

Chapter 2

Phage Conversion and the Role of Bacteriophage and Host Functions in Regulation of Diphtheria Toxin Production by *Corynebacterium diphtheriae*

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Abstract *Corynebacterium diphtheriae* is the etiologic agent of diphtheria. Toxinogenic isolates of *C. diphtheriae* produce diphtheria toxin, a protein that inhibits protein synthesis in susceptible eukaryotic cells, whereas nontoxinogenic isolates of *C. diphtheriae* do not produce diphtheria toxin. The characteristic local and systemic manifestations of diphtheria are caused by diphtheria toxin. The toxinogenic phenotype of *C. diphtheriae* is determined by temperate corynephages whose genomes carry the *tox* gene that encodes diphtheria toxin. Toxinogenesis in *C. diphtheriae* is a paradigm for phage conversion, defined as a change in the phenotype of a bacterial host resulting from infection by a bacteriophage. In *C. diphtheriae*, transcription of the phage gene *tox* is negatively regulated by the diphtheria toxin repressor (DtxR), a corynebacterial metalloregulatory protein that requires intracellular Fe^{2+} as a cofactor under physiological conditions. This repressor is the master global regulator of iron-dependent gene expression in *C. diphtheriae*, and it controls intracellular iron homeostasis in *C. diphtheriae* by repressing under high-iron growth conditions and derepressing under low-iron growth conditions the transcription of genes that are essential for function of its multiple iron-acquisition systems. Production of diphtheria toxin by *C. diphtheriae*, therefore, reflects complex interactions between the *tox* operon on a coryneophage, the bacterial regulatory protein DtxR, and the intracellular Fe^{2+} level which controls activity of DtxR and is, in turn, determined both by bioavailability of iron in the extracellular environment and activity of multiple DtxR-regulated systems that contribute to iron assimilation by *C. diphtheriae*.

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2.1 Ubiquitous Bacteriophages and Their Roles in Evolution of Bacterial Genomes

Bacteriophages, also known as phages, are viruses that infect bacteria. Since their discovery by Twort (1915) and d'Herelle (1917), bacteriophages have been shown to represent the most diverse and abundant microbial entity in the biosphere, having an estimated magnitude of 10^{31} viral particles (Wommack and Colwell 2000). They can be found in a multitude of locations, from the oceans (Suttle 2005, 2007) to animal guts (Breitbart et al. 2003, 2008). Not surprisingly, because of their prevalence, bacteriophages have been shown to play an important role in microbial evolution. Through horizontal transfer of genetic material via transduction or lysogeny, bacteriophages contribute to overall fitness, adaptation to new environments, or pathogenicity of the recipient bacteria. Most bacteriophages are classified as either lytic (virulent) or temperate (Guttman et al. 2005). Replication by lytic phages results in lysis of their bacterial hosts at the end of phage replication cycles. In contrast, temperate phages can replicate either lytically or by integrating their genomes as prophages and replicating as part of their host's chromosomes. Bacteria that contain prophages are referred to as lysogens.

In recent years, investigation of the presence of prophages and the overall impact of bacteriophages on bacterial genome evolution has skyrocketed. Evaluation of genomes from gamma-proteobacteria and (G+C)-rich Gram-positive bacteria revealed that two-thirds of the genomes harbor prophages (Canchaya et al. 2003; Casjens 2003). Pangenomic studies showed that prophage genes comprise approximately 13.5 % of *Escherichia coli* and 5 % of *Salmonella* genomes (Bobay et al. 2013; Touchon et al. 2009). Furthermore, it is estimated that the global rate at which phages influence genetic composition in bacteria is through approximately 20×10^{15} gene transfer events per second (Bushman 2002). Many temperate phages integrate into bacterial chromosomes at transfer RNA (tRNA) genes, and either the integration event regenerates the tRNA coding sequence (Campbell 1992) or a phage-encoded tRNA complements the inactivated bacterial tRNA gene (Ventura et al. 2003). However, other temperate phages can integrate at non-tRNA sites on bacterial chromosomes and can inactivate genes located at their insertion sites, which may or may not have functional consequences (Coleman et al. 1991; Goh et al. 2007; Lee and Iandolo 1986).

