BACTERIOPHAGES ENCODING BOTULINUM AND DIPHTHERIA TOXINS

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Bacteriophages have long been recognized to harbor genes that encode various classes of virulence factors, including protein toxins. The transfer of virulence genes by bacteriophages has been postulated to be a primary mechanism for the emergence of new pathogens (2, 16, 20, 39, 49, 86). Upon infection of a susceptible host, bacteriophages can persist in the host by integration into the chromosome (lysogeny) or by extrachromosomal replication in a subgroup of the host population (pseudolysogeny) (1, 7, 83). Phage conversion will persist in the bacterial population if the infection enhances the genetic fitness of the host (39, 49, 58, 79, 131). Phage conversion involves a significant change in the phenotype of a susceptible host, including the acquisition of virulence properties. Virulence is generally a polygenic trait that is integrated within the cellular physiology, including nutrition, the growth rate, stress responses, and response to host factors (140). Thus, in most cases the successful acquisition of virulence by phage conversion requires that the phage function within the biology of the host. Some classic examples of phage conversion of gram-positive hosts to toxinogenesis are the production of diphtheria toxin (DT) in *Corynebacterium diphtheriae* and the production of botulinum neurotoxins (BoNTs) in types C and D of *Clostridium botulinum*.

C. DIPHTHERIAE AND DT

Diphtheria is a classic infectious disease, and many landmark discoveries have resulted from its study (5, 11, 60, 103, 104). Diphtheria was demonstrated to be a distinct respiratory disease in the 1820s (50, 60, 62), and it was in the 1820s that *C. diphtheriae* was observed by Klebs in culture smears, while it was isolated in the 1880s by Loeffler (11, 60). During this period, *C. diphtheriae* was demonstrated by Roux and Yertsin to produce a cell-free heat-labile toxic antigen that, when injected intradermally or parenterally into guinea pigs or rabbits, caused characteristic symptoms of the disease (103). The organism was subsequently shown to produce a highly potent toxin, designated DT, with an estimated lethal dose for humans of ~0.1 μg per kg of body weight (41, 50). Evidence that DT is a protein toxin with a high

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potency was demonstrated by its filtration away from bacteria in culture fluids and particularly by its neutralization by antitoxins prepared against DT toxoids in experimental animals (7, 103). A case of diphtheria was successfully treated by the use of antitoxin by Von Behring, who received the Nobel Prize in 1901 for the introduction of antitoxin therapy to clinical medicine (60). Treatments of DT with formalin eliminated its toxicity but not its antigenicity, and the toxoid has been successfully used as a vaccine. A strain (PW8) isolated by Park and Williams in 1896 that hyperproduces DT is used for the production of toxoid for vaccines (60).

Diphtheria causes disease in many animals, including horses, cows, rabbits, birds, alligators, guinea pigs, and dogs, while other animals, such as mice, rats, and cats, are quite resistant to infection by *C. diphtheriae*. Susceptible animals can serve as vectors for transfer of the pathogen to humans (62), either directly in aerosols or by transmission in contaminated foods. Infected cows were found in the early 1900s to shed virulent diphtheria bacilli into raw milk (67), which caused numerous disease outbreaks of diphtheria in the United States and other countries. In the United States, no outbreaks of diphtheria transmitted in milk have been reported since 1948, when pasteurization was mandated within the dairy industry (67). Other species of *Corynebacterium*, such as *C. ulcerans* and *C. pseudotuberculosis* (*C. ovis*), have been associated with infections and the formation of DT-like toxins (81, 112, 117), suggesting that horizontal toxin gene transfer to normally nonpathogenic corynebacteria can occur.

Complete protection against diphtheria can be attained by surveillance and immunization programs (37, 38, 107, 138). Unfortunately, due to the lack of such programs in regions of countries faced with economic hardship, the disease has seen a resurgence in recent years. The largest outbreak in the 1990s occurred in Russia and other countries of the former Soviet Union (93), and epidemics have also taken place in Southeast Asian and South American countries (37, 38, 107, 130, 138). Tragically, diphtheria can be prevented by surveillance and immunization, yet it remains endemic in several countries (37, 118, 130, 138).

C. diphtheriae is a gram-positive non-sporeforming pleomorphic rod belonging to the actinomycete lineage of bacteria (5, 112). The pathogen generally colonizes wounds or mucosal surfaces and produces DT, which destroys host tissue (50). Extrapharyngeal infections are also known to occur (60, 117). Generally, *C. diphtheriae* can only be isolated from the throats of infected persons in the thick gray-green growth that occurs on the tonsils and mucosal pharyngeal surfaces. Although the throat is the primary colonization site of the pathogen, DT causes widespread tissue damage. Symptoms include neuritis and myocarditis, and death is generally due to myocardial failure (50, 60). Since the disease is caused by DT, antibiotics generally have little utility once the disease has progressed, but the passive administration of antitoxins and especially immunization against DT toxoid are able to control the disease (60).

