8 Bacteriophages and Bacterial Virulence

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CONTENTS

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8.1. INTRODUCTION

In general, differences in pathogenic potential among bacterial species and among strains within a species are due to the presence and expression of virulence genes (i.e., genes that encode virulence factors) in pathogenic strains and to their absence in related nonpathogenic strains. Many virulence factor-encoding genes of facultative pathogens (e.g., *Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Streptococcus* spp., *Staphylococcus aureus*, and *Vibrio cholerae*) are present in various mobile genetic elements, such as transposons, plasmids, bacteriophages, and pathogenicity islands. In that regard, it has become increasingly evident that one group of mobile genetic elements, the bacteriophages, plays an important role in the evolution and emergence of pathogenic bacteria. In a process called phage conversion, bacteriophage-encoded virulence genes can convert their bacterial host from a nonpathogenic strain to a virulent strain, or to a strain with increased virulence, by providing mechanisms for the invasion of host tissues and the avoidance of host immune defenses (Table 8.1). Indeed, the loss of those bacteriophages may render the bacteria nonpathogenic. However, the majority of bacteriophages possessing virulence genes are temperate bacteriophages that form stable lysogens. Therefore, lysogenic conversion does not result in bacterial lysis but, instead, allows both vertical and horizontal gene transfer—which may confer a selective advantage to the host and result in clonal expansion. This hypothesis is supported by the recent epidemic spread of cholera from Asia to South America after nearly a century of absence. The *ctxAB* genes, which encode cholera toxin (CT), reside in the genome of a filamentous bacteriophage CTXϕ (Waldor and Mekalanos, 1996). Therefore, *V. cholerae*'s lysogenic conversion by the CTXϕ enables it to produce CT, the main cause of the secretory diarrhea so characteristic of cholera, thus facilitating the spread of *V. cholerae* to new hosts and enhancing its pandemic spread.

The location, within the bacteriophage genome, of numerous genes encoding bacterial virulence factors also suggests an evolutionary advantage to the bacteriophage, by enhancing survival of the lysogen. Hence, the ecological success of lysogenic bacteria contributes to the dissemination of bacteriophage genes. Also, the phenomenon may be considered to indicate the co-evolution of bacteriophages and bacteria. In this chapter, the various classes of bacteriophage-encoded virulence factors and the evolutionary distribution and diversity of the bacteriophages among pathogenic bacterial

TABLE 8.1
Bacteriophage-Encoded Virulence Factors Involved in Various Stages of Bacterial Pathogenesis

Bacterial Host	Bacteriophage	Virulence Factor (gene)	Reference
Proteins required for host attachment			
E. coli	λ	OMP (*lom*)	(Pacheco et al., 1997)
M. arthritidis	MAV1	OMP (*vir*)	(Voelker and Dybvig, 1999)
S. mitis	SM1	Coat protein (*pblA*, *pblB*)	(Bensing et al., 2001b)
V. cholerae	VPIϕ	TCP pilin (*tcp*)	(Karaolis et al., 1999)
Proteins that alter antigenic recognition			
N. meningitidis	Mu-like	Membrane proteins	(Masignani et al., 2001)
S. enterica	ε^{34}	O-antigen (*rfb*)	(Wright, 1971)
S. enterica	P22	O-antigen (*gtr*)	(Vander Byl and Kropinski, 2000)
S. flexneri	Sf6	O-antigen (*oac*)	(Clark et al., 1991)
S. flexneri	SfII, SfV, SfX	O-antigen (*gtrII*)	(Allison and Verma, 2000)
Proteins involved in cellular invasion			
S. enterica	SopEϕ	Type III effector (*sopE*)	(Mirold et al., 1999)
S. enterica	Gifsy-1	Type III effector (*gogB*)	(Figueroa-Bossi et al., 2001)
S. enterica	Gifsy-1	IS-like sequence (*gipA*)	(Figueroa-Bossi et al., 2001)
S. enterica	Gifsy-2	Type III effector (*gtgB*)	(Figueroa-Bossi et al., 2001)
S. enterica	Gifsy-3	Type III effector (*sspH1*)	(Figueroa-Bossi et al., 2001)
S. pyogenes	H4489A	Hyaluronidase (*hylP*)	(Hynes and Ferretti, 1989)
Proteins required for intracellular survival			
E. coli	λ	OMP (*bor*)	(Barondess and Beckwith, 1990)
E. coli	λ-like	OMP (*eib*)	(Sandt et al., 2002)
E. coli O157	Sp4, 10	Superoxide dismutase (*sodC*)	(Ohnishi et al., 2001)
S. enterica	Gifsy-2	Superoxide dismutase (*sodCI*)	(Figueroa-Bossi et al., 2001)
S. enterica	Fels-1	Superoxide dismutase (*sodCIII*)	(Figueroa-Bossi et al., 2001)

(*Continued*)

TABLE 8.1
(Continued)

Bacterial Host	Bacteriophage	Virulence Factor (gene)	Reference
Extracellular toxins			
C. botulinum	C1	Neurotoxin (c1)	(Barksdale and Arden, 1974)
C. diphtheriae	ß-phage	Diphtheria toxin (*tox*)	(Freeman, 1951)
E. coli	H-19B	Shiga toxins (*stx1,2*)	(O'Brien et al., 1984)
E. coli	ϕFC3208	Enterohemolysin (*hly*)	(Beutin et al., 1993)
V. cholerae	ϕCTX	Cytotoxin (*ctx*)	(Nakayama et al., 1999)
V. cholerae	CTXϕ	Cholera toxin (*ctxAB*)	(Waldor and Mekalanos, 1996)
V. cholerae	CTXϕ	Ace toxin (*ace*)	(Trucksis et al., 1993)
V. cholerae	CTXϕ	ZOT toxin (*zot*)	(Fasano et al., 1991)
S. aureus	NA	Enterotoxin (*see, sel*)	(Betley and Mekalanos, 1985)
S. aureus	ϕN315	Enterotoxin P (*sep*)	(Kuroda et al., 2001)
S. aureus	ϕ13	Enterotoxin A (*entA*)	(Coleman et al., 1989)
S. aureus	ϕMu50A	Enterotoxin A (*sea*)	(Kuroda et al., 2001)
S. aureus	ϕ13	Staphylokinase (*sak*)	(Coleman et al., 1989)
S. aureus	ϕETA	Exfoliative toxin A (*eta*)	(Yamaguchi et al., 2000)
S. pyogenes	T12	Toxin type A (*speA*)	(Weeks and Ferretti, 1984)
S. pyogenes	CS112	Toxin type C (speC)	(Goshorn and Schlievert, 1989)
Putative virulence factors			
S. enterica	Fels-1	Neuraminidase (*nanH*)	(Figueroa-Bossi et al., 2001)
S. enterica	Gifsy-1,2	Hemolysin (*ehly*)	(McClelland, 2001)
S. enterica	Gifsy-1,2	Serum-resistance (*ail*)	(McClelland, 2001)
S. enterica	Gifsy-2, Fels-1	Antivirulence gene (*grvA*)	(Ho and Slauch, 2001)
V. cholerae	K139	G-protein-like (*glo*)	(Reidl and Mekalanos, 1995)

TABLE 8.2
Mechanisms of Virulence Gene Acquisition by Bacteriophages

Mechanism	Virulence Gene	Bacteriophage	Bacterium
Imprecise excision	*see, sea, sek*		*S. pyogenes*
Transferable module	*stx1, stx2*	Sp5, Sp15	*E. coli* O157
Phage component	*pblA, pblB*	SM1	*S. mitis*
	Ace, zot	CTXϕ	*V. cholerae*

isolates are discussed. The virulence genes are categorized on the basis of the gene products' roles in the various stages of pathogenesis: attachment and colonization, host immune avoidance, cellular invasion, intracellular survival, and toxin production (Table 8.1). The prevalence and evolutionary distribution of the bacteriophages and virulence genes are examined in terms of their occurrence within a species. Studies of the diversity of bacteriophages encoding virulence factors indicate that some are morphologically diverse and consist of members of a number of viral families, while others have mosaic genomes possessing characteristics of more than one viral family (reviewed in Canchaya et al., 2003). Three possible mechanisms by which bacteriophages acquire virulence genes are also examined; they (i) are acquired by imprecise prophage excision from the bacterial host genome, (ii) are transferable modules within the bacteriophage genome, or (iii) are integral components of the bacteriophage genome (Table 8.2). The indirect and direct roles that bacteriophages play in bacterial virulence (i.e., as passive vectors for the transfer of virulence genes, and as active components in the regulation of bacterial pathogenesis, respectively) also are explored. In addition, the role of phage-phage interactions in bacterial pathogenesis is examined, including the requirement for a helper phage for the mobilization and functioning of another bacteriophage. Finally, the relationship and common features between bacteriophages and pathogenicity islands are discussed.

8.2. BACTERIOPHAGE-ENCODED VIRULENCE FACTORS

In this section, several categories of bacteriophage-encoded virulence factors are discussed based on their roles in bacterial pathogenesis (Table 8.1). The bacteriophages include members of the viral families *Inoviridae* (filamentous), *Myoviridae* (contractile tails), *Podovirdae* (short tail stubs), and *Siphoviridae* (long flexible tails); (for detailed descriptions of the phage families/phage taxonomy, please refer to Chapter 4).

8.2.1. Extracellular Toxins

The best characterized group of bacteriophage-encoded virulence factors are extracellular toxins expressed in various Gram-negative and Gram-positive bacteria, including *E. coli*, *Shigella* spp., *P. aeruginosa*, *V. cholerae*, *S. aureus*, and *Streptococcus pyogenes*. The bacteriophage-encoded toxins are functionally diverse and include some of the most potent bacterial toxins ever described; e.g., tetanus toxin, botulinum toxin, diphtheria toxin, which are encoded on prophages harbored by *Clostridium tetani*, *C. botulinum*, and *Corynebacterium diphtheriae*, respectively.

The phage-encoded bacterial toxins may be cytotoxic, enterotoxic, or neurotoxic, and they enable the bacteria to cause an astonishing array of diseases ranging from mild gastrointestinal disease to life-threatening toxemia and sepsis. Another remarkable feature of the toxins is that although all of them were acquired by lateral gene transfer from unknown sources, all are controlled by bacterial host chromosomal regulatory factors, which suggests some common ancestry.

8.2.1.1. *Streptococcus* and *Staphylococcus*

Streptococcal and staphylococcal isolates contain bacteriophage genes encoding superantigen toxins, exfoliative toxin A, enterotoxin P, hyaluronidase, and staphylokinase. *S. pyogenes* causes a wide range of infections (e.g., fasciitis, rheumatic fever, pharyngitis, pyoderma, scarlet fever, and toxic shock syndrome), and the diverse disease syndromes it elicits probably reflects the various mechanisms of action of its phage-encoded toxins. In view of the variety of bacteriophage-encoded superantigen toxins produced by *S. pyogenes*, it appears likely that at least part of the specific pathogenic potential of distinct clinical *S. pyogenes* isolates is determined by a specific combination of lysogenic conversion genes (Desiere et al., 2001; Banks et al., 2002), located between the phage lysin gene and the right attachment site of the prophages closely related to *cos*-site and *pac*-site *Siphoviridae* (Ferretti et al., 2001; Smoot et al., 2002). *S. pyogenes* M1 and M18 serotype strains associated with invasive wound infections and rheumatic fever, respectively, have highly homologous genomes, and the differences between the strains are accounted for by their bacteriophage content (Desiere et al., 2001). This finding is consistent with the recent observation in Japan that the replacement of older strains by newer M3 strains can be traced to the acquisition of a prophage encoding a new combination of superantigens (Inagaki et al., 2000). A similar scenario is responsible for the diversity among *S. aureus* isolates, which cause a range of illnesses with diverse clinical outcomes (Table 8.3). For example, various staphylococcal phage-encoded genes are required for the bacteria to express (i) enterotoxins that cause acute gastroenteritis, (ii) leucocidin causing leukocytolysis, (iii) SPEA toxin that causes scarlet fever and tissue necrosis, (iv) exfoliative

TABLE 8.3
Bacteriophage- and Phage-Like Element-Encoded *S. aureus* Toxins and Their Associated Diseases

Toxin	Disease	Element	Size
TSST-1	Toxic Shock Syndrome (TSS)	PAI	15 kb (ϕ-like)
Enterotoxin A	Food poisoning, TSS	Phage	30 kb
Enterotoxin B,C	Food poisoning, TSS	PAI	15 kb (ϕ-like)
Enterotoxin D	Food poisoning, TSS	Plasmid	30 kb
Exfoliatin A	Blistering skin disease	Phage	45 kb
Exfoliatin B	Scalded-skin syndrome	Plasmid	30 kb
Leukocidin	Necrotizing pneumonia	Phage	45 kb
SPEA, C	Scarlet fever	Phage	45 kb

toxin causing "scalded-skin" syndrome, and (v) TSST toxin that elicits toxic shock syndrome (Table 8.3). Comparative genomic analysis of *S. aureus* has revealed closely related bacterial genomes, with differences between strains confined mostly to their bacteriophage content (Kuroda et al., 2001). Interestingly, the bacteriophages involved are related to *cos*-site and *pac*-site temperate *Siphoviridae* from streptococci and lactobacilli, which indicates that a pool of bacteriophages may circulate among the Gram-positive bacteria with low GC content. All of the lysogenic conversion genes present in the bacteriophages—including those encoding exfoliative toxin A, enterotoxin P, staphylokinase, superantigen toxins, and leukocyte toxins (some related to streptococcal bacteriophage-encoded proteins)—are located near the right bacteriophage attachment site (Kaneko et al., 1998; Yamaguchi et al., 2000).