Prophages are major contributors to genetic diversity in bacteria and through phage conversion contribute to virulence of numerous pathogens, including *Vibrio cholerae* (Boyd et al. 2000a, b; Boyd and Waldor 1999; Davis et al. 2000; Mekalanos et al. 1997; Waldor and Mekalanos 1994, 1996), *Escherichia coli* (Mead and Griffin 1998; Ohnishi et al. 1999, 2001, 2002; Hayashi et al. 2001; Yokoyama et al. 2000), *Salmonella enterica* (Cooke et al. 2007; Figueroa-Bossi et al. 2001; Hermans et al. 2005, 2006; Thomson et al. 2004), *Streptococcus pyogenes* (Aziz et al. 2005; Banks et al. 2002; Cleary et al. 1998), *Staphylococcus aureus* (Baba et al. 2008; Bae et al. 2006; Goerke et al. 2009; Rahimi et al. 2012), and *Corynebacterium diphtheriae* (Freeman and Morse 1952; Trost et al. 2012).

While many of the phage-encoded factors that contribute to the virulence of these pathogens were shown to be toxins, numerous other phage-encoded virulence determinants have also been identified including, but not limited to, hydrolytic enzymes, antibiotic resistance determinants, superantigens, adhesins, serum resistance factors, detoxifying enzymes, LPS-modifying enzymes, mitogenic factors, and type III effector proteins (Boyd 2012; Brussow et al. 2004; Fortier and Sekulovic 2013). Although phage conversion contributes to the virulence of many pathogens on multiple levels, we will restrict our discussion to several examples of phage-encoded toxins that have been well characterized.

2.2 Phage Conversion and Toxinogenicity in Medically Important Bacterial Pathogens

In this section, we will briefly review a few medically important pathogens whose virulence is enhanced by production of phage-encoded toxins, including *V. cholerae*, Shiga toxin-producing *E. coli*, and *Clostridium botulinum*. The production of phage-encoded diphtheria toxin by *C. diphtheriae* will be discussed in detail in later sections.

While there are over 200 serogroups of *V. cholerae*, the two serogroups O1 and O139 are the causative agents of Asiatic cholera, a gastrointestinal disease characterized by profuse watery diarrhea and severe dehydration that can progress rapidly to death (Faruque 2014; Faruque et al. 1998; Kaper et al. 1995). The principal virulence factor for these serogroups of *V. cholerae* is cholera toxin, which is encoded by the lysogenic filamentous phage CTX ϕ (Waldor and Mekalanos 1996). Orogastric administration of cholera toxin to human volunteers can induce a diarrheal response characteristic of cholera (Levine et al. 1983). Cholera toxin is an AB₅ protein toxin, with a single A subunit (CTA) and a pentameric B subunit (CTB), and details of its mode of action are reviewed elsewhere (Wernick et al. 2010; Bharati and Ganguly 2011). Briefly, cholera toxin binds to ganglioside GM1 receptors on the surface of enterocytes in the small intestine. The receptor-bound toxin enters the enterocytes by endocytosis and traffics through the retrograde pathway via endosomes and the Golgi apparatus to the lumen of the endoplasmic reticulum (ER). The reduced CT-A1 fragment is removed from the holotoxin by a chaperone-facilitated process and retrotranslocated from the ER lumen into the cytosol. In the cytosol, CT-A1 interacts with small GTPases called ADP-ribosylation factors (ARFs), leading to allosteric activation of the catalytic activity of CT-A1. The activated CT-A1 ADP ribosylates the α -subunit of the heterotrimeric stimulatory G-protein (Gs α), ultimately causing constitutive activation of adenylate cyclase in the basolateral cell membrane, increased production of intracellular cyclic adenosine-3', 5-monophosphate (cAMP), and cAMP-dependent stimulation of secretion of fluid and electrolytes into the lumen of the small intestinal, resulting in diarrhea (Galloway and van Heyningen 1987; Field et al. 1972).