The discovery of DT and its characterization have been described in several excellent reviews (11, 22, 23, 60, 103, 104, 132, 137), and only selected findings are mentioned here. DT was originally isolated and characterized in the 1930s and 1940s, and elegant studies have continued through the present (23, 60, 104). DT is a dichain molecule consisting of DTA, which is 193 amino acids long, and DTB, which is 342 amino acids long (43, 44, 92). It is among the most thoroughly studied protein toxins from biochemical and biophysical perspectives, and its investigation has provided a paradigm for many aspects of the molecular toxinogenesis of the A-B toxins (22, 23, 104, 132). These include the identification of its receptor, determinations of its structure, mechanism of internalization by endocytosis, mechanism of translocation into the cytosol of the target tissue, and the identification of its catalytic activity. A single molecule of the catalytic moiety is sufficient to kill a cell upon entry (141). The diphtheria model has provided a basis for the study of many other toxins of the A-B class.

DT has generally been considered mainly responsible for the gross symptoms of diphtheria, and the lack of genetic systems for *C. diphtheriae* has prevented the identification of other virulence factors associated with its pathogenicity (19). The recent sequencing of the genome of a clinical isolate in the United Kingdom led to the suggestion that its pathogenicity is also dependent upon adhesins, fimbrial proteins, and iron uptake systems (19). The genomic analysis of *C. diphtheriae* has suggested that certain of these virulence traits were acquired by horizontal DNA acquisition (19). This is in striking contrast to its most closely related pathogenic organism, *Mycobacterium tuberculosis*, in which the acquisition of virulence traits does not appear to have been acquired by lateral gene transfer. From the analysis of the genome of *C. diphtheriae*, it was found that the G+C mol% was not constant across the genome, and 13 regions, including the integrated corynephage β, which harbors the *tox* gene for DT, had a strong GC skew or dinucleotide bias (19). This bias was not present in the nonpathogenic corynebacteria *Corynebacterium glutamicum* and *Corynebacterium efficiens* and was postulated to reflect different environmental mutational pressures between the pathogenic and environmental groups. These GC-skewed regions were suggested to be indicative of a relatively recent acquisition of DNA. Several of these 13 regions were near tRNA genes, including the corynephage and DT genes, which were flanked by two tRNA genes. Other putative virulence genes, including those for fimbriae, metal transport systems, possible lantibiotic synthesis proteins, DNA methylases, transposases, insertion sequence elements, and sortase homologs also had the anomalous G+C mol%, GC skew, or dinucleotide frequency. Cerdeño-Tárraga et al. (19) hypothesized that compared to the nonpathogenic *Corynebacterium* spp., *C. diphtheriae* has recently acquired a sortase-related fimbrial system that may be involved in pathogenicity. These data clearly illustrate the value and future of functional genomics for the analysis of pathogenesis in organisms for which genetic tools are not yet developed.

CORYNEPHAGES AND DT

Several excellent reviews have described the genetics of toxinogenesis in *C. diphtheriae* (6, 7, 11, 60, 82), and only selected highlights are described here. *C. diphtheriae* has long been known to harbor a variety of phages, and >33 phage types have been observed (11). Phage infection was initially detected in 1918 by d'Herelle (136), who successfully showed that corynephages formed plaques on nontoxinogenic strains of *C. diphtheriae* (60, 136). Pathogenic isolates of *C. diphtheriae* were shown in the late 1940s and 1950s to harbor specific tox^+ bacteriophages (1, 33, 34, 45–47). These investigators found that nonimmune, susceptible, avirulent strains of *C. diphtheriae* could be converted to toxin production through infection by a temperate phage, later referred to as corynebacteriophage β (reviewed in references 7, 11, and 60). Other phages can also carry the *tox* gene and can bestow immunity (11, 60), but phage β is the most extensively studied corynephage. Natural isolates of *C. diphtheriae* that were resistant to phage infection were demonstrated to harbor phage β or closely related phages. From single cell isolation studies, evidence indicated that the conversion of nontoxigenic strains to their toxigenic counterparts was not due to spontaneous mutation or nonphage components in the culture filtrate (34). These early studies strongly suggested that phage conversion to toxigenicity or lysogenic conversion was caused by infection of a susceptible strain by a tox^+ phage.

Phage β has an icosahedral head and a noncontractile tail, similar to many lambdoid phages (11). Phage β contains double-stranded DNA (85), and electron microscopy analyses revealed linear and circular double-stranded DNA molecules of approximately 2.3×10^7 Da with a length of 35 kb (11). Like those of many other lysogenic phages, its lytic growth cycle is induced by mitomycin C or UV light. After a 1-h latency period following induction, a burst of 30 to 60 PFU per cell is obtained (8).

The association of DT with phages provides a classic example of lysogenic conversion, whereby the bacterial cell acquires traits as a consequence of phage infection (12, 83). This

discovery of lysogenic transduction or phage conversion occurred in 1951 (33), prior to the classic paper on lysogeny by Lwoff (83) and the demonstration of transduction in *Salmonella* by Zinder and Lederberg (12). However, the realization that the transduction involved the transfer of genetic information was not fully realized upon this discovery (12). This remarkable finding raised considerable interest from the medical community, but surprisingly, did not stimulate similar studies by notable phage biologists studying lysogenic conversion in gram-negative systems (12).