8.2.1.2. *E. coli*

Diversity in the number and type of toxins among strains, as well as in the bacteriophages involved, is not restricted to streptococci and staphylococci. *E. coli*, whose natural habitat is the gastrointestinal tract of warm-blooded animals, is the most common facultative anaerobe in the human intestine. However, many pathogenic *E. coli* strains cause enteric diseases (e.g., severe watery diarrhea, dysentery, and hemorrhagic colitis) and extraintestinal infections (e.g., cystitis, septicemia, and meningitis). In enterohemorrhagic *E. coli* strains (EHEC, including O157:H7) and Shiga toxin *E. coli* (STEC) strains responsible for hemorrhagic colitis and hemolytic uremic syndrome, the shiga-toxins (Stx1, Stx2) and enterohemolysins (Hly) are encoded by a diversity of lambda-like bacteriophages (Wagner et al., 1999; Unkmeir and Schmidt, 2000; Johansen et al., 2001; Recktenwald and Schmidt, 2002). Stx1 and Stx2 are A-B toxins consisting of one active A-subunit and five identical B-subunits. The B subunits bind to glycolipids on the host cells, and the A-subunit is taken up by the cell and causes apoptosis by disrupting protein synthesis. A recent study (Wagner et al., 1999) examining seven different Stx2-encoding bacteriophages isolated from multiple STEC isolates discovered striking differences in bacteriophage titers and in the amount of toxin produced. Also, structural analysis of bacteriophage-borne *stx1, stx2* and flanking sequences in *E. coli* O157, STEC and *Shigella dysenteriae* type 1 strains demonstrated significant amounts of bacteriophage genomic variation, which indicated that the shiga toxin genes were encoded on unrelated bacteriophages (Unkmeir and Schmidt, 2000; Recktenwald and Schmidt, 2002). The diversity of Stx-encoding bacteriophages is, evolutionarily speaking, an important mechanism for the spread of toxin genes among isolates, because it may inhibit bacteriophage exclusion due to superinfection immunity, competition for integration sites and restriction-modification systems.

A study (Perna et al., 2001) comparing the genomic sequences of a pathogenic *E. coli* O157 strain and a laboratory-maintained *E. coli* K12 strain showed that 4.1 million base pairs of core sequences are conserved between the genomes, but that this backbone sequence is interspersed with sequences unique to one strain. The *E. coli* O157 strain contained 1.3 million base pairs of strain-specific DNA, most of which encompassed bacteriophage and pathogenicity island DNA (Fig. 8.1). A comparative genomic analysis (Ohnishi et al., 2001) of *E. coli* O157 strain Sakai and EDL933 also revealed that both have very similar bacterial DNA content but

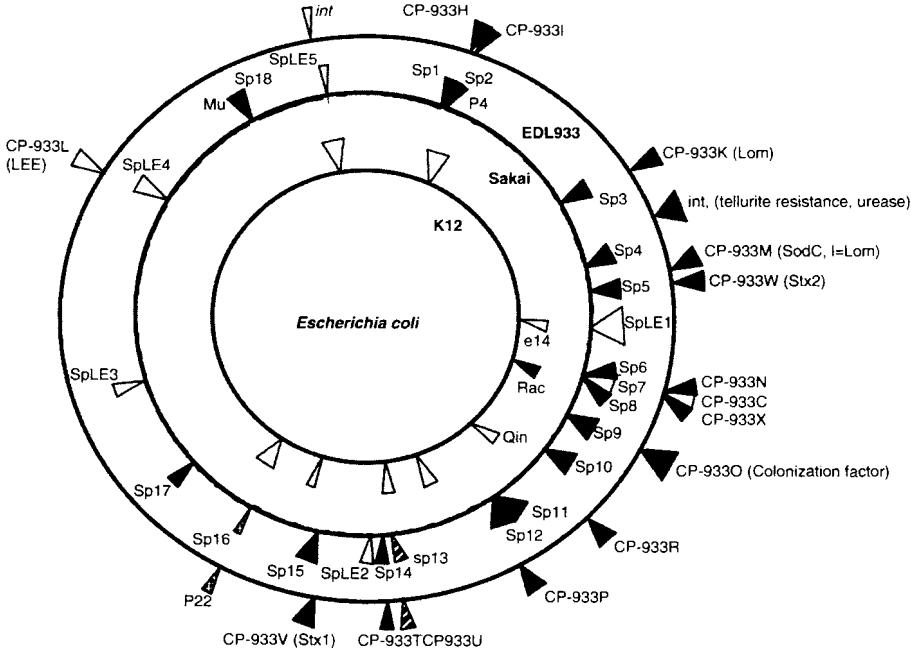

FIGURE 8.1 Comparative genomic maps of the prophage and prophage-like elements of *E. coli* strains EDL933, Sakai, and K12. The outer circle shows the prophages and prophage remnants (triangles) in strain EDL933 of *E. coli* serotype O157 (Perna et al., 2001), the middle circle represents the Sakai strain of *E. coli* serotype O157 (Yokoyama et al., 2000), and the inner circle is the genomic map of the nonpathogenic laboratory strain *E. coli* K12 (Blattner et al., 1997). Lambdoid-like prophages are indicated as black triangles, PAIs encoding integrases are indicated as white triangles, P2 prophages are indicated as striped triangles, P4 prophages are indicated as dotted triangles, P22 prophages are indicated as checkered triangles, and a Mu prophage is indicated in gray.

differ in their prophage content. In strain EDL933, 12 prophage sequences were identified; whereas, in strain Sakai, 18 prophages were identified, 13 of which were lambda-like (Fig. 8.1). A dotplot matrix of the different lambda-like prophages revealed large regions of high sequence identity (Boyd and Brüssow, 2002). Also, *in silico* genomic analysis of the two *E. coli* O157 serotypes strains identified numerous virulence factors encoded by their prophages (Ohnishi et al., 2001). They included (i) Stx-1, a cytotoxin, and the Bor and Lom proteins (conferring serum resistance and cell adhesion, respectively) encoded by bacteriophages Sp3-Sp5, Sp8-12, Sp14, and Sp15, (ii) an intestinal colonization factor encoded by bacteriophages 933 and O, and (iii) tellurite resistance gene products and superoxide dismutase (SodC) encoded by bacteriophages Sp 4 and Sp10 (Ohnishi et al., 2001). It is tempting to speculate that the pathogenic potential of the two isolates correlates with their prophage content, and that acquisition of the bacteriophage-encoded virulence factors may have played a decisive role in the emergence of O157 as a

foodborne pathogen. The laboratory *E. coli* K12 strain contains the inducible lambda phage as well as numerous lambda prophage remnants (DLP-12, e14, Rac and Qin) (Fig. 8.1). Phage lambda contains both *bor* and *lom* genes, which have been associated with virulence.

8.2.1.3. *V. cholerae*

A recent addition to the list of bacteriophage-encoded toxins is *V. cholerae*'s CT, which is the only known toxin that is encoded by a filamentous bacteriophage (CTXϕ) (Waldor and Mekalanos, 1996). CT, similar to Shiga toxin, is an A-B toxin consisting of one active subunit and five binding subunits. However, CT is an enterotoxin that affects membrane permeability by disrupting the normal flow of ions in the small intestines. The A-B subunits, encoded by the *ctxAB* genes, share extensive sequence homology with heat-labile enterotoxins produced by enteropathogenic *E. coli* isolates. Also, the CTXϕ, which carries *ctxAB*, is similar in size, structure, and synteny to other filamentous coliphages, such as M13 and f1 (Fig. 8.2). CTXϕ has the typical gene organization of about ten DNA replication, coat, and morphogenesis genes with sequence homology. However, CTXϕ differs from M13 and other filamentous coliphages because it lacks a putative bacteriophage export gene (gene IV) (Fig. 8.2). Instead, CTXϕ and CT exploit the same pathway for export out of the cell, a type II extracellular protein secretion system (Davis et al., 1999). In addition, CTXϕ unlike other coliphages, is maintained as a prophage within the *V. cholerae* chromosomes (Waldor and Mekalanos, 1996). The CTXϕ genome can be divided into two functional domains: the 4.7 kb core region and the 2.4-kb repeat sequence (RS2) region (Fig. 8.3). The CTXϕ core region genes (*cep*, *orfU*, *ace* and *zot*) correspond to gVIII, gIII, gVI, and gI of M13; whereas *ctxAB* do not share sequence similarity with known genes and are not essential for phage production (Fig. 8.2). The genes in the RS2 region (*rstR*, *rstA* and *rstB*) encode regulation, replication, and integration functions (Waldor and Mekalanos, 1996; Waldor et al., 1997). The Ace and Zot proteins are also enterotoxins (Trucksis et al., 1993; Fasano et al., 1991). However, since Ace and Zot are primarily structural and assembly proteins of CTXϕ enterotoxicity is likely a secondary trait, a side consequence of bacterial invasion of the human host. An analysis (Davis et al., 1999) of the CTXϕ repressor gene (*rstR*) involved in superinfection immunity showed considerable sequence variation in closely related strains, which suggests a possible mechanism for polylysogeny in *V. cholerae*. Interestingly, unlike other integrated prophages, CTXϕ does not excise from the chromosome to form extrachromosomal CTXϕ particles. Instead, CTXϕ is generated by a replicative process that requires tandem elements, either CTX-CTX prophages or CTX-RS1 prophages, within the chromosome (Fig. 8.3) (Davis et al., 2000).

RS1ϕ is a satellite filamentous phage that (i) contains *rstR*, *rstA* and *rstB* genes homologous to the RS2 region genes of CTXϕ (ii) encodes an additional gene (*rstC*), and (iii) depends on the CTXϕ for packaging and transmission (Faruque et al., 2002; Davis et al., 2002). Examination of the diversity of CTXϕs derived from a variety of *V. cholerae* natural isolates revealed the presence of two distinct lineages of CTXϕs within the classical and El Tor biotypes of *V. cholerae* O1 serogroup isolates, the

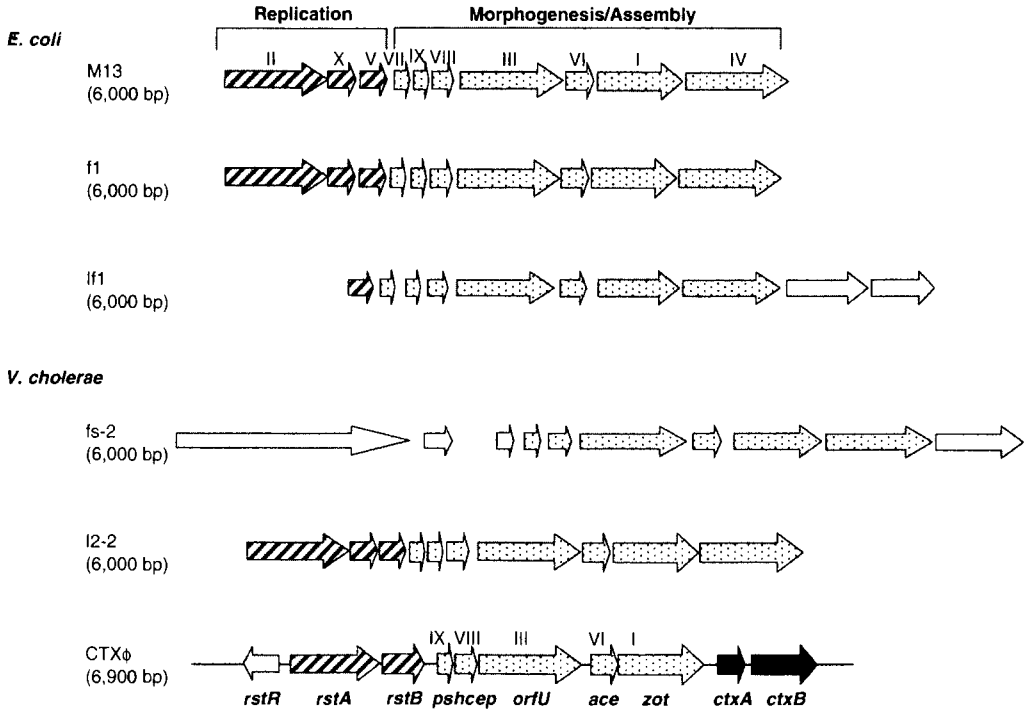

FIGURE 8.2 Schematic representation of ssDNA filamentous bacteriophages (f1 and M13) for *E. coli* and *V. cholerae*. Arrows indicate open reading frames (ORFs), and the direction of the arrows indicates the direction of transcription. The Roman numerals above the ORFs indicate gene designations. Genes encoding similar proteins have identical pattern arrows among the phages. DNA replication genes are represented as striped arrows, phage morphogenesis and assembly genes are represented as dotted arrows, and toxin genes are represented as black arrows. The Ff class of the filamentous phages are 98% homologous at the nucleotide level; therefore, the amino acid sequences of their proteins are virtually identical.