Strains of *V. cholerae* that are deficient in cholera toxin production exhibit attenuation of virulence in animals and humans (Guinee et al. 1985, 1987, 1988).

In some cases, phage conversion has been shown to generate a potent pathogen from an avirulent bacterium, which is the case with the Shiga toxin-producing *E. coli* strain O157:H7 and the recently identified strain, O104:H4 (Beutin and Martin 2012; Hunt 2010). In fact, comparing the genomes of pathogenic *E. coli* strain O157:H7 and the laboratory strain *E. coli* K12 reveals that most of the differences are due to prophages (Blattner et al. 1997; Hayashi et al. 2001; Ohnishi et al. 2001). While *E. coli* is a common commensal bacterium found in the intestinal tracts of humans and animals, individuals infected with Shiga toxin-producing strains can develop diseases ranging from mild diarrhea to severe hemorrhagic colitis and hemolytic uremic syndrome (Karch et al. 2012; Mellmann et al. 2011; Beutin and Martin 2012; Hunt 2010). The production of Shiga toxins 1 and 2 (Stx1 and Stx2) by O157:H7, and Shiga toxin 2, in the case of O104:H4, is the result of the bacteria being lysogenized by one or more of the Stx-phage group of bacteriophages (Allison 2007; Laing et al. 2012). Shiga toxins are characteristic AB₅ toxins with a single A subunit and a pentameric B subunit (Law 2000). After binding to globotriaosylceramide or to globotetraosylceramide receptors, the Shiga toxins enter the target cells by endocytosis and traffick to the ER in a manner similar to that described previously for cholera toxin. Their A1 fragments are retrotranslocated into the cytosol, where their RNA N-glycosidase activity irreversibly inactivates protein synthesis by removing an essential adenine residue from the 28S rRNA of the 60S ribosomal subunit (Endo et al. 1988; Furutani et al. 1992; Saxena et al. 1989). The Stx toxins can cause systemic pathology in addition to colonic pathology, and their receptors are present on endothelial cells throughout the body, including in the kidney and brain (Brigotti et al. 2010; te Loo et al. 2000).

C. botulinum is a strictly anaerobic bacterium capable of producing botulinum neurotoxin (BoNT), and isolates of *C. botulinum* are classified by the serotype of the BoNT (A, B, C1, D, E, F, or G) that they produce. Botulinum neurotoxins are potent toxins that can cause disease in humans and animals, but only BoNTs A, B, E, and F are associated with disease in humans. The primary manifestation of botulism is flaccid paralysis caused by inhibition of the release of the neurotransmitter acetylcholine from motor neurons at myoneural junctions (Montecucco et al. 2004). Botulinum neurotoxins have three structural domains: a C-terminal domain responsible for binding to presynaptic terminals, a middle domain responsible for translocation of the third domain, and an N-terminal domain that has a Zn²⁺-dependent and sequence-specific endopeptidase activity (Lacy and Stevens 1999). Upon binding and internalization into presynaptic cholinergic neurons, BoNTs cleave at least one of three proteins involved in neuroexocytosis: synaptic vesicle-associated membrane protein (VAMP), 25 kDa synaptosomal-associated protein (SNAP-25), or syntaxin. Serotype B, D, F, and G BoNTs cleave VAMP (Schiavo et al. 1993a, b, 1994); serotype A and E BoNTs cleave only SNAP-25 (Schiavo et al. 1993a; Simpson 1979, 2004); and serotype C BoNT cleaves both SNAP-25 and syntaxin (Schiavo et al. 1995; Simpson 1979, 2004). While serotype A, B, and F BoNTs are chromosomally encoded and serotype G BoNT

is plasmid encoded (Barksdale and Arden 1974; Hutson et al. 1996; Zhou et al. 1993, 1995), serotype C1, D, and possibly E BoNTs are encoded on CE β and DE β phages, and curing of these phages results in loss of virulence of the bacteria (Barksdale and Arden 1974; Eklund et al. 1971, 1972; Zhou et al. 1993). Analysis of the genetic organization of the C1 and D loci of CE β and DE β phages shows genes for both toxin secretion and regulation (Hauser et al. 1992; Tsuzuki et al. 1990).