Although phage infection studies supported the hypothesis that the temperate phage β carries the DT structural gene, the conclusive piece of evidence was the isolation of phage mutants that produced nontoxigenic DT derivatives or cross-reacting materials (CRM) that reacted serologically with antibodies to active DT but were nonlethal in a guinea pig model (40, 133). The isolation of additional mutants with mutated toxin genes further supported the theory that the *tox* gene resided on the phage genome (59, 71, 135). In vitro experiments showed that a mixture of CRM45 and CRM197 relinked by a disulfide bond was toxic in vitro and in guinea pigs, suggesting that a single corynephage carried the *tox* gene (134). It was subsequently shown that DT was produced when purified phage β DNA was added to an in vitro *Escherichia coli* protein synthesizing extract (71, 78, 92). In certain *C. diphtheriae* strains, DT was produced only when phage β was present as a prophage, while in other strains, DT was also produced by phage β as a nonreplicating exogenous element in a host with homologous lysogenic immunity (42, 60, 84, 85). These observations showed that the *tox* gene is not coordinately regulated by genes responsible only for the growth or lysogeny of phage β (60).

Holmes and Barksdale (61) developed a mating system utilizing extrachromosomal vegetative corynephages. The development of a genetic system for the analysis of recombination between tox^+ phage β and tox^- phage β showed that the recombined *tox* markers segregated as alleles at a distinct locus. This system enabled the identification and mapping of genes in the corynephage genome, including *h* (host range), *imm* (lysogenic immunity), *tox*, *c* (clear plaque), and *h′* (extended host range). Further definition of the gene locations and functions was achieved by the use of temperature-sensitive mutants of virulent phage β (11). Laird and Groman (76, 77) isolated monolysogenic recombinants from heteroimmune double lysogens and showed that the prophage map is a circular permutation of the vegetative map (11). This led to the observation that phage β has cohesive ends (*cos*) that circularize the DNA, allowing either vegetative extrachromosomal replication or integration into the host chromosome by the Campbell model (15). Fine-structure mapping confirmed that the prophage map of β was circularly permuted and that *tox* occupies a central location in the vegetative map but a terminal location at one end of the prophage map, adjacent to the phage attachment site (*attP*) (11, 59, 60, 76). Two functionally equivalent *attB* sites are located within an Arg-tRNA$_2$ present at two chromosomal locations (110, 111). Integration of the prophage genomic DNA (34 to 36 kb) occurs at these two equivalent *attB* sites (110, 111).

The *tox* gene is located immediately upstream of the phage DNA integration site (*attP*) (111). The orientation of the *tox* gene is such that the amino terminus of the encoded toxin is directed inwards and the carboxyl terminus is located near the *attP* site, with transcription proceeding towards the terminus. The availability of the CRM mutants showed that the ADP-ribosyltransferase enzymatic portion of the toxin resides at the amino terminus of the toxin. The bacterial (*attB*) and corynephage (*attP*) sites for lysogenization have a homologous core sequence (110, 111). Site-specific recombination between a toxinogenic corynephage and a nontoxinogenic *C. diphtheriae* strain yielded a virulent strain in which the *tox* gene was expressed from a phage promoter. The control of expression was shown to be dependent on bacterial control elements, as described below. Isolation of the phage β genome facilitated the construction of detailed restriction maps and sequence analyses (11). *tox* alleles were also cloned

and sequenced from *tox*$^+$ corynephages (11, 13, 60). The cloned *tox* gene was heterologously expressed in *E. coli*, allowing analyses of structure and function by random and site-directed mutagenesis (44, 71, 92). The structures of DT and its mutant derivatives, as well as cellular mechanisms of toxicity in eukaryotic systems, have been extensively studied from biochemical and structural perspectives (22, 23, 104).

In retrospect, the elucidation of phage conversion and toxinogenicity in *C. diphtheriae* was facilitated by the development of certain genetic tools, including the isolation of plaque-susceptible indicator strains (8, 21, 34, 72), the availability of a phage mating system (6, 42, 61, 85), the isolation of corynephage mutants and CRMs (40, 133, 134), the development of methods for the analysis of nucleic acids, the cloning and heterologous expression of the phage β genome (43, 44, 91), molecular genetic studies (44, 71, 78, 110), and recently, the availability of the genome sequence (19). Excellent reviews describe aspects of these developments in the discovery of lysogenic conversion in *C. diphtheriae* (4, 11, 12, 60, 103).

Like those of many toxin genes, the function of DT in the host cell is not known. The *tox* gene is stable in phage β, but it does not appear to bestow any selective advantage on the bacterial host (5). Pappenheimer (103) proposed that the function of the toxin could be learned if it was studied in relation to the physiology of *C. diphtheriae*, and nutritional studies provided evidence of its function. DT production was known for many years to be affected by the culture medium and method of fermentation (90, 91, 105). The most-studied factor affecting the expression of DT is the level of iron in the medium, and inorganic iron or heme present at moderate levels in the medium decreases expression. This regulation was demonstrated to be due to genetic determinants on the bacterial genome and not the phage genome (60). Extensive molecular studies have led to the characterization of the DT repressor (DtxR) and the mechanism of repression by iron (48, 60, 126, 139). These studies have also contributed to the discovery of a unique class of metal-dependent virulence factors (called IdeR) in different species of bacteria (60).

Since the *tox* gene resulting in DT gene expression and associated necrotic lesions is expressed only when iron levels drop to a low level, one function of the *tox* gene may be to act as a specific nutrient acquisition factor for scavenging of iron by the bacterium (103). Thus, the corynephage genome contains at least one gene that is advantageous for *C. diphtheriae*, and stable integration and propagation of the phage would provide a selective advantage to the organism. The need for iron limitation can also be thought of in terms of the host response, for which the toxin essentially serves as a biosynthetic and survival mechanism by making iron available to the bacterium. Phage genomes can undergo mutation, deletion, and transposition events and evolve into constituent parts of their hosts' genomes (111).