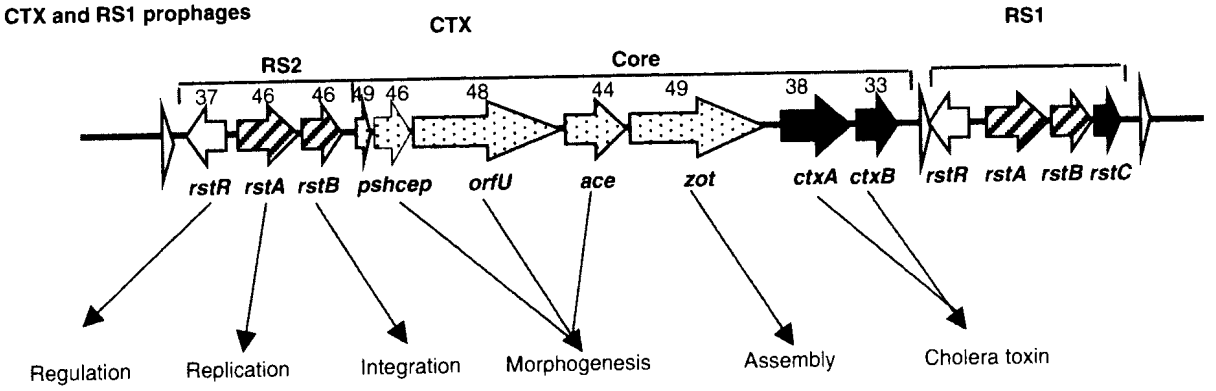

FIGURE 8.3 Gene organization and gene designation of the El Tor-derived CTXϕ and RS1ϕ genomes (Waldor and Mekalanos, 1996; Waldor et al., 1997). Open arrows represent CTXϕ ORFs and the direction of transcription of each gene. White triangles indicate end repeats. The numbers underneath the arrows indicate each gene's percent GC content. The *rstR, rstA* and *rstB* genes of both RS1ϕ and RS2 regions are almost identical. The *rstC* and *ctxAB* genes indicated in black encode an antirepressor and CT, respectively.

predominant cause of epidemic cholera (Boyd et al., 2000a). These data indicate independent acquisition of CTXϕ among isolates (Boyd et al., 2000a; Waldor and Mekalanos, 1996; Waldor et al., 1997), which is not surprising because CTXϕ transduction is readily detectable *in vitro* and *in vivo* (Waldor and Mekalanos, 1996; Waldor et al., 1997). Nonetheless, the distribution of CTXϕ among *V. cholerae* natural isolates is sporadic; CTXϕ is present in most O1 and O139 serogroup isolates, but it is only occasionally isolated from non-O1/non-O139 serogroup isolates (Faruque et al., 1998a; 1998b). CTXϕ has been isolated from environmentally occurring isolates of *V. mimicus* (Boyd et al., 2000b), a close relative of *V. cholerae*, which indicates an additional reservoir for this bacteriophage. Also, a comparative sequence analysis (Boyd et al., 2000b) of several genes of CTXϕ from *V. cholerae* and *V. mimicus* showed near sequence identity, indicating recent transfer, evolutionary speaking, of this bacteriophage between the species.

8.2.2. Proteins Involved in Bacterial Attachment/Colonization

Proteins involved in bacterial attachment to host cells, a critical step in the establishment of a bacterial infection, are novel additions to the list of bacteriophage-encoded virulence factors (Table 8.1). In *V. cholerae*, the toxin co-regulated pilus (TCP) plays an essential role in intestinal colonization of the human host (Taylor et al., 1987). Therefore, the recent proposal that TCP genes are encoded on a phage (Karaolis et al., 1999) is highly significant and has been followed with great interest (Lee, 1999; Davis and Waldor, 2003; Miller, 2003; Faruque et al., 2003). Initially, the *tcp* gene cluster, which encodes TCP, was defined as part of a pathogenicity island (PAI), the *Vibrio* pathogenicity island (VPI) (Karaolis et al., 1998). The VPI conforms to the definition of a PAI; i.e., it is a large chromosomal region (39.5 kb) that encodes several virulence gene clusters and a phage-like integrase, it inserts adjacent to a tRNA-like gene (*ssrA*), and it has a GC content that differs from the host chromosome (Fig. 8.4). Subsequently, Karaolis and colleagues (Karaolis et al., 1999) found that TCP is encoded by a filamentous phage, VPIϕ, and that the major pilus protein (TcpA), monomers of which make up the TCP pilus structure, is also the VPIϕ coat protein. The filamentous phage itself appeared to be very different from other filamentous phages based on the nucleotide sequences (Fig. 8.2 and Fig. 8.4). In addition, the authors have been unable to show transfer of VPIϕ among *V. cholerae* O1 serogroup isolates, the main cause of epidemic cholera. Furthermore, Faruque et al. (2003a) recently were unable to confirm the existence of VPIϕ when they examined a collection of TCP-positive, VPI-containing, clinical and environmental isolates of *V. cholerae* under the same conditions (which included mitomycin- and UV-induction) reported by Karaolis et al. (1999). Although minor differences between strains and growth conditions may account for the discrepancy between the two reports, the study by Faruque et al. (2003a), together with the lack of confirmatory evidence to the existence of VPIϕ, suggests that additional studies are required to confirm or refute the existence of VPIϕ and to better understand its role in the pathogenesis of *V. cholerae*.

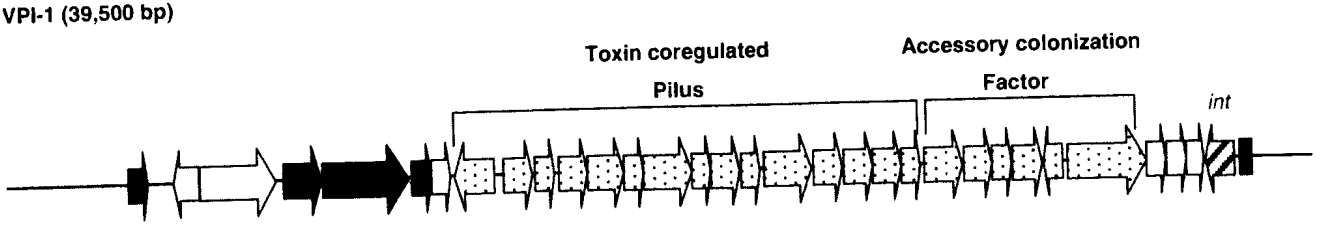

FIGURE 8.4 Schematic representation of the 39.5-kb VPI-1 (VC0819-VC0847) regions of *V. cholerae* strain N16961 (Heidelberg et al., 2000). ORFs are indicated by arrows that point in the direction of transcription. Virulence genes are colored black, *int* genes are indicated as striped arrows, proteins of known function are indicated as dotted arrows, hypothetical proteins are indicated as black arrows, and genes without a significant match are indicated as white arrows.

The distribution of the VPI region among *V. cholerae* is similar to the distribution of CTXϕ; i.e., VPI is present in most O1 and O139 serogroup isolates, and it is absent from most *V. cholerae* non-O1 and non-O139 serogroup isolates. Interestingly, several *V. mimicus* isolates carrying VPI have been identified, and their VPI genes show remarkable sequence identity to those of the VPI in *V. cholerae* biotype El Tor (Boyd et al., 2000b). These data suggest that there was recent interspecies lateral gene transfer of this region between *V. cholerae* and *V. mimicus*; thus, indicating that the VPI region was mobile in the recent past.

Another example of bacteriophage-encoded proteins involved in bacterial attachment and colonization is found in *S. mitis*, a leading cause of infectious endocarditis. Infection of the endocardium commences with the attachment of bloodborne organisms to deposits composed of platelets, fibrin, and extracellular matrix proteins on the damaged heart valve surface (Durack and Beeson, 1975; McGowan and Gillett, 1980). Recently, Bensing and colleagues (Bensing et al., 2001a; Bensing et al., 2001b) identified two distinct loci, *pblT* and a polycistronic operon *pblA* and *pblB*, in *S. mitis* strain SF100. The latter two genes encode large surface proteins that promote binding to human platelets. However, PblA and PblB are unusual since neither protein shows a strong similarity to known bacterial adhesins; instead, both resemble structural components of bacteriophages. Further analysis showed that *pblA* and *pblB* are clustered with genes showing significant homology to genes in streptococcal bacteriophages r1t, 01205 and Pp-1 (Bensing et al., 2001b). To determine whether *pblA* and *pblB* reside within a prophage, cultures of *S. mitis* strain SF100 were treated with either UV light or mitomycin C, both of which induce the lytic cycle in temperate bacteriophages. Both treatments resulted in significant increases in the transcription of *pblA*, and Southern hybridization analysis of bacteriophage DNA revealed that *pblA* and *pblB* were contained in the SM1 phage genome. Further experimentation indicated that the genes encoded proteins present in SM1 phage particles (Bensing et al., 2001b).

8.2.3. Proteins Required for Host Immune Avoidance

Once a pathogen has entered a human host, the first line of defense against bacterial infection is the innate immune response. Several lysogenic conversion genes encode proteins that alter bacterial recognition by the host immune system or that confer serum-resistance (Table 8.1). In this category are the O-antigen modification genes whose proteins alter bacterial antigenicity, thus facilitating bacterial evasion of the host immune system. The O-antigen modification genes include *rfb*, *oac*, and *gtr* of *E. coli* and *Shigella* spp., which express lipopolysaccharide O-antigen glucosylating, acetylating, and transferase proteins, respectively. The genes are located in several morphologically diverse bacteriophages of the families *Podoviridae*, *Siphoviridae*, *Myoviridae*, and *Inoviridae*. Genetically, the bacteriophages share several common features, such as the location of the O-antigen modification genes immediately downstream of the bacteriophage attachment site *attP*, which is preceded by the integrase (*int*) and excisionase (*xis*) genes in bacteriophages SfV, SfX, SfII, and Sf6 from *E. coli*, and in bacteriophage P22 from *S. enterica* (Allison and Verma, 2000; Vander Byl and Kropinski, 2000)) (Fig. 8.5). The integrase proteins of SfV, SfX,

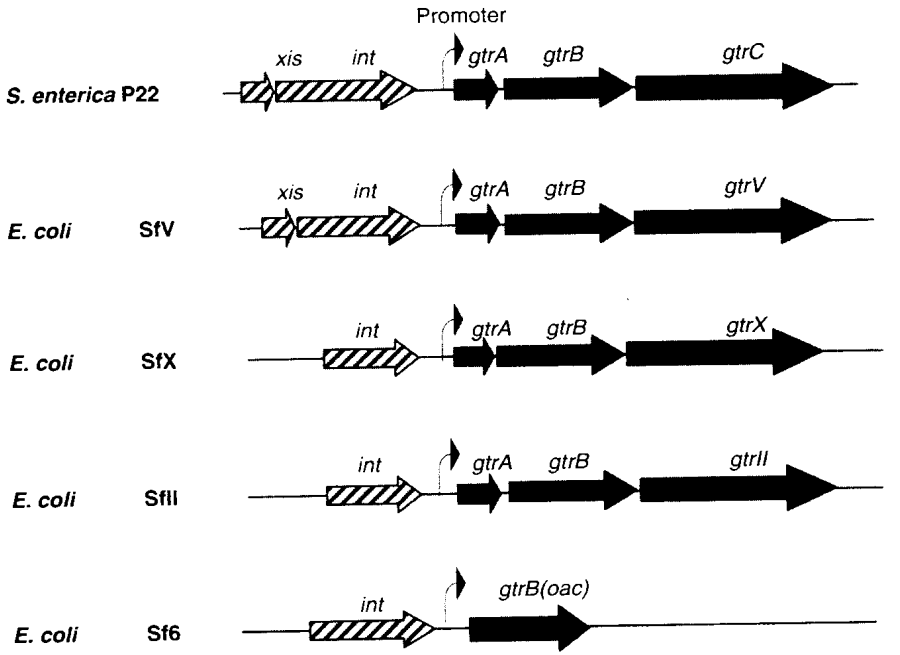

FIGURE 8.5 The O-antigen modifying, lysogenic conversion genes located between the phage lysin gene and the right attachment site in *Siphoviridae* phages for *E. coli* and *Salmonella*. The *int* and *xis* genes are are represented as striped arrows and the lysogenic conversion genes are black arrows. The promoter start point is indicated by a vertical arrow.