2.3 A Brief History of *Corynebacterium diphtheriae*

C. diphtheriae is the etiological agent of diphtheria (von Graevenitz and Bernard 2006), an acute, communicable, infectious disease associated with characteristic pseudomembranes that often form at primary sites of infection on mucous membranes in the respiratory tract (respiratory diphtheria) or in ulcerating skin lesions (cutaneous diphtheria). Diphtheria toxin produced at the site of primary infection can spread throughout the body to cause polyneuritis, myocarditis, or other systemic toxic effects. In addition, *C. diphtheriae* sometimes causes systemic infections (Hadfield et al. 2000).

In 1883, Klebs visualized *C. diphtheriae* in stained pseudomembranes taken from patients with diphtheria (Klebs 1883). In 1884, Loeffler isolated *C. diphtheriae*, injected it into susceptible experimental animals, caused local infections with widespread tissue damage, and hypothesized that *C. diphtheriae* produced a toxic substance capable of spreading throughout the body (Loeffler 1884). In 1888, Roux and Yersin found that sterile culture filtrates from *C. diphtheriae* contained a potent heat-labile toxin (diphtheria toxin [DT]); they subsequently showed that injection of such culture filtrates into susceptible experimental animals caused pathological changes resembling those seen in diphtheria (Roux and Yersin 1888). In 1890, von Behring and Kitasato (von Behring 1890; von Behring and Kitasato 1890) showed that animals injected with *C. diphtheriae* developed antitoxin that could protect susceptible animals by neutralizing the effects of diphtheria toxin. In 1893, von Behring successfully treated a child afflicted with diphtheria by administering an antitoxic antiserum prepared in experimental animals (von Behring 1893). In 1901, von Behring received the first Nobel Prize in Physiology or Medicine for his contributions to the development of serum therapy of toxin-mediated diseases (Holmes 2000).

In 1896, Park and Williams isolated a strain of *C. diphtheriae* (named PW8) that produced an unusually large amount of DT in comparison with other strains (Park and Williams 1896). A little over a decade later, Theobald Smith proposed using nontoxic complexes of DT with anti-DT as a vaccine to protect against diphtheria, and in 1922, W. H. Park successfully conducted a large-scale immunization trial in New York City using a toxin-antitoxin vaccine (Holmes 2000). In 1923, Ramon reported that treatment of DT with formalin reduced its toxicity without affecting its immunogenicity; this product, called diphtheria toxoid, is now used worldwide

for active immunization against diphtheria (Holmes 2000). However, despite the demonstrated ability of vaccines to prevent diphtheria cases and deaths in well-immunized populations, diphtheria continues to occur in regions of the world where immunization rates are too low or levels of population immunity decline to low levels for any reason, including prolonged disruptions of systems for delivering preventive health care (Golaz et al. 2000; Mattos-Guaraldi et al. 2003).