BoNT AND PSEUDOLYSOGENY IN *C. BOTULINUM* TYPES C AND D

C. botulinum has attracted much interest in recent years, largely due to advances in the study of the structure, function, and genetics of the neurotoxins it encodes (87, 113, 116, 120) and in the therapeutic applications of these toxins for the treatment of several neurologic disorders (69, 115). *C. botulinum* is an important pathogen of humans and animals, causing serious food-borne outbreaks, intestinal toxemias, and extensive outbreaks in wild and domestic animals (68, 119).

The clostridia produce more protein toxins than any other bacterial genus (52, 137), and pathogenic clostridia are usually identified on the basis of the characteristic toxins that they produce. More than 20 toxins, as well as other extracellular proteins contributing to virulence, such as spreading factors and proteolytic enzymes, have been identified in *Clostridium* spp. (52, 137). Two of the toxins, the botulinum and tetanus neurotoxins, are the most potent poisons known, as the lethal doses of BoNT and tetanus neurotoxin (TeNT) for various animals are ≤1 ng per kg by intravenous injection (41, 115) and the lethal human oral dose for BoNT type A is

estimated to be 10 to 70 μg for an ~70-kg adult based on primate feeding studies (3, 115). The oral toxin lethal dose would be considerably lower for a baby or young child. There is considerable interest in developing countermeasures and treatments for botulinum neurotoxins, but currently only passive administration of antitoxin and immunization are used (3).

BoNTs and TeNT act by selectively blocking the neurotransmission of presynaptic nerve terminals in the peripheral and central nervous systems (113, 116). Once the toxins bind to nerve endings and the internalization process begins, it is currently not possible to treat botulism. Intoxicated individuals are kept alive by supportive care, including intragastric feeding and artificial respiration in an intensive care unit. One of the hallmarks of botulism is the long duration of action, which is often several months, depending on the serotype, and thus a large botulism outbreak could be a devastating event (3).

C. botulinum is an extremely heterogenous species from phylogenetic and metabolic perspectives (52, 68), and the "species" actually comprises several genera of bacteria that are linked and currently classified into one species based on their ability to produce a characteristic neurotoxin with extraordinary potency (Table 1). *C. botulinum* produces seven serologically distinguishable BoNTs (A to G) as well as a binary cytotoxin (C_2) and an ADP-ribosylating toxin (C_3) (87, 115, 116, 120). The BoNTs of *C. botulinum* are produced as protein complexes consisting of various combinations of BoNT, hemagglutinin, nontoxic nonhemagglutinin (NTNH), and possibly other proteins (87, 115, 120). With the exception of types C and D, strains of *C. botulinum* generally only produce one toxin type. Rare strains have been demonstrated to produce two toxin types, with one type being produced at a higher titer than the other (32, 52, 68, 120). Studies have indicated that many type A strains possess the gene or gene remnant for BoNT type B (32, 52, 63). Additionally, strains of normally nonpathogenic *Clostridium butyricum* and *Clostridium baratii* have been isolated that produce type E and F BoNTs, respectively (52). The surprisingly high frequency of more than a single BoNT toxin gene cluster on the chromosome, as well as the isolation of new neurotoxigenic clostridial species, supports the hypothesis that certain BoNT genes may be associated with transferable genetic elements and may have a biological function other than killing and colonization of eukaryotic prey.

A greater understanding of the organism's basic biology will likely result from genomic analysis, and a fully annotated genome sequence for *C. botulinum* ATCC 3502 will soon

TABLE 1 Properties of neurotoxigenic clostridia[a]

Species	Group	Toxin type	Proteolysis	Glucose	Lipase	Related species
C. botulinum	I	A	+	+	+	*C. sporogenes*
		B	+	+	+	*C. sporogenes*
		F	+	+	+	Not known
C. botulinum	II	B	−	+	+	Not known
		E	−	+	+	"E-like" *Clostridium* sp.
		F	−	+	+	Not known
C. botulinum	III	C	±	−	−	*C. novyi*
		D	±	−	−	*C. novyi*
C. argentinense (nontoxinogenic)		G	+	−	−	*C. argentinense*
C. butyricum (nontoxinogenic)		E	−	−	−	*C. butyricum*
C. baratii (nontoxinogenic)		F	−	+	−	*C. baratii*
C. tetani		TeNT	−	+	−	*C. tetanomorphum*

[a]Adapted from references 52 and 87 with permission of the publishers.

be available from the Sanger Centre. This information will facilitate genomic studies for defining gene expression and potentially the functions of BoNT and other gene products in vitro and in vivo. However, the study of *C. botulinum* is seriously compromised at present by the lack of genetic tools to manipulate the genome, particularly the absence of gene replacement and knockout methods to facilitate the understanding of gene functions and the biology of the pathogen (87). There are currently no effective clostridial integration vectors and transposons for mutational studies with *C. botulinum*. This inability to easily generate stable mutants (directed or random) severely hinders the ability of the scientific community at large to fully exploit clostridial genome information to better understand and control *C. botulinum* in foods and as an animal and human pathogen.