SfII, and SfI are very similar to the corresponding protein in phage P22, and the *attP* site of SfV, SfX, and SfII is identical to that of P22. The SfV phage's DNA packaging and head genes have the genetic organization of a lambda-like Siphovirus; however, its tail genes resemble those of a Mu-like Myovirus (Allison et al., 2002). Morphologically, SfV is a Myovirus; thus, chimeras between different classes of defined bacteriophages can also be carriers of lysogenic conversion genes.

The O-antigen plays a significant role in pathogenesis and is a major protective antigen, and the ability to switch O-antigen types has been acquired by many bacterial pathogens, such as *S. enterica*, *E. coli*, *V. cholerae*, *S. pneumoniae*, and *Neisseria meningitidis*. This capability can deleteriously affect vaccine development and efficacy, since vaccines may be rendered ineffective due to O-antigen switching. In *V. cholerae*, horizontal transfer of the O-antigen genes via a bacteriophage has been implicated in the emergence of the novel serogroup O139 that replaced the O1 serogroup as the major cause of epidemic cholera in Asia in 1992. Cholera caused by *V. cholerae* serogroup O139 infects more adults than the O1 serogroup, due to a lack of immunity in the adult population (Faruque et al., 2003c). Several studies have shown that the O139 serogroup strain was derived from an El Tor biotype *V. cholerae* O1 serogroup strain by the acquisition of O139 antigen genes (Bik et al., 1995; Waldor and Mekalanos, 1994; Mooi and Bik, 1997; Stroeher and Manning, 1997). The origin

of the O139 serogroup biosynthesis genes is unknown; however, it has been suggested (Dumontier and Berche, 1998; Yamasaki et al., 1999) that an O22 serogroup strain may have been the donor responsible for the emergence of the Bengal O139 serogroup strain. Analyses of the organization and nucleotide sequence of the O-antigen biosynthesis genes of *V. cholerae* O1, O139, and O22 (Dumontier and Berche, 1998; Yamasaki et al., 1999) revealed a similar organization, and O139 and O22 serogroups shared extensive sequence homology. Indeed, there is evidence for the emergence of several non-O1/non-O139 *V. cholerae* strains with pathogenic potential by exchange of O-antigen biosynthesis regions in O1 El Tor and classical isolates (Li et al., 2002). The authors analyzed 300 *V. cholerae* strains representing all of the 194 known serogroups, and they identified several strains carrying *ctxAB* and *tcp* genes. DNA sequencing of the O-antigen cluster in one O37 serogroup strain revealed that most of the O1 serogroup *wbe* region was replaced by a novel *wbe* gene cluster, thus indicating a possible mechanism for the emergence of epidemic *V. cholerae* strains. O-antigen switching in *V. cholerae* has been proposed (Mooi and Bik, 1997; Stroeher and Manning, 1997) to be most likely mediated by a bacteriophage, as has been found with *E. coli* and *S. enterica*.

8.2.4. Proteins Essential for Bacterial Invasion of Host Cells

After bacterial attachment to the host cell, a pathogen either remains extracellular or invades the host cell to become intracellular. One example of an intracellular pathogen is *S. enterica*, a facultative pathogen that causes numerous infections, including typhoid fever, gastroenteritis, and septicemia. Many *S. enterica* serotypes are host-adapted; for example, serotype Typhi is restricted to humans, serotype Pullorum is strongly associated with chickens, and serotype Choleraesuis is strongly associated with pigs. The majority of human *S. enterica* isolates belong to subspecies I, whereas the other subspecies are mainly found in cold-blooded animals. *S. enterica* employs two specialized type III secretion systems that translocate effector proteins directly into the cytosol of its eukaryotic host cells, where they play a key role in the bacterial invasion process (Hansen-Wester et al., 2002). The type III secretion systems are encoded by two PAIs, *Salmonella* PAIs 1 and 2 (SPI-1 and SPI-2), which are found in all seven *S. enterica* subspecies (Hansen-Wester et al., 2002). Various combinations of effector proteins have been identified among *Salmonella* isolates, and many of those proteins, which are translocated either *via* SPI-1 (effector proteins SopB, SopD, SopE, SopE2, SspH1, and SlrP) or SPI-2 (effector proteins SspH1, SspH2, SlrP, GtgB, SseJ, SifA, and SifB), are encoded either adjacent to phage-like sequences (SopE2 and SspH2) or are found on lysogenic bacteriophages (SopE, GogB, SseI, and SspH1) (Fig. 8.6). The SopEϕ, a P2-like phage, encodes the effector protein SopE, which promotes invasion of tissue culture cells by *Salmonella* (Mirold et al., 1999). Among the lambda-like phages, Gifsy-1 encodes GogB, Gifsy-2 encodes GtgB (also called SseI or SfrH), and Gifsy-3 encodes SspH1 (Figueroa-Bossi et al., 2001). Also, the *sopE, gogB, gtgB*, and *sspH1* genes are located in the tail fiber-encoding regions of their respective bacteriophage genomes (Fig. 8.7). Lysogenic conversion by these and similar bacteriophages is probably responsible for the diversity of effector proteins observed among *S. enterica*, and the acquisition

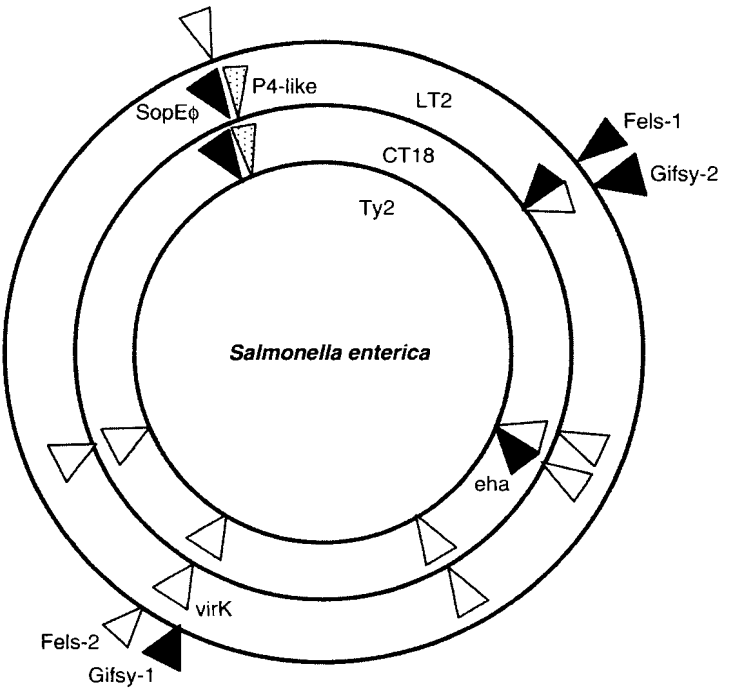

FIGURE 8.6 Distribution of prophages possessing virulence genes in *S. enterica* genomes. The outer circle represents the genome of serotype Typhimurium strain LT2, and the inner circles represents the genomes of serotype Typhi strain CT18 and Ty2, respectively. The prophages and suspected prophages are indicated as colored triangles (black triangles represent lambda-like phages and dotted triangles represent P4-like phages).

of various repertoires of lysogenic bacteriophage genomes could be an important step in the emergence of new epidemic clones and, perhaps, in the adaptation to new hosts.

In view of the many bacteriophages that encode effector proteins, it is not surprising to find a reassortment of bacteriophages among strains and a reassortment of effector proteins among bacteriophages (Hansen-Wester et al., 2002; Mirold et al., 2001). A case in point is the P2-like SopEϕ, which—over large parts of its genome—is closely related to Myovirus P2 from *E. coli*. At the *sopE* position, P2 encodes the bacteriophage resistance gene for protecting the lysogen against superinfection with coliphage T5 (Boyd and Brüssow, 2002). However, in other *Salmonella* strains, the *sopE* gene is found associated with lambda-like *Siphoviridae* (Mirold et al., 2001). The *sopE* integration site is occupied in other lambdoid *Salmonella* bacteriophages by still other lysogenic conversion genes (e.g., the type III effector *sseI*, the phagocytosis-activated *pagJ*, and the neuraminidase-encoding *nanH*) (Figueroa-Bossi et al., 2001). Sequence comparisons defined a *sopE* transfer cassette and *pagJ* flanked by a transposase (Mirold et al., 2001). Although there are a number of PAI-phage interactions among *S. enterica* serovar Typhimurium

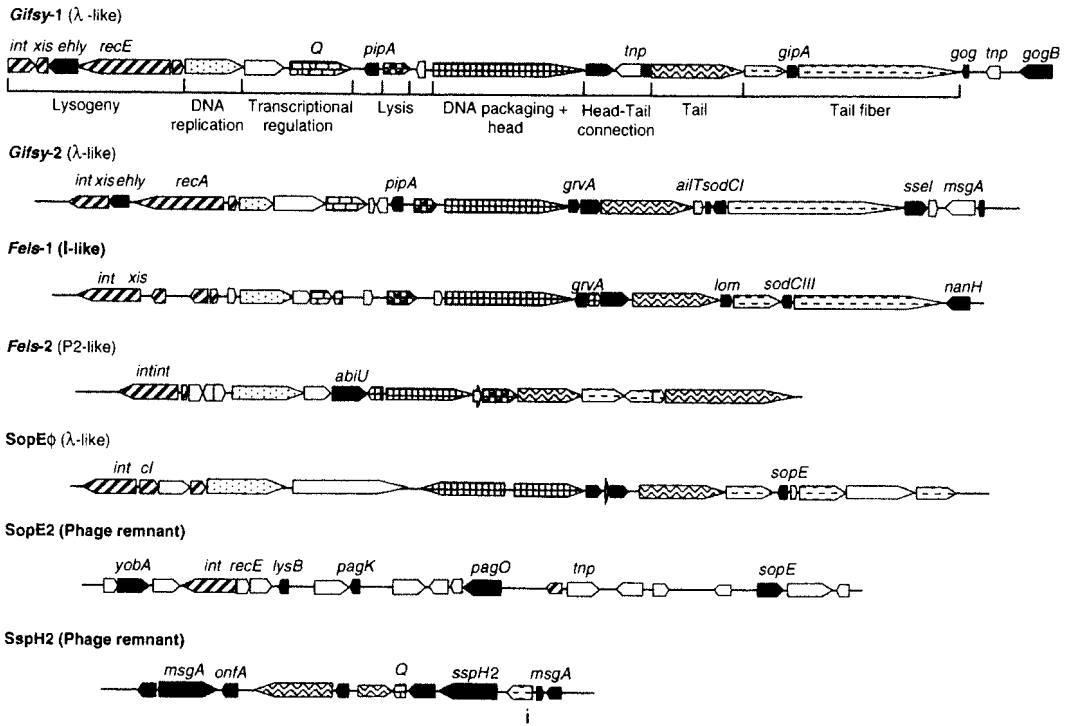

FIGURE 8.7 Genomic maps of five *S. enterica* prophages and two prophage remnants possessing genes that encode virulence factors. Prophages Fels-1 (STM0894-STM0928), Gifsy-2 (STM1005-STM1056), Gifsy-1 (STM2636-STM2584), and Fels-2 (STM2740-STM2694), and prophage remnants SspH2 (STM2230-STM2245) and SopE2 (STM1853-STM1870) are from the genome of *S. enterica* serotype Typhimurium (McClelland et al., 2001). The SopE (STY4645-4600) prophage is from the genome of serotype Typhi (Parkhill et al., 2001). To aid the comparison of the prophage maps, the encoding genes that may be attributed to modules are patterned: striped, for lysogenic conversion; dotted, for DNA replication; bricked, for transcription regulation; checkered, for DNA packaging and head proteins; gray, for head-to-tail proteins; wavy lined, for tail proteins; dashed lined, for tail fibers; bubbled, for lysis modules. Unattributed genes are not colored. Proven or suspected virulence genes are colored black and are annotated.

subspecies I isolates, in the other serovars and subspecies of *S. enterica* most of the Typhimurium-specific bacteriophages are absent or highly divergent (Porwollik et al., 2002; Boyd et al., 2003). This observation suggests that other serovars and subspecies of *S. enterica* may contain unique repertoires of phages that interact with SPI-1 and SPI-2. Analogous to the reassortment of *stx* genes among diverse bacteriophages in *E. coli*, reassortment of virulence genes among *Salmonella* bacteriophages may be an important mechanism for avoiding restrictions for bacteriophage-mediated gene transfer by superinfection immunity, competition for insertion sites, and restriction-modification systems.

