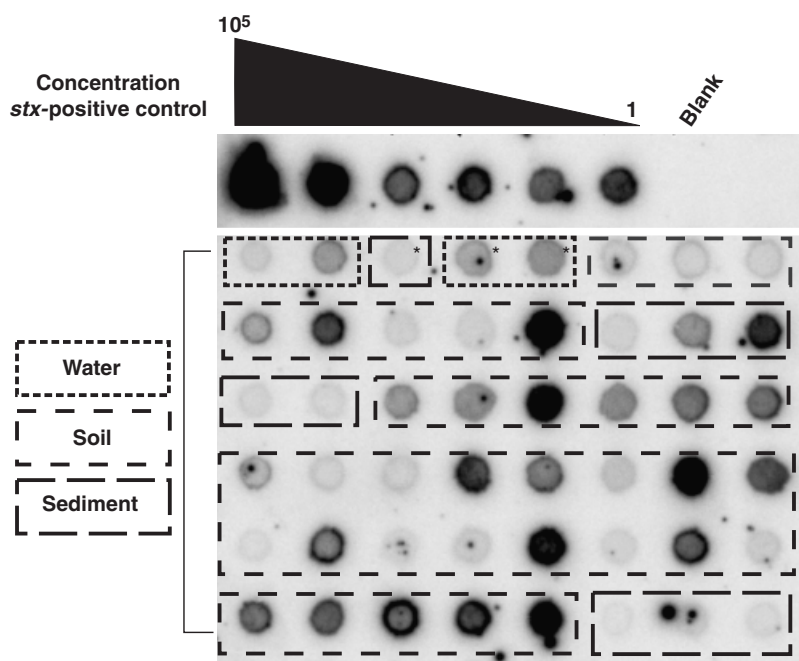


Fig. 2. Southern dot blot of *stx* in environmental samples. Total DNA from 45 soil, sediment, and water samples were fixed on a nylon membrane and probed with a γ -[32 P]ATP-labeled *stx* probe. Samples denoted with an asterisk (*) are phage DNA fixed onto the membrane. The top portion of the blot shows 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) [†]	Positive	Positive	(-)
	Mission Beach (seawater) [†]	Negative	Negative	(-)
	Torrey Pines (seawater) [†]	Positive	Positive	(-)
	Salton Sea (hypersaline) [†]	Negative	(-)	(-)
	Scripps pier May 2001 (seawater) [†]	Positive	Positive	(-)
Sediment	Scripps pier June 2001 (seawater) [†]	Negative	Positive	(-)
	Mission Beach (seawater) [†]	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: 1×10^4 gene copies for *ctx* and *sea*, 1×10^3 gene copies for *ctx*, and 100 gene copies for *stx*.

*Samples processed by PEG precipitation followed by CTAB DNA extraction

[†]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*, *ctx*, and *sea* genes in environmental samples

QPCR assays were developed for *ctx*, *ctx*, 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 *ctx* 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*, *dtx*, 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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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.

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