2.4 Phage Conversion and Toxinogenicity in *C. diphtheriae*

In 1951 Freeman reported the paradigm-shifting discovery that a nontoxinogenic isolate of *C. diphtheriae* acquired the ability to produce DT after contact with a specific corynebophage called B (Freeman 1951), and in 1952, Freeman and Morse proposed a possible relationship between lysogeny and toxinogenicity in *C. diphtheriae* (Freeman and Morse 1952). In 1953, Groman showed that the ability to produce DT is induced and involves a bacteriophage (Groman 1953). In 1954, Barksdale and Pappenheimer showed that Freeman's phage B stock contained both a temperate phage β and its non-lysogenizing variant B, and their quantitative studies of infection of the nontoxinogenic *C. diphtheriae* C4 isolate by phage β demonstrated a one-to-one correlation between lysogenization by phage β and becoming toxinogenic (Barksdale and Pappenheimer 1954). Based on these findings, Barksdale and Pappenheimer introduced the term "conversion" to indicate that toxinogenicity was conferred via lysogeny (Barksdale and Pappenheimer 1954). In 1955, Groman showed that curing *C. diphtheriae* C4(β) of its β -prophage produced a nonlysogenic C4 variant that was identical to the ancestral *C. diphtheriae* C4 isolate in being nonlysogenic, susceptible to infection by phage β , and nontoxinogenic (Groman 1955). Subsequently, Matsuda and Barksdale showed that DT is also produced during lytic replication of a virulent mutant of phage β in a nontoxinogenic *C. diphtheriae* host (Matsuda and Barksdale 1967). Because the genes in temperate bacteriophages responsible for conversion of bacterial phenotypes can be expressed either in bacterial lysogens (from prophages, as described above, or from superinfecting, non-replicating, phage exogenotes) (Gill et al. 1972) or in lytically infected bacteria (from actively replicating phage genomes), the more general term "phage conversion" has replaced the earlier and more restrictive term "lysogenic conversion" in the microbiology literature.

The ability to convert susceptible nontoxinogenic isolates of *C. diphtheriae* to toxinogenicity is a genetic determinant in some corynebphages that are designated *tox*⁺ (Barksdale 1955). Other corynebphages do not confer toxinogenicity and are designated *tox*⁻ (Barksdale 1955; Groman and Eaton 1955). Holmes and Barksdale developed a system for genetic analysis of β -related phages and used it to map several genetic loci that determine immunity specificity (*imm*), host range (*h* and *h'*), and toxinogenicity (*tox*) (Holmes and Barksdale 1969). They also assembled a set of temperate corynebphages (including the *tox*⁺ phages α , β , δ , L, P, and π and the *tox*⁻ phages γ , K, and ρ) and performed comparative studies of their virion

morphology, plaque morphology, single-step growth parameters, immunity specificity, and neutralization by homologous and heterologous antiphage sera (Holmes and Barksdale 1970). Shortly thereafter, Uchida et al. provided direct genetic and biochemical evidence that the *tox* determinant of phage β is the structural gene for diphtheria toxin (Uchida et al. 1971). Buck et al. used DNA hybridization techniques to evaluate further the relationships among the corynephages described above and showed that most of the *tox*⁺ strains contained DNA sequences related to corynephage β (Buck et al. 1985). Furthermore, they showed that this family of related phages, referred to as the β -family, contains not only *tox*⁺ converting phages but also non-converting phages like γ and some other *tox*⁻ phages (Buck and Groman 1981a; Groman et al. 1983; Michel et al. 1982; Buck et al. 1985; Groman 1984). A recent study evaluating the genomic diversity of 13 strains of *C. diphtheriae* identified a new *tox*⁺ corynephage that is similar in its *tox* gene region to corynephage β but has genes for phage structural components that are homologous to a different cryptic prophage from *C. ulcerans* (Trost et al. 2012; Sekizuka et al. 2012).

2.5 Establishment and Maintenance of Lysogeny by β - and Related Corynephages

Phage β has a polyhedral head with a 270-nm long, slender tail and a linear double-stranded DNA genome of approximately 34.7 kbp with terminal cohesive ends (Freeman 1951; Mathews et al. 1966; Buck et al. 1978; Holmes and Barksdale 1970). Lytic replication of phage β in *C. diphtheriae* C7 in rich medium yields an average burst size of 35 plaque-forming units after a minimum latent period of 65 min (Holmes and Barksdale 1970). Most susceptible cells infected by phage β undergo lysis, but a small number survive and become lysogenic and toxinogenic (Barksdale and Pappenheimer 1954). As a consequence of being able to produce lysogens, phage β forms turbid plaques on lawns of susceptible *C. diphtheriae* C7. Two different classes of mutant β -phages that can form clear plaques on *C. diphtheriae* C7 have been characterized. The first class, called clear (c) mutants, is unable to lysogenize C7, but they respect homologous lysogenic immunity, do not form plaques on C7(β), and are likely unable to make the functional immunity repressor needed to establish lysogeny. The second class, called hypervirulent (*hv*) mutants, produce clear plaques either on C7 or C7(β) and presumably have operator mutations that render them unresponsive to the activity of a homologous immunity repressor (Matsuda and Barksdale 1967; Holmes and Barksdale 1969). In *C. diphtheriae* C4(β) or C7(β) lysogens, the β -prophages can be induced to enter the lytic cycle of phage replication by exposing the lysogenic bacteria to ultraviolet light (Groman and Lockart 1953; Barksdale and Pappenheimer 1954; Matsuda and Barksdale 1967). Limited information is