8.2.5. Proteins Involved in the Survival of Intracellular Bacteria

Bacteriophages Gifsy-2 and Fels-1 encode an additional potential virulence factor, a periplasmic copper- and zinc-cofactored superoxide dismutase (Cu,Zn-SodC) (Fig. 8.7). The enzyme catalyzes the conversion of superoxide to hydrogen peroxide and molecular oxygen, thus protecting the bacterium from oxidation stress (Figueroa-Bossi et al., 2001). In *S. enterica*, three Cu,Zn-SodC-encoding genes, *sodCI*, *sodCII*, and *sodCIII*, have been identified (Figueroa-Bossi et al., 2001), and the amino acid sequence of SodCI shows 57% and 60% homology to that of SodCII and SodCIII, respectively. The chromosomally-encoded *sodCII* gene is present in all *Salmonella* isolates, and *sodCI* is carried on phage Gifsy-2 and is confined to strains belonging to the most pathogenic serotypes, which are exclusively subspecies I isolates (Fang et al., 1999; Figueroa-Bossi et al., 2001). The *sodCIII* gene was recently identified within the genome of phage Fels-1, in a location similar to that of *sodCI* in Gifsy-2 (Fig. 8.7) (Figueroa-Bossi et al., 2001). Carriage of SodC-encoding genes by *S. enterica* bacteriophages is not unique; e.g., a *sodC* gene has been identified (Ohnishi et al., 2001) in two lambda-like phages located at identical positions in the bacteriophage genome in *E. coli* O157 strain Sakai.

8.2.6. Putative Virulence Proteins

Neuraminidase (encoded by *nanH*) is another example of a possible phage-encoded protein that may play a role in bacterial virulence, by enhancing cellular survival of the bacterium. Phage Fels-1 from *S. enterica* serotype Typhimurium carries the *nanH* gene, which is located adjacent to the phage attachment site (Fig. 8.7) (Figueroa-Bossi et al., 2001). At the present time, the role of neuraminidase in the virulence of that species has not been rigorously evaluated. In the *V. cholerae* genome, the *nanH* gene is encoded on a 59-kb region designated VPI-2, which is unique to pathogenic isolates (Jermyn and Boyd, 2002). Interestingly, the VPI-2 region has a G+C content of 42%, which differs from the rest of the *V. cholerae* genome (47%), thus suggesting that the region was acquired by horizontal transfer. In addition, *nanH* is located adjacent to several genes that show significant homology to phage Mu (Fig. 8.8) (Jermyn and Boyd, 2002). The role of *nanH* in the pathogenesis of cholera has not yet been clearly defined either; however, the neuraminidase has been proposed (Galen et al., 1992) to act synergistically with CT (by converting higher-order

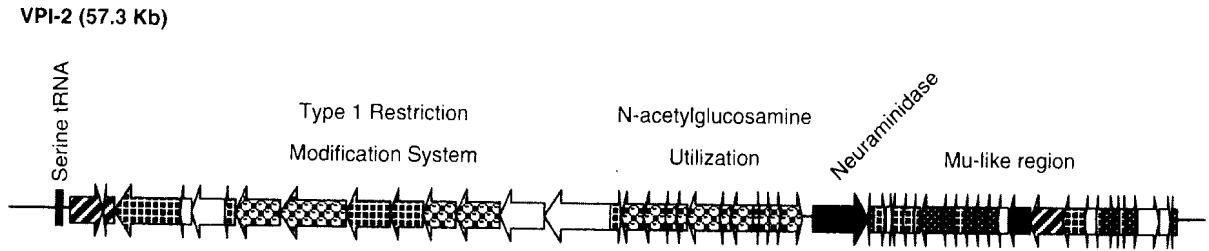

FIGURE 8.8 Schematic representation of the 57.3-kb VPI-2 (VC1758-VC1809) regions of *V. cholerae* strain N16961 (Heidelberg et al., 2000). ORFs are indicated by arrows that point in the direction of transcription. Virulence genes are black, *int* genes are striped, phage genes are dotted, proteins of known function are bubbled, hypothetical proteins are checkered, and genes without a significant match are white.

gangliosides to ganglioside GM1 [CT's receptor site]), thus increasing the binding of the toxin to host enterocytes. The *nanH* gene also is found in other bacterial genomes, where it also appears to have been acquired by horizontal transfer. For example, in *Clostridium perfringens, nanH* is integrated near an *attP* site, and it has a G+C content of 32%, compared to 27% for the *C. perfringens* genome.

8.3. MECHANISMS OF VIRULENCE GENE ACQUISITION BY BACTERIOPHAGES

In this section, three possible scenarios for the acquisition of virulence genes by bacteriophages are discussed (Table 8.2). As noted earlier in this chapter, the genes encoding the virulence factors may be (i) products of imprecise prophage excision from an ancestral host bacterium, (ii) transferable modules of extra bacterial DNA with their own promoters and terminators, or (iii) integral components of the bacteriophage genome.

8.3.1. Imprecise Prophage Excision

Most bacteriophage genes encoding bacterial virulence factors are not essential for bacteriophage replication, morphogenesis, or assembly. Additionally, since many virulence genes show an independent evolutionary history compared to the rest of the bacteriophage genome and are located near the bacteriophage attachment site, it is likely that bacteriophages acquired them by imprecise excision of the prophage from the bacterial host's genome. Imprecise prophage excision of toxin genes has been demonstrated for *S. pyogenes*; e.g., the genes encoding exotoxins SpeA and SpeC, encoded by phages T12 and CS112, respectively (Zabriskie, 1964; Goshorn and Schlievert, 1989), are located directly adjacent to the *attP* site, and they have their own promoter regions separate from the rest of the bacteriophages' regulatory control. Indeed, all sequenced *S. pyogenes* prophages possess lysogenic conversion genes at a specific position between the phage lysin and the right attachment site. A similar situation is found in *S. aureus* isolates, where the lysogenic conversion genes are located between the phage lysin-encoding site and the right attachment site (Desiere et al., 2001; Ferretti et al., 2001; Smoot et al., 2002). Further evidence in support of this model of independent acquisition of virulence genes via imprecise prophage excision comes from studies (Nakayama et al., 1999) of ϕCTX, a cytotoxin-converting bacteriophage of *P. aeruginosa.* The toxin gene *ctx* is located at the right bacteriophage attachment site and has a GC content that differs from the rest of the bacteriophage's genome. A potential variation of imprecise excision as a mechanism for virulence gene acquisition is imprecise prophage replication. In the CTXϕ genome, the *ctxAB* genes, which encode CT, are located adjacent to the bacteriophage's *attRS* site, and they have a distinct GC-content compared to the rest of the bacteriophage's genome (Fig. 8.3). However, since CTX virion production does not involve excision of the CTX prophage from the chromosome (Davis and Waldor, 2000), the pre-CTX genome most likely integrated adjacent to the *ctxAB* gene—and a CTXϕ possessing the *ctxAB* genes may have been generated *via* imprecise prophage replication. A precursor CTXϕ (pre-CTXϕ) has been identified in nontoxigenic

isolates of *V. cholerae*, and it did not contain the *ctxAB* genes or the upstream control region containing the *ctxAB* promoter normally found 5′ of *ctxA* (Boyd et al., 2000a). Each pre-CTX prophage gave rise to a replicative plasmid form of the CTXϕ whose genomic organization was identical to that of the CTXϕ replicative form, except that it lacked the latter's *ctxAB* (Boyd et al., 2000a). The existence of both pre-CTXϕ and CTXϕ in *V. cholerae* populations raises the question of whether gene products other than CT encoded on CTXϕ impart a selective advantage to cells that carry the phage; i.e., perhaps the role of Ace and Zot in enterotoxicity is greater than previously appreciated.

8.3.2. Transferable Virulence Gene Cassettes

In contrast to the toxin-encoding genes described above, the putative virulence genes in *E. coli* and *Salmonella* prophages are not exclusively concentrated near the *att* sites; i.e, they are found inside the bacteriophage genome (Fig. 8.7). However, their position is not random, and most are found downstream of (i) anti-termination bacteriophage lambda N or Q homologs (Fig. 8.9), (ii) the lysis cassette (located at the center of the prophage map of lambda-like phages), (iii) the tail fiber-encoding genes involved in host recognition (which are frequently the target of recombination reactions), or (iv) the genes encoding the phage capsid protease. The virulence genes frequently represent separate transcriptional units flanked by their own promoter and terminator, which prevents transcription of parts of the prophage genome that have to be transcriptionally silent in order to maintain the prophage state. Since those virulence genes represent more genetic material than is found in comparable bacteriophages, the term *moron* has been proposed for this extra DNA (Hendrix et al., 1999).

DNA sequence analysis of the H-19B phage genome (Neely and Friedman, 1998b) revealed that the *stxAB* genes are downstream and in the same transcriptional orientation as a λ Q homology, which functions as a transcriptional activator at the late promoter P_R' of late phage genes. The *stx* genes either represent tightly controlled transcription units regulated by nutritional signals (low iron concentration, contact with eukaryotic host cells, etc.) or are coordinated with prophage induction. For example, Shiga toxin does not have transport signals but depends on bacterial lysis, after prophage induction in a small number of lysogenic cells, for export out of the cell (Wagner and Waldor, 2002). Thus, the location of *stx* between the Q antiterminator and the lysis cassette makes sense genetically (Fig. 8.9).

8.3.3. Integral Components of the Bacteriophage Genome

As stated previously, many bacteriophage genes that encode bacterial virulence factors appear to be remnants of an ancestral bacterial host, and they are not required for phage regulation and morphogenesis. An interesting exception is the PblAB phage's structural proteins that are also involved in attachment of *S. mitis* to human platelets, which may represent an example of proteins evolving dual functions (Bensing et al., 2001a; Bensing et al., 2001b). Similarly, in *V. cholerae* the CTXϕ's *ace* and *zot* gene products, which are phage morphogenesis and assembly proteins

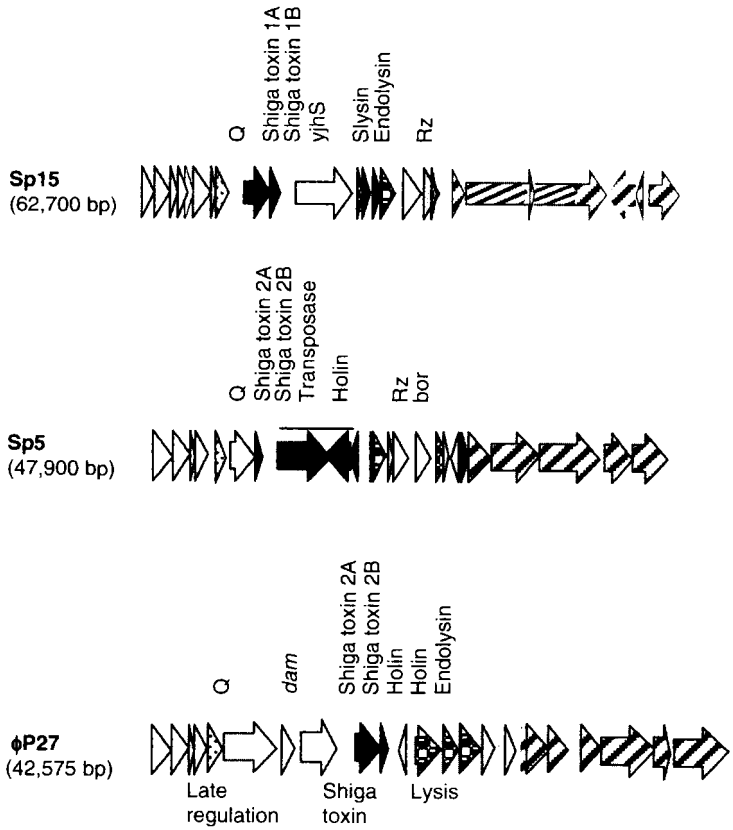

FIGURE 8.9 Genome location of the Shiga toxin genes (*stx1, stx2*) in three prophages of two *E. coli* O157 isolates (Recktenwald and Schmidt, 2002; Yokoyama et al., 2000). The antiterminator gene Q is indicated as a dotted arrow, the shiga toxin 1- and shiga toxin 2-encoding genes are black arrows, and the lysis genes are bubbled arrows.