The characterization of genes for BoNT and associated proteins of the toxin complexes indicated that these genes are located on the chromosome, on pseudolysogenic bacteriophages, or on plasmids, depending on the serotype (Table 1) (52, 80, 87, 115). The chromosomal location of the gene for BoNT/A in *C. botulinum* type A has been conclusively identified by the isolation of Tn*916* mutants (in 1995) (70, 80) and by the genome sequencing project. Evidence from cloning and sequencing studies suggests that the genes for BoNT/B (groups I and II), BoNT/E, and BoNT/F (groups I and II) are also likely located on the chromosome. The BoNT genes for *C. botulinum* types C and D are present on pseudolysogenic bacteriophages, as discussed below, while the genes for the BoNT/G toxin complex are carried by a very large plasmid (31, 143). The genetic locations for *C. butyricum* and *C. baratii* producing BoNT/E and BoNT/F are not known, although two independent studies indicated that the BoNT/E gene in *C. butyricum* may be present on a phage (142) or on a plasmid (54).

Nearly all strains of *Clostridium* spp. examined have been shown to harbor bacteriophages (10, 24–26, 51, 64, 96, 108, 109, 121). Most phages isolated from toxigenic clostridia have been reported to have a double-stranded DNA genome with a low G+C mol% (~24 to 30%), which is typical of toxin-producing clostridia (52). The low G+C mol%'s are among the lowest reported for phages (96). Most phages from *C. botulinum*, *Clostridium novyi*, *Clostridium sporogenes*, and *Clostridium tetani* that have been observed microscopically have a hexagonal head, distinct capsomere structures, and a tail with a contractile sheath (10, 24–26, 64, 96). The genome of the phage F1 in *C. sporogenes* was reported to be 33 kb long (127, 128). Two inducible phages from *C. botulinum* type A strain 190L were observed to be infectious in *C. botulinum* strain Hall A (74, 125). These phages contained double-stranded DNAs with molecular sizes of 31.9 and 23.5 kb, as estimated by their sedimentation rates (14, 74). Since two nontoxigenic mutants of *C. botulinum* 190L still produced these two phages, a lysogenic conversion to toxinogenicity seemed unlikely for this *C. botulinum* type A strain.

Despite their prevalence, clostridial phages are very poorly characterized, and little structural, genomic, or molecular information is available for these phages (144). None of the genomes from phages infecting *C. botulinum* have been isolated and characterized, and infection and mating systems are also not available except for types C and D. Only one *Clostridium* phage, from *Clostridium perfringens*, has been thoroughly characterized (144). Two temperate bacteriophages were isolated from *C. perfringens*. The genome sequence of the phage ϕ3626 was determined, providing the first complete nucleotide sequence of a bacteriophage infecting a *Clostridium* sp. The bacteriophage was from the *Siphoviridae* family of tailed phages in the order *Caudobirales* (144). Its genome consisted of a linear double-stranded DNA molecule of 33,507 nucleotides. The genome had 3′ protruding single-stranded cohesive ends of nine nucleotides. The average G+C content was 28.4 mol%, which was slightly higher than the 24 to 27 mol% reported for the host but very similar to the 28.6 mol% reported for *C. perfringens* strain 13, whose genome sequence is available.

Fifty open reading frames were identified in the ϕ3626 genome, and these were organized into three life cycle-specific gene clusters. Gene products were assigned to 19 genes, including

genes putatively involved in lysogeny, replication, recombination, and modification of phage DNA, packaging, and a dual lysis system consisting of a holin (Hol) and an endolysin (Ply). Interestingly, a sigma factor-like gene (*C. perfringens* sigma K) was identified; it was postulated to integrate into a *guaA* homolog and to affect sporulation, analogous to certain other endospore formers. The sigma factor also potentially induced genes whose products could increase heat resistance. The ϕ3626 attachment site *attP* resided in a noncoding region immediately downstream of *int*. Overall, similarities in the protein complement were found with phages from other low-G+C-content gram-positive bacterial genera, including *Bacillus, Staphylococcus, Lactococcus, Listeria*, and *Lactobacillus*, and also, to a limited degree, with phages from *E. coli* and *Pseudomonas* (144). It was noted that the order of the genes encoding the terminases, portal, prohead protease, and major head protein were analogous to phages from low-G+C-content lactic acid bacteria and lambdoid phages in general. Since the relatedness of other phages from low-G+C gram-positive bacteria was observed at the protein level but not at the nucleic acid level, it was concluded that the evolution of phage ϕ3626 did not involve a recent horizontal transfer and that the mosaicism observed at the nucleic acid level of clostridial phages suggested considerable recombination within the phage gene pool (57, 58). No association of the phage with toxigenicity or pathogenicity was noted for phage ϕ3626 from *C. perfringens*.