(Waldor and Mekalanos, 1996), are also potent enterotoxins (Fasano et al., 1991; Trucksis et al., 1993).

8.4. ROLE OF BACTERIOPHAGES IN THE PATHOGENESIS OF BACTERIAL INFECTIONS

8.4.1. Indirect Involvement of Bacteriophages

Bacteriophages that encode virulence genes are generally considered passive vectors for the dissemination of those genes among bacterial populations, and they have been implicated in the dispersal of virulence factors and the emergence of new pathogenic strains in both Gram-positive and Gram-negative bacteria. For example, the CTXϕ is responsible for the horizontal transfer of the *ctxAB* genes between

TABLE 8.4
***V. cholerae* Bacteriophages Involved in Lateral Transfer of Virulence Factors**

Bacteriophage	Virulence Genes	Role
CTXϕ	*ctxAB, ace, zot*	Encodes CT, ACE, and ZOT toxins
RS1ϕ	*rstC*	Encodes antirepressor
KST-1ϕ	Helper phage	Transmits RS1ϕ
K139	*glo*	Encodes G-protein-like
CP-T1	Helper phage	Transmits RS1ϕ, CTXϕ, and VPI

V. cholerae isolates, and it is one of the key factors in the emergence of toxigenic strains. Indeed, to date at least five *V. cholerae* bacteriophages have been identified that contribute to lateral transfer of virulence factors between strains (Table 8.4). In addition, the *stxAB* genes in most *E. coli* STEC isolates reside in the genome of lambdoid prophages, and this probably accounts for the wide distribution of *stxAB* in more than 60 serotypes of *E. coli*. It is also possible that some of those strains arose via antigen switching, in which case a common pathogenic strain acquired a novel serotype. Also, *Shigella dysenteriae* strains with a clonal lineage of *E. coli* (i.e., they phylogenetically group with *E. coli* strains) have shiga toxin genes encoded on a lambdoid phage. Furthermore, the presence of numerous superantigen toxin-encoding genes in many streptococcal and staphylococcal prophages likely accounts for their wide dissemination and is a possible mechanism for the emergence and spread of new pathogenic strains. For example, the dissemination of the novel superantigen gene *speL*, in recent invasive and noninvasive *S. pyogenes* M3/T3 isolates in Japan, is likely to have been associated with bacteriophage carriage (Ikebe et al., 2002).

8.4.2. Direct Involvement of Bacteriophages

The bacteriophage life cycle can also be important in the pathogenesis of bacterial infections (reviewed in Wagner and Waldor, 2002). For example, phage-controlled regulation of the production and export of Stx toxins directly affects the pathogenesis of disease caused by STEC. In the *E. coli* O157 phage H 19B, the *stx* genes are located directly downstream of the PR′ promoter and upstream of the phage lysis genes (Neely and Friedman, 1998a; 1998b). Also, prophage induction by exogenous (e.g., mitomycin C and antibiotics) and endogenous (hydrogen peroxide released from human neutrophils) agents increases Stx production by their host strains (Wagner and Waldor, 2002). Functional studies (Neely and Friedman, 1998a) indicated that the Q protein of phage H-19B, acting in trans, directs high-level expression of Stx from repressed H-19B and 933W prophages. Transcription from the late phage promoter P'_R, resulting from prophage induction, also is important for Stx production by the O157:H7 *E. coli* clinical isolate 1:361 (Wagner and Waldor, 2002).

CTX prophage induction also results in high toxin production by *V. cholerae*. CTXϕ either integrates into the bacterium's genome or replicates as an episome,

and its replicative form (RF) yields high virion titers. Skorupski and Taylor (1997) have demonstrated that expression of *ctxAB* from CTX prophages is dependent on the transcriptional activators ToxR and ToxT. However, it has also been shown that *in vitro* and *in vivo* expression of CT by the CTXϕ RF does not require ToxR and ToxT (Wagner and Waldor, 2002). This observation suggests that CTX prophage induction may up-regulate toxin production during intestinal infection, by increasing the *ctxAB* copy number and by eliminating the requirement for ToxR and ToxT. This observation may also indicate the presence of as-yet-unidentified, alternative induction-related phage regulators of *ctx* transcription (Wagner and Waldor, 2002).

8.5. ROLES OF PHAGE-PHAGE INTERACTIONS IN BACTERIAL VIRULENCE

The previous section discussed the roles bacteriophages play in the pathogenic process, either as passive vectors for the dissemination of genes or as regulators of virulence gene expression. This section presents information concerning the roles of phage-phage interactions in bacterial virulence (Table 8.5) (reviewed in Boyd et al., 2001). The phage-phage interactions examined are divided into three categories, based on the requirement of a helper phage for (i) mobilization of another unrelated

TABLE 8.5
Various Categories of Phage-Phage Interactions Based on the Role of the Helper Bacteriophage

Helper Phage	Associated Phage	Bacterial Host	Reference
Helper phage supplies morphogenesis genes			
P2	P4	*E. coli*	(Lindqvist et al., 1993)
CTXϕ	RSIϕ	*V. cholerae*	(Faruque et al., 2002)
KST-1ϕ	RSIϕ	*V. cholerae*	(Faruque et al., 2003b)
Generalized transduction by helper phage			
80α	*tst* element	*S. aureus*	(Ruzin et al., 2001)
CP-T1	CTXϕ	*V. cholerae*	(Boyd and Waldor, 1999)
CP-T1	VPIϕ	*V. cholerae*	(O'Shea and Boyd, 2002)
Phage's host receptor is another phage			
VPIϕ	CTXϕ	*V. cholerae*	(Karaolis et al., 1999)
Helper phage potentates expression of virulence genes			
VPIϕ	CTXϕ	*V. cholerae*	(Lee et al., 1999)
RS1ϕ	CTXϕ	*V. cholerae*	(Davis et al., 2002)
Gifsy-2	*Gifsy*-1	*S. enterica*	(Figueroa-Bossi et al., 2001)
λ-like	λ-like	*E. coli*	(Sandt et al., 2002)

bacteriophage, (ii) entry of another bacteriophage into the host cell, or (iii) expression of bacteriophage-encoded virulence genes.

8.5.1. Helper Phage Facilitation of Bacteriophage Mobilization

8.5.1.1. *V. cholerae* CTXϕ Supplies Morphogenesis Genes for RS1ϕ

The classical example of the mobilization of a defective phage by a helper phage is the interaction between the *E. coli* lambdoid phages P2 (helper) and P4 (defective). P4 is dependent on P2, or on a related lambdoid bacteriophage, to supply the capsid, tail, and lysis genes required to assemble its capsid, package its DNA, and lyse the bacterial host cell. These requirements are essential for P4 to complete a lytic cycle and produce virions (Christie and Calendar, 1990; Lindquist et al., 1993). Therefore, P4 is a defective phage, which, once released as a virion, can—in the absence of P2 .—replicate and integrate its DNA within a host bacterium.

The requirement of RS1ϕ for the morphogenesis proteins of *V. cholerae*'s CTXϕ is analogous to the P2/P4 interaction in *E. coli*. *V. cholerae* strains responsible for contemporary cholera epidemics (i.e., biotype El Tor O1 serogroup and O139 serogroup strains) contain fully functional prophages that can produce the infectious RF of CTXϕ (Davis et al., 1999). In those isolates, the CTX prophage is always flanked by another genetic element, RS1. RS1 is identical to the RS2 region but contains an additional open reading frame, *rstC*, that encodes an antirepressor that causes the RstR repressor to aggregate (Fig. 8.3), thus affecting the regulation and transmission of the CTXϕ (Davis et al., 2002). The production of CTXϕ virions by a lysogen depends on the presence of either a tandem array of CTX prophages or a single prophage followed by an RS1 element (Davis et al., 2000a). A recent study of RS1 demonstrated that it propagates horizontally as a filamentous bacteriophage that exploits the morphogenesis genes of CTXϕ (Faruque et al., 2002). The authors also confirmed the presence of an excised copy of RS1, and they showed that a kanamycin (Kn)-marked copy of that RF of RS1, pRS1-Kn, transduced *V. cholerae* isolates to Kn^r (Faruque et al., 2002). The isolates carried a single-stranded form of pRS1-Kn; therefore, they resembled the genome of a filamentous bacteriophage (RS1-Knϕ). Similar to CTXϕ, the RS1ϕ genome uses the *attRS* sequence to integrate site-specifically into the *V. cholerae* genome. However, only transductants of RS1-Knϕ, which already harbor the CTXϕ genome, can produce detectable RS1-Knϕ (Faruque et al., 2002). A second filamentous phage, KSF-1ϕ, which also provides functions required for RS1ϕ production, has been identified (Faruque et al., 2003b). However, unlike the CTXϕ, it does not require TCP for entry into *V. cholerae*. Thus, a hypothetical scenario for the evolution of CTXϕ would appear to have involved three main steps (Fig. 8.10): (i) a precursor-CTXϕ—pre-CTX) emerged when the RS1ϕ integrated adjacent to the core region sequence (presumably with the loss of *rstC*), thus forming the RS2 and core domains, (ii) the pre-CTXϕ infected an unknown bacterial host that contained the *ctxAB* genes, and (iii) the toxigenic CTXϕ was formed by a mechanism similar to imprecise excision. CTXϕ propagation does not involve excision

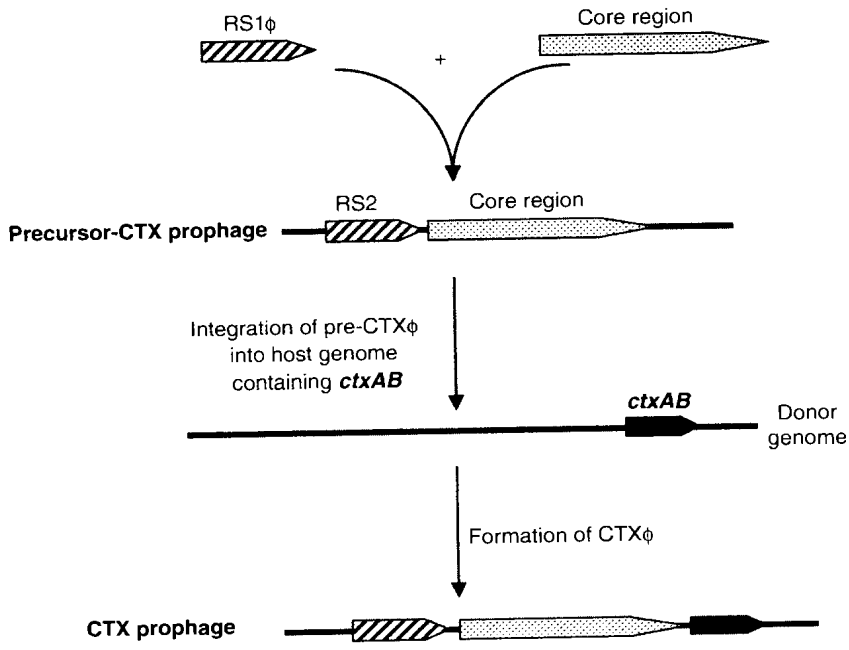

FIGURE 8.10 Model for the emergence of precursor-CTXϕ and CTXϕ. The 2.7-kb *rstR*, *rstA* and *rstB* genes of the RS1ϕ share near sequence identity with the 2.4-kb *rstR*, *rstA*, *and rstB* genes in the RS2 region of the CTXϕ. The RS1ϕ contains an additional gene (*rstC*) not present in the RS2 region. The precursor-CTXϕ probably arose when an RS sequence integrated next to the core region sequence in an unknown host. The pre-CTXϕ then infected and integrated next to the *ctxAB* genes in a new, unknown donor host. The RS region is indicated by an orange-filled arrow, the core region is indicated by a dotted arrow, and the CT-encoding genes are indicated by a black arrow.

of the CTX prophage from the chromosome but it is similar to propagation of replicative transposons or Mu phage (Davis and Waldor, 2000). As stated earlier, production of CXTϕ requires the presences of tandem elements, either CTX-CTX prophages or CTX-RS1 prophages. Hence, the CTXϕ is not present in isolates of the classical *V. cholerae* biotype, which do not contain tandem CTX prophages or the RS1 prophage (Davis et al., 2000b). However, the RS1 prophage is present in all *V. cholerae* El Tor isolates, which may explain the predominance of this biotype in current cholera epidemics. The interaction between CTX and RS1 prophages, which promotes the production of infectious CTXϕ, is important because it increases the dissemination of *ctxAB* among *V. cholerae* isolates.

8.5.1.2. *S. aureus* ϕ80 Generalized Transduction of the tst Element

The *tst* gene in *S. aureus* encodes toxic shock syndrome toxin-1 (TSST-1), a potent superantigen responsible for toxic shock syndrome associated with some *S. aureus*

infections. In *S. aureus* strain RN4282, the 15-kb *tst* element is not capable of self-mobilization. Rather, it is mobilized by propagation of the staphylococcal generalized transducing phages 13 and 80α, which efficiently encapsidate and transduce the *tst* element (Lindsay et al., 1998; Ruzin et al., 2001). Novick and colleagues (Lindsay et al., 1998; Ruzin et al., 2001; Novick, 2003) have proposed that the 15-kb *tst* element in *S. aureus* RN4282 is a PAI, which they called SaPI-1, a term which has recently been extended to several related elements encoding superantigens in *S. aureus* isolates (Fig. 8.11). At the present time, it is unclear whether the SaPI-1 truly falls under the rubric of PAIs, because the *tst* elements it contains are characterized by certain phage-related features, such as encoding integrases, helicases and terminases, and the presence of flanking direct repeats (Novick, 2003). In strain RN4282, phage 80α induces the SaPI-1 to excise, replicate and encapsidated, at high efficiency, into phage-like infectious particles with heads about one-third the size of the phage helper head. This results in very high frequencies of transfer. In the absence of a helper phage the island is highly stable in the *S. aureus* genome. The high frequency, 80α-dependent mechanism outlined for SaPI-1's transfer in strain RN4282 probably is responsible for the horizontal spread of the *tst* gene among clinical isolates of *S. aureus*. The close association between SaPI-1 and its helper phage suggests that they are genetically related. Indeed, SaPI-1 may be a defective phage that requires a helper phage, similar to the P2/P4 interaction and the CTXϕ/RS1ϕ interaction (Boyd et al., 2001; Ruzin et al., 2001; Faruque et al., 2002). The SaPI-1 in *S. aureus* isolates is one of the first PAIs characterized in Gram-positive bacteria.

8.5.1.3. *V. cholerae* CP-T1 Generalized Transduction of VPI

Recently, O'Shea and Boyd (2002) reported that transfer of the VPI region between O1 serogroup strains could be mediated by generalized transduction with phage CP-T1. A Kn-labeled copy of the VPI was transferred, with high efficiency, from a *V. cholerae* El Tor biotype strain to four VPI-negative *V. cholerae* O1 serogroup strains via generalized transduction with phage CP-T (the entire 39.5-kb VPI region was transduced and integrated site-specifically at the same chromosomal position in the four recipient strains). These observations suggest that generalized transduction is the main mechanism for the transfer of VPI under natural conditions (O'Shea and Boyd, 2002).

8.5.1.4. *V. cholerae* CP-T1 Generalized Transduction of CTXϕ

Several studies (Faruque et al., 1998a; 1998b; Mekalanos et al., 1997; Waldor and Mekalanos, 1996) indicate that two sequential steps are essential for the evolution of pathogenic *V. cholerae* strains. The bacteria must first acquire the *tcp* operon, which encodes TCP (the receptor for CTXϕ, and they must then be lysogenized by CTXϕ (Fig. 8.12). A few *V. cholerae* O1 and non-O1 isolates that lack the *tcp* genes but contain the CTX prophage have been described (Ghosh et al., 1997; Said et al., 1995). Some authors (Faruque et al., 1998a; 1998b) have suggested that those isolates

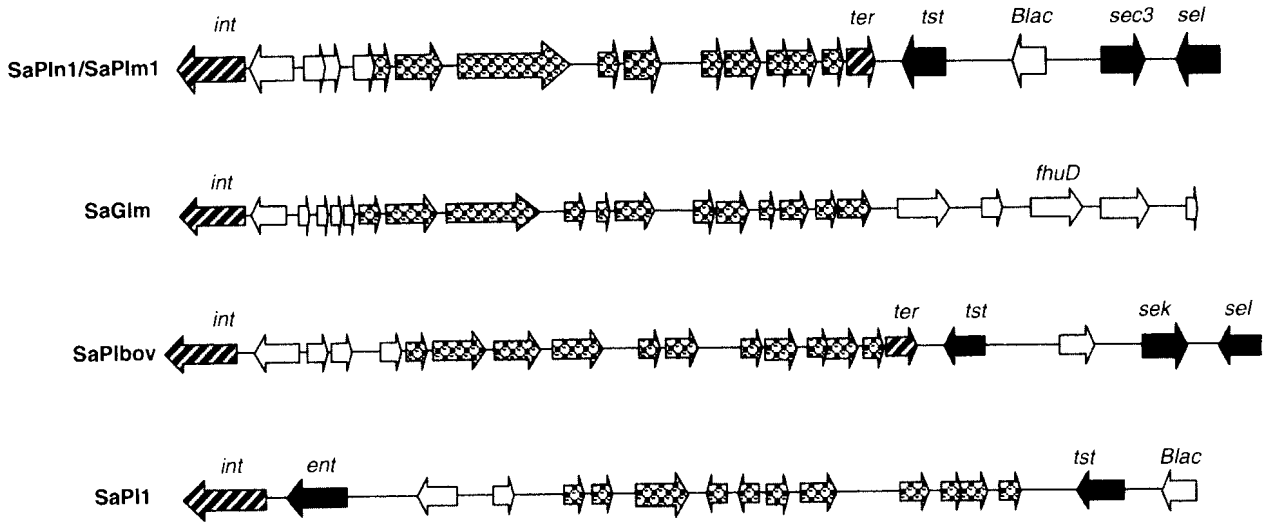

FIGURE 8.11 Genomic maps of three PAIs encoding the TSST1 toxic shock toxins of *S. aureus* (Kuroda et al., 2001). The *int* genes are represented by striped arrows, virulence genes as black arrows, and bacteriophage-like genes as dotted arrows. Genes of unknown function are indicated as white arrows. The SaGIm element does not encode any known virulence factor but it contains a central genomic region (indicated as bubbled arrows) that shares sequence identity with SaPI-1.

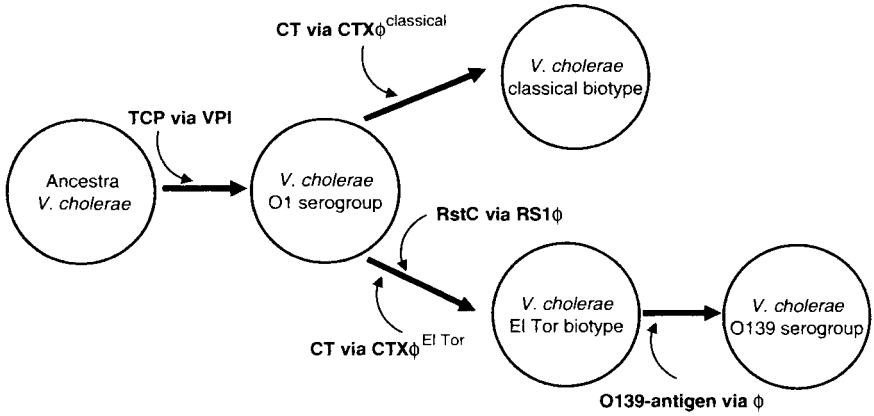

FIGURE 8.12 Evolutionary scenario for the emergence of epidemic *V. cholerae* isolates. From left to right, circles represent the core genome common to all *V. cholerae*. Points of acquisition of prophage-encoded virulence factors that define the major epidemic serogroups of *V. cholerae* are indicated by curved arrows. The model begins (on the left) with an ancestral *V. cholerae* progenitor, which gave rise to contemporary *V. cholerae* O1 serogroup isolates. The *V. cholerae* O1 progenitor first acquired VPI-1, which encodes the TCP (the CTXϕ receptor), and were then infected by CTXϕ. In addition, *V. cholerae* biotype El Tor isolates also acquired RS1ϕ. The *V. cholerae* Bengal O139 serogroup strain emerged from an El Tor strain by O-antigen switching probably mediated *via* a bacteriophage.

arose by TCP-mediated CTXϕ infection, with the subsequent loss of the TCP region. However, CP-T1 phage-mediated generalized transduction of the entire CTXϕ genome to both classical and El Tor isolates, including TCP-negative strains, has been reported (Boyd and Waldor, 1999). Interestingly, those strains also served as CTXϕ donors, even though classical biotype strains do not produce infectious CTXϕ (Boyd and Waldor, 1999; Davis et al., 2000b). The data indicate that expression of a specialized CTXϕ receptor is not always essential for converting nontoxigenic strains to toxigenic strains. Also, the fact that the genomes of most toxigenic isolates of *V. cholerae* encode both TCP and CTXϕ suggests that most of the strains did not arise as a result of generalized transduction, and it favors the model of sequential evolution of virulence.

8.5.2. Helper Phage Source of Bacteriophage Host Receptor

As stated previously, the TCP is *V. cholerae*'s receptor for CTXϕ (Taylor et al., 1987; Waldor and Mekalanos, 1996). Thus, if the VPI region encodes a phage receptor, it appears that one bacteriophage's receptor is encoded within the genome of another bacteriophage. Thus far, the finding that the VPI region encodes the receptor (and perhaps is the receptor) for CTXϕ is the only example of this type of phage-phage interaction, and this example may prove to be unique (reviewed in Lee, 1999).

8.5.3. Helper Phage Requirement for Virulence Gene Expression

8.5.3.1. RS1ϕ Encodes an Antirepressor Involved in CT Production Mediated by CTXϕ

As discussed above, the CTXϕ-related element RS1ϕ is a satellite phage whose transmission depends upon proteins produced by a helper CTX prophage. Unlike other satellite phages, RS1ϕ aids the CTX prophage, due to the RS1ϕ-encoded protein RstC. RstC is an antirepressor that counteracts the activity of the CTX repressor RstR. Therefore, RstC promotes transcription of CTX genes required for phage production and, hence, promotes transmission of both RS1ϕ and CTXϕ. In addition, RstC induces expression of *ctxAB* (which encode CT), thus contributing to *V. cholerae*'s virulence (Davis et al., 2002).

8.5.3.2. The VPI Encodes Regulatory Genes Required for CT Expression

The expression of *V. cholerae* genes in the CTX prophage and the VPI are regulated by a complex interaction of VPI genes (*toxT, tcpP,* and *tcpH*) and chromosomal genes (*toxS* and *toxR*) controlled by growth conditions and environmental signals. The three proteins ToxR, TcpP, and ToxT coordinately regulate transcription of the structural genes for CT and TCP. ToxR, a transcription activator, works synergistically with ToxS to activate expression of the VPI loci *tcpP, tcpH,* and *toxT,* and the CTXϕ loci *ctxAB* (reviewed in Davis and Waldor, 2003). ToxT amplifies its own expression and, together with TcpP and TcpH, directly activates production of CT and TCP (Davis and Waldor, 2003). Most of the experimental evidence for this virulence-regulating cascade is based on *in vitro* data. In that regard, *in vivo* studies (Lee et al., 1999) showed that the *in vivo* requirement for ToxR and TcpP, to regulate expression of CT and TCP, differs significantly from the *in vitro* requirement. An intimate interaction between VPI and CTXϕ gene expression has been demonstrated (Lee et al., 1999) using recombinase-based *in vivo* expression technology (RIVET) to monitor transcription of *ctxA* (encodes the catalytic subunit of CT) and *tcpA* (encodes the major TCP subunit) during infection in the infant mouse model of cholera. Production of TCP preceded CT production, and CT expression was induced only after increased levels of ToxR and ToxT appeared. The observed temporal progression suggests a mechanism that enables *V. cholerae* to delay CT release until the bacterium is close to host cells. The above-cited study (Lee et al., 1999) highlights the co-evolution of CTXϕ and VPI, and the importance of phage-phage interactions in the pathogenesis of cholera.