BACTERIOPHAGES AND TOXIGENICITY IN *C. BOTULINUM* TYPES C AND D

Phages and their relationship to toxinogenicity in *C. botulinum* serotypes C and D have been extensively investigated compared to other serotypes (26). With *C. botulinum* types C and D, Inoue and Iida (65, 66) and Eklund et al. (27, 28) initially demonstrated a relationship between toxigenicity and pseudolysogeny in specific strains. Nontoxigenic mutants were readily isolated after treatments of vegetative cells with mitomycin C, and the derivative strains appeared to be nonlysogenic by further phage induction experiments (26) (Fig. 1). Phage conversion to toxigenicity in *C. botulinum* types C and D was obtained by infections of nonlysogenic strains by phage lysates prepared from toxigenic strains (27, 28, 65, 66, 114). Toxin conversion by the filtrate was not prevented by a treatment with trypsin or DNase, and converted strains stably gained the ability to produce toxin, supporting a lysogenic conversion. Eklund et al. (29) also showed that *C. botulinum* types C and D could be interconverted to produce the opposite toxin from that of the original strain. A *C. botulinum* type C isolate that was cured of phage and nontoxinogenic could be infected with lysates of type D, with subsequent production of the type D neurotoxin (29). Thus, the tox^+ phages from *C. botulinum* types C and D have a rather broad host range and can interconvert type C to D or vice versa. Infections of *C. botulinum* types C and D with phages induced from the closely related organism *C. novyi* enabled the recipients to produce the lethal alpha toxin of *C. novyi* (26). *C. novyi* could also be infected with lysates from *C. botulinum* types C and D, with resultant BoNT/C or BoNT/D formation (Fig. 1).

Shortly after phage conversion to BoNT production for *C. botulinum* types C and D was reported, the C_2 toxin (cytotoxin) was demonstrated in culture filtrates of *C. botulinum* type C (26, 106), but the gene encoding the C_2 toxin did not appear to be associated with specific bacteriophages (26). Similar procedures demonstrated that the genes for the hemagglutinin of the toxin complex (98, 115) and the C_3 exoenzyme (26, 55, 106) of *C. botulinum* types C and D are located on pseudolysogenic phages (26, 101, 106). Hemagglutinin production can be transmitted separately or concomitantly with toxin production (101). It is not clear if the gene encoding the NTNH protein present in all BoNT toxin complexes (98, 115) is harbored on pseudolysogenic phages in *C. botulinum* types C and D.

Converting and nonconverting phages from *C. botulinum* types C and D have been isolated and partially characterized. Depending on the source strain, they vary considerably in their antigenicities, host specificities, and stabilities of toxin production (97, 99, 100, 102, 122–124).

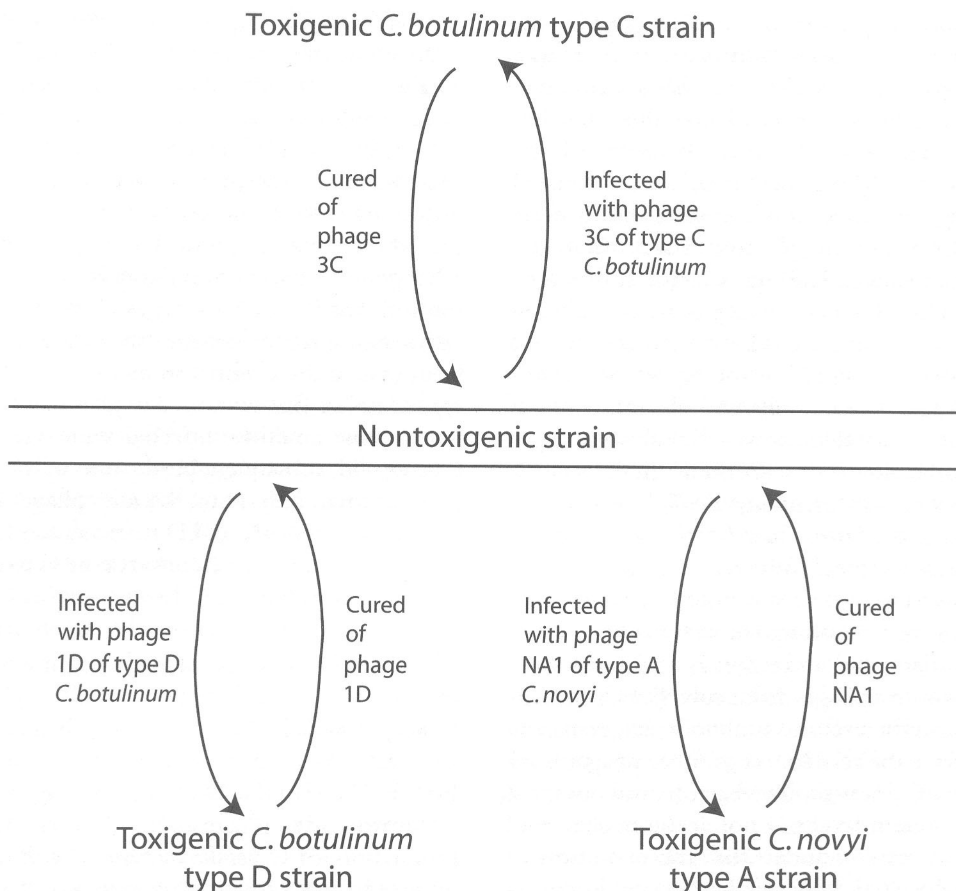

FIGURE 1 Relationships of bacteriophages and toxins produced by *C. botulinum* types C and D. Adapted from reference 26 with permission of the publisher.