8.5.3.3. Interactions of *S. enterica* Gifsy-1 and Gifsy-2 Phages

As discussed in previous sections, the phages Gifsy-1 and Gifsy-2 encode several virulence genes essential for the pathogenesis of *Salmonella* infections (Figueroa-Bossi et al., 2001). Gifsy-2 encodes GtgB (a translocated effector protein) and SodC1, a superoxide dismutase. Curing *S. enterica* of the Gifsy-2 prophage has been reported (Figueroa-Bossi et al., 2001) to result in substantial attenuation of its mouse

virulence, but under most circumstances, Gifsy-1-cured strains were fully virulent for mice. However, another study found that Gifsy-1 lysogens that contained *sodCI*, but lacked the remainder of Gifsy-2's genes, were more virulent than were isogenic strains lacking a Gifsy-1 prophage (Figueroa-Bossi et al., 2001). Also, the authors proposed that Gifsy-1 might encode virulence factors whose effects are dependent upon *sodCI*; that is, Gifsy-1 may contribute to virulence simply by enhancing expression of SodCI. Interestingly, four potential virulence genes are found in Gifsy-1: (i) *gogB*, a type III secretion system protein, (ii) *gogD*, a *pagJ* homolog that functions to increase *Salmonella* survival after phagocytosis, (iii) *gipA*, required for *in vivo* survival of *Salmonella* in Peyer's patches (Stanley et al., 2001), and (iv) *ehly*, an enterohemolysin (Fig. 8.7). The *gogD*, *pagJ*, and *gogD* genes are integrated adjacent to the right attachment site, and *pagJ* and *gogB* are on either side of a transposase, which indicates a possible mechanism of acquisition (Fig. 8.7). The *ehly* gene is integrated downstream of the *int* and *xis* genes, the same location where a similar gene is located in *E. coli* O157 prophage 933W (Plunkett et al., 1999). An antivirulence gene (*grvA*) is also carried on Gifsy-2, and both its deletion and overexpression increases virulence in a mouse model (Ho and Slauch, 2001). The complex interplay of partial redundancy (Gifsy-1 and Gifsy-2 prophages), and the possible interactions among the virulence factor-encoding genes (*grvA* and *sodC*), complicated a study (Ho and Slauch, 2001) to determine the contribution of each factor to animal virulence. Nevertheless, the results obtained by Ho and Slauch (2001) illustrate the potentially subtle ways in which interactions between phage-encoded proteins may contribute to bacterial virulence.

8.5.3.4. *E. coli* Prophage Enhancement of *eib* Expression

Four distinct *E. coli* immunoglobulin-binding (*eib*) genes are carried by separate prophages in *E. coli* strain ECOR-9 (Sandt et al., 2002). The Eib proteins confer resistance to human serum complement, and the expression of the *eib* genes is significantly enhanced by the overlapping genes *ibrA* and *ibrB*. The IbrA and IbrB proteins are very similar to proteins encoded within a prophage-like element in *E. coli* strain Sakai, and the genome segment containing *ibrA* and *ibrB* in strain ECOR-9 contains regions homologous to the Shiga-toxin-converting prophage (Sandt et al., 2002). These observations indicate an interdependence of gene products encoded by separate bacteriophages in *E. coli*. The *ibrAB* genes were found in all strains encoding Eib, and in most strains of the B2 phylogenetic lineage, which suggests that this phage-phage interaction is not a unique event.

8.6. BACTERIOPHAGES AND PATHOGENICITY ISLANDS (PAIS)

Many pathogenic bacteria encode PAIs (Hacker and Kaper, 2000), which possess a set of common unifying characteristics; for example, they are large chromosomal regions (35–200 Kb) encoding several virulence factors that, in general, are present in pathogenic strains and are absent from nonpathogenic strains. In many cases, they also encode a phage-like integrase (e.g., P4-like integrases (Table 8.6), integrate

TABLE 8.6
Bacteriophage-Related Integrases Associated with PAI and Genetic Islands

Phage-related Integrase	tRNA	Gene	PAI	Species	Reference
ϕR73, P4	*serV*	*vap*	PI	*D. nodosus*	(Cheetham et al., 1995)
SF6, P4, ϕR73	*selC, pheU*	*eaeB, esp*	PAI III	*E. coli*	(McDaniel et al., 1995)
ϕR73	*selC*	*hly*	PAI I	*E. coli*	(Blum et al., 1994)
P4	*leuX*	*hly, prf*	PAI II	*E. coli*	(Blum et al., 1994)
P4	*pheV*	*hly, pap*	PAI IV	*E. coli*	(Swenson, 1996)
P4	*pheR*	*hly, prs*	PAI V	*E. coli*	(Swenson, 1996)
P4	*pheR*	*afa*	PAIAL862	*E. coli*	(Lalioui et al., 2001)
P4	*asnT*	*fyuA-irp*	HPI-2	*E. coli*	(Schubert et al., 1998)
CP4	*ser*	Iha, urease	SpLE1	*E. coli* O157	(Ohnishi et al., 2001)
CP4	*ser*	LEE	SpLE4	*E. coli* O157	(Ohnishi et al., 2001)
P4	*pheU*	*aer*	SHI-3	*S. boydii*	(Purdy and Payne, 2001)
P4	*selC*	*aer*	SHI-2	*S. flexneri*	(Moss et al., 1999)
P4	*selC*	*aer*	SHI-2	*S. sonnei*	(Vokes et al., 1999)
CP4-57	*leu*	NA	HiGI1	*H. influenzae*	(Chang et al., 2000)
P4	*phe*	Symbiotic genes		*M. loti*	(Sullivan and Ronson, 1998)
Mu	NA	Phage genes	Region 3	*N. meningitidis*	(Klee et al., 2000)
L54a	-	*tst*	SaPI1	*S. aureus*	(Lindsay et al., 1998)
T12, T270	-	*tst, sec, sel*	SaPIbov	*S. aureus*	(Fitzgerald et al., 2001)
ϕR73	*selC*	Aerobactin	SHI-2	*S. flexneri*	(Moss et al., 1999)
P22	NA	O-antigen	NA	*S. flexneri*	(Adhikari and Berget, 1993)
Mu-like	*ser*	*nanH*	VPI-2	*V. cholerae*	(Jermyn and Boyd, 2002)
P4	*asnT*	*fyuA-irp*	HPI-1	*Y. enterocolitica*	(Carniel et al., 1996)
CP4	*asp*	*pgm,hms*	HPI-2	*Y. pestis*	(Cheetham et al., 1995)

adjacent to tRNA genes, and are flanked by repeat sequences). The percent G+C contents of PAIs usually differ from those of the host genome, which is indicative of foreign DNA acquired from another source (Hacker and Kaper, 2000). The mechanism(s) by which PAIs are transferred from one bacterial strain to another has not been elucidated, and PAIs do not encode gene products required for self-mobilization. Among *V. cholerae* isolates, the VPI region, which encodes TCP, has been proposed to correspond to the genome of filamentous phage named VPIϕ (Karaolis et al., 1999). However, as noted above, the existence of VPIϕ is a matter of controversy, and even the authors of the original report (Karaolis et al., 1999) did not observe VPIϕ transfer to *V. cholerae* O1 serogroups strains, the predominant cause of epidemic cholera. Nonetheless, the horizontal transfer of this region among strains is not in question, and recent sequence homology studies (Boyd et al., 2000) strongly suggest that the region was recently transferred between *V. cholerae* and *V. mimicus*. In addition, O'Shea and Boyd (2002) showed that a Kn-marked copy of the VPI from several *V. cholerae* isolates could be transferred to *V. cholerae* recipient strains by the generalized transducing phage CP-T1. Another example of phage-mediated transfer of a PAI involves SaPI-1, which encodes the toxic shock toxin in *S. aureus*. Staphylococcal phage 80a is required for excision, replication, and encapsidation of SaPI-1 (Lindsay et al., 1998). The observation that generalized transduction is the preferred mode of transfer for the SaPI-1 of *S. aureus* is consistent with the idea that generalized transduction of PAIs is the most plausible mechanism of transfer in nature (Lindsay et al., 1998; O'Shea and Boyd, 2002).

Incorrectly designating PAIs as phages (and vice-versa) is an impediment to our understanding of the origins and evolution of PAIs. The VPI clearly falls into the PAI category but the SaPI-1 element does not clearly conform to PAI criteria. Since many of the characteristics of PAIs can also be attributed to bacteriophages (for example, encoding an integrase, integration adjacent to a tRNA gene, flanking by repeat sequences, aberrant GC contents), it is possible that some PAIs may have a bacteriophage origin; however, most do not.

Some PAIs that are mosaic structures containing PAI and phage characterisitics have been identified; e.g., the VPI-2 region of *V. cholerae* (Fig. 8.8) (Jermyn and Boyd, 2002). The VPI-2 region encodes a restriction modification gene cluster, a gene cluster required for amino sugar utilization, and numerous genes encoding hypothetical proteins in the 5′ region of the island. Also, a 20-kb region in the 3′ end of the island contains several genes that show homology to Mu phage. Interestingly, only the amino sugar utilization genes are present in *V. mimicus* and *V. vulnificus*, and the only VPI-2 region present in most *V. cholerae* O139 isolates is the 20-kb region containing Mu-remnants (Jermyn W.S., personal communication). An additional factor complicating the separation of bacteriophages and PAIs is the interdependence between the two elements in many bacterial species (Boyd et al., 2001). For example, the effector protein GtgB in *S. enterica* is encoded by the Gifsy-2 phage, and it is secreted by the type III secretion system encoded by the SPI-2 PAI (Figueroa-Bossi et al., 2001).

A confusing aspect of the debate regarding the relationship between bacteriophages and PAIs is the tendency of some researchers to label cryptic prophages and prophage remnants as islands. Regions that predominantly encode phage structural,

morphogenesis, and regulatory gene products should not necessarily be considered to be PAIs. The common features of PAIs and bacteriophages (e.g., they insert at tRNA sites, and they both contain a phage-like integrase) may reflect a general mode of transfer and a common integration mechanism rather than a common origin. For example, the fact that many bacteriophages integrate adjacent to tRNA genes may reflect the abundance and sequence conservation of those regions rather than some common ancestry with PAIs. Indeed, the presence of P4-like integrases within PAIs has led to the suggestion (Boyd et al., 2001) that some P2/P4 type interaction may have contributed to the integrases' acquisition and transfer. Finally, the ability of generalized transducing phages to transfer large regions of DNA in somePAIs may explain the close association between phages and PAIs.

8.7. CONCLUSIONS

This chapter focuses on the significant contributions of various bacteriophages to bacterial virulence and bacterial evolution. In recent years, our increased interest in the important roles that bacteriophages play in bacterial virulence has been driven by two very different fields of research. First, molecular microbial pathogenesis studies have discovered novel virulence factors and have elucidated their regulatory factors and mechanisms of action. Second, bacterial whole genome and comparative analyses have led to the discovery of numerous prophages within bacterial genomes (reviewed in Canchaya et al., 2003), many of which encode bacterial virulence factors. Our appreciation for the range of phage-encoded bacterial virulence factors, and the distribution and diversity of the bacteriophages involved, will increase as more bacteriophage genomes are characterized.

Two of the major themes emerging from comparative genomic analyses of both Gram-negative and Gram-positive bacteria are that prophages are quantitatively an important component of bacterial genomes, and that they are the major cause of strain differences in pathogenic isolates of various bacteria, including *E. coli*, *S. enterica* serovar Typhimurium and Typhi, *S. pyogenes*, and *S. aureus*. In addition, bacteriophages acting as vectors in the lateral transmission of bacterial virulence factors are potent factors in the emergence of new strains with pathogenic potential (Table 8.3 and Table 8.4). Although the phages' presence may potentially increase bacterial fitness, especially in certain niches such as the human intestines, the advantages or benefits of phage-encoded virulence factors for phages or bacteria are often unclear. Therefore, it is important to recognize that what we label as phage-encoded virulence factors may, in fact, have evolved for alternative and unrelated functions, and their roles in virulence may just be accidental consequences of their presence.

The advancement of knowledge concerning the roles of bacteriophages in the pathogenesis of bacterial infections also may be of significant practical importance. In that regard, the emergence of many antibiotic-resistant mutants of pathogenic bacteria recently has rekindled interest in using phages prophylactically and therapeutically in a variety of agricultural and clinical settings (for more details on this subject, refer to Chapters 13 and 14). Thus, improving our understanding of the roles of various phages and phage-encoded genetic loci in bacterial pathogenesis

can be invaluable for developing safe and effective phage preparations free of "undesirable genes" (i.e., genes encoding bacterial virulence factors; see Table 8.1). Phage preparations developed for use in agricultural and clinical settings must be free of undesirable genes, in order to prevent or limit phage-mediated emergence of new pathogenic bacterial strains.

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