Biological and biophysical characteristics of phages isolated from *C. botulinum* type C and D strains have been studied by Inoue and coworkers (122–124). The G+C mol% of the phages ranged from 26 to 29%, which is similar to that of genomic DNAs of *C. botulinum* types C and D. The nucleotide bases in the phage DNAs did not appear to be methylated. The converting phages were more susceptible than the nonconverting phages to physical and chemical inactivation by temperature, pH, UV irradiation, and organic solvents. The genome sizes of the converting and nonconverting phages were estimated to be approximately 110 kb for two converting phages and approximately 65 kb for two nonconverting phages. The DNA homologies estimated by dot blots were estimated to be 50 to 75% for the converting phages and 100% for the nonconverting phages. Converting and nonconverting phages did not show DNA-DNA hybridization (123).

The characteristics and life cycle of the *C. botulinum tox*$^{+}$ phages have not been elucidated in detail at the molecular level, but the phages harboring genes of the BoNT toxin complex have been reported to be pseudolysogenic for their host (26). Pseudolysogenic phages (1, 83) have an unstable relationship with the chromosome, and the phage genome has been suggested not to integrate into the chromosome and to reside in the cytoplasm for most or all of its life cycle. In these pseudolysogenic or carrier strains (1,

83), phage propagation involves an equilibrium within the bacterial population between resistant and sensitive cells, with the latter being susceptible to infection by free phage (1). The pseudolysogenic phage relationship probably contributes to the instability of toxin production and consequent low titers in many strains (97). For highly toxigenic strains of *C. botulinum* type C, it was reported that the phage-bacterium relationship constitutes a stable lysogenic type of interaction (51). In the pseudolysogenic life cycle, phages and host strains undergo repeated cycles of curing and reinfection in their natural habitat (1, 83). During these growth cycles, double lysogenization and recombination of phage genomes probably occur in the host strains (122). In these double lysogens, considerable recombination and subsequent diversification of the phage genome would be expected to occur, particularly in nonessential genes such as those coding for BoNT and other proteins of the toxin complex (98, 115, 120).

Analyses of the genes for BoNT/C and BoNT/D have shown that the predicted amino acid sequences vary considerably compared to lineages of other *C. botulinum* serotypes (36, 53, 56, 73, 88, 123). The BoNT from a *C. botulinum* type C strain was shown to have a mosaic structure consisting of a type C light chain and a type D heavy chain (89, 98, 123). Thus, both conserved sequences and mosaic-like sequences have been observed in various *C. botulinum* strains of serotypes C and D. Phage recombination may also explain the heterogeneity of the NTNH genes present in the toxin complexes of all seven serotypes of *C. botulinum*, but an association of phage with NTNH has not been shown. The transfer of Tn*916* into *C. botulinum* type A has been associated with large deletions (~31 kb), including the loss of the entire BoNT toxin gene cluster (70, 80). Such a large deletion event may result from phage induction and recombination events, but this has not been demonstrated.

Although phages are abundant in *C. botulinum* types A, B, E, F, and G, phage involvement in toxin production in these strains has not been demonstrated (26). The evidence that phages, plasmids, or other extrachromosomal elements are not involved in toxinogenicity is indirect and based on continued toxin production in strains that have been cured of phages and plasmids after treatments with such agents as UV light, mitomycin C, and acridine orange. Experiments to obtain plaque formation have been attempted with *C. botulinum* serotypes other than C and D, but these have generally been unsuccessful, hindering investigations of a possible association between phages and toxinogenicity in these types. Phage remnants have commonly been observed in cultures of neurotoxigenic clostridia, particularly after induction by stress agents (mitomycin C, UV light, and acridine orange) (24–26, 108). Certain of these phage remnants in the culture medium have been associated with a bacteriocin-like activity (94, 96) resembling that of pyocins from *Pseudomonas*.

The isolation of neurotoxigenic *C. butyricum* and *C. baratii* strains (52, 56) supports the hypothesis that genes of the toxin complex have been transferred to these normally nontoxigenic clostridia. Hauser et al. (53, 54) reported that a large extrachromosomal plasmid was present in a toxigenic *C. butyricum* strain. When plasmid or chromosomal DNA was used as a template for PCRs with primers corresponding to DNA sequences at the 5′ and 3′ termini of the BoNT/E light chain, amplification products were obtained from both plasmid (strong signal) and chromosomal (weaker signal) DNAs. The chromosomal amplification product was attributed to contamination with plasmid DNA. The isolated plasmid was not reported to react directly with a toxin gene probe. These results are suggestive, but not conclusive, of a plasmid localization of the type E BoNT gene, since PCR amplification was required to detect the presence of the toxin gene. Zhou et al. (142) reported that a toxin gene probe hybridized directly to chromosomal DNAs of toxigenic strains of *C. butyricum* and to *C. botulinum* type E, but not to plasmid DNA. All toxigenic and most nontoxigenic strains were lysogenized by a prophage on the chromosome. Prophages of toxigenic strains, irrespective of the host species, had related or identical DNA sequences which differed from

the genomes of prophages found in nontoxigenic strains. The prophages were located close to the toxin gene on the chromosome. DNAs from phages induced by mitomycin C and purified by cesium chloride density centrifugation did not hybridize with the toxin gene probe. These results suggested that a mixed population of phages was induced and that the phage carrying the toxin gene was present at a low level and was undetectable by direct hybridization. Phage DNA, but not chromosomal DNA, purified from toxinogenic strains served as a PCR template to amplify the toxin gene. This amplification was not negated by first incubating the phage particles with DNase and RNase, indicating that the toxin gene was sheltered in the phage particle (142).

These findings supported the occurrence of natural interspecies transfer of the BoNT/E gene from *C. botulinum* to *C. butyricum*. Interspecies transfer of the toxin gene could not be obtained in the laboratory when *C. botulinum* type E or phage lysates from type E were used as a gene donor. Interspecies transfer of the toxin gene did occur when a nontoxigenic *C. botulinum* type E-like strain was used as a recipient and phage lysates induced from toxigenic *C. butyricum*, together with phage lysates from nontoxigenic *C. butyricum*, were used as gene donors. The toxin gene was detected in about 1 in 10^4 colonies obtained by plating the recipient strain. Hence, the gene for BoNT/E appears to be associated with a prophage, and transfer to the nontoxigenic strain resulted from transduction by a defective phage which required a helper phage for infectivity (142).

The natural transfer of the BoNT toxin genes to nontoxinogenic clostridia is also supported by the isolation of *C. baratii* strains that produce BoNT/F (52). BoNT gene transfer to *C. baratii* appears to have occurred independently at different times, since toxigenic *C. baratii* strains have been isolated from humans with intestinal botulism in distinct geographic locations within the United States (52). The natural interspecies transfer of toxin genes may be significant for the food industry and for medical microbiology. However, an understanding of the mechanisms of toxin gene transfer is hindered by our rudimentary knowledge of molecular genetics and gene transfer in *C. botulinum* and by the lack of a positive genetic selection marker for the BoNT phenotype.

The study of phage conversion and toxigenicity in *C. botulinum*, particularly in types A, B, E, F, and G, is very rudimentary at present, and its development has been hindered by the lack of certain methodologies, including the isolation of indicator strains, phage mating systems, the cloning and expression of clostridial DNAs, and genetic tools. Indicator strains that are susceptible to infection and that generate plaques are not available for most serotypes of *C. botulinum* other than types C and D. The use of the double-layer method (1) has been of limited utility for plaque formation on clostridia due to their anaerobic requirements and production of gases. For types C and D, it has been possible to obtain turbid plaques on solid media with recipient strains by the agar layer method (1). Other approaches, such as the microplaque method, are needed to detect phage infections in *C. botulinum* (72). Current evidence, including multiple toxin production by certain isolates, genotypic analyses showing multiple gene clusters in single strains, and the isolation of toxinogenic strains of *C. butyricum* and *C. baratii*, supports the occurrence of natural transfer of the BoNT genes. However, genetic tools and developed phage systems are needed to more fully investigate these phenomena.

BACTERIOPHAGES AND EVOLUTION OF TOXIGENICITY

Genetic analyses of many genera of bacteria have indicated that bacteriophages, transposons, and other transferable elements comprise a prominent proportion of genomes (17, 18, 57). Pathogenic lineages arose from nonpathogenic bacteria by various mechanisms, including lysogenic conversion by temperate bacteriophages and the transfer of plasmids and pathogenicity islands (7, 9, 16, 20, 49, 58, 79, 86, 95, 111, 129). The horizontal transfer of sequences mediated by bacteriophages has been called "rampant" (57) and is mediated by both homologous and nonhomologous recombination (57). It is in-

triguing that the acquisition of pathogenicity islands from plasmids or toxinogenic phages (49) often occurs within tRNA genes. Ratti et al. (111) demonstrated that toxinogenic corynephages also occur within a $tRNA_2^{Arg}$ sequence, supporting a common theme in the evolution of pathogens. The transfer of bacterial virulence factors on bacteriophages has repeatedly been demonstrated for bacteria of distant evolutionary lineages. The integration of phages resulting in lysogenic conversion occurs at *att* sites and is often associated with tRNA genes due to the specificity of phage-derived integrase genes (20). Bacteriophage survival depends on mechanisms for avoiding nucleic acid restriction systems of the hosts (57, 75). The presence of virulence factors on bacteriophages may allow the infected bacterium to amplify its host range and to increase its fitness in environmental niches (9, 16, 35, 49, 58, 79, 86). The locations of bacterial virulence determinants on the lysogenic coliphage lambda have been demonstrated to increase fitness by providing direct protection from host immune defenses (9, 86). As predicted (9), phage-mediated adaptive evolution has developed into a general theme, and it is now clear that phages as agents of lateral gene transfer have had a major impact on microbial evolution (2, 58, 79). It is intriguing that double-stranded DNA tailed phages from a wide diversity of host bacteria share a common ancestry and that all double-stranded DNA tailed phages may actually comprise a large global genetic pool facilitating the wide-ranging exchange of genomic information (58, 79).

In consideration of this theme, it is apparent that the evolution of gram-positive toxinogenic pathogens has depended extensively on bacteriophage infections and exchanges of genetic information. Some of the most poisonous toxins, including DT and BoNTs, have evolved and been dispersed in association with phages. However, due to a lack of genetic and phage systems, the surface has barely been scratched with regards to understanding the mechanisms of toxin gene transfer, particularly for the genus *Clostridium*. It is anticipated that this situation will advance with the development of genetic tools and genomic information for the gram-positive toxinogenic bacteria combined with advances in our knowledge of phage molecular biology and evolution.

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