available about the process of maturation and release of mature virions during lytic replication of phage β .

A variety of genetic and molecular studies suggest that the genome of phage β integrates into the chromosome of *C. diphtheriae* in a manner similar to integration of the λ -phage genome into the chromosome of *E. coli* (Laird and Groman 1976; Buck and Groman 1981b; Michel et al. 1982) (see Fig. 2.1). The genetic map of

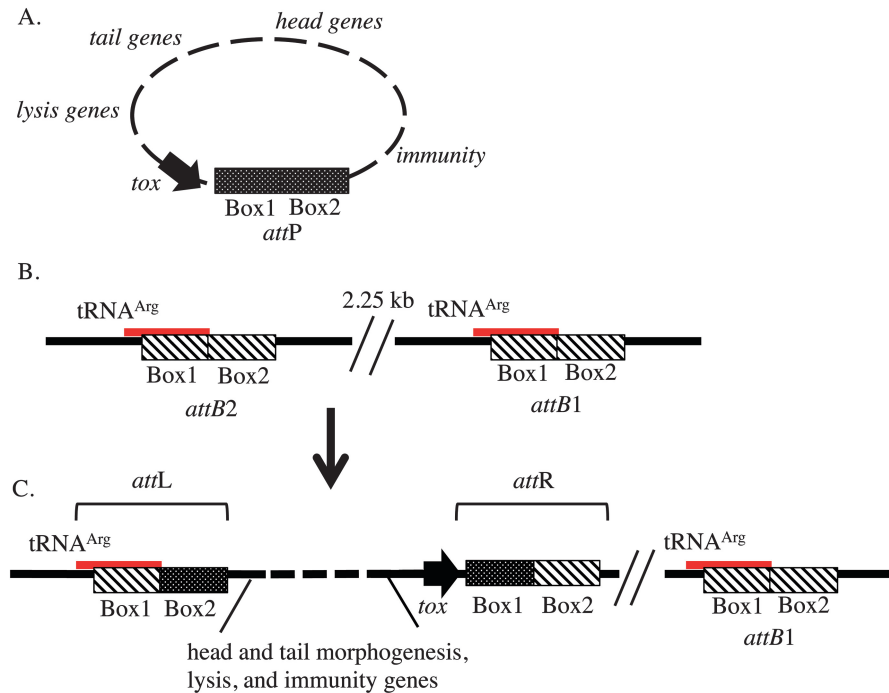


Fig. 2.1 Site-specific integration by corynebacteriophage. Panel (a) shows a schematic representation of a circularized corynebacteriophage β -genome and indicates the relative positions of some representative phage genes/gene clusters (Buck and Groman 1981b; Trost et al. 2012). Integration occurs via site-specific recombination between the phage attachment site (*attP*) (panel a) and either of two equivalent bacterial attachment sites (*attB1* and *attB2*) in the chromosome of *C. diphtheriae* C7 (–) (panel b) (Rappuoli and Ratti 1984). The two *attB* sites overlap with two Arg-tRNA₂ genes that are 2.25 kilobases apart on the *C. diphtheriae* C7(–) chromosome, and they share core sequences of approximately 93 bp that have high homology with the corresponding core sequence of the *attP* site in β -phage (Ratti et al. 1997; Buck et al. 1985). Furthermore, each core sequence consists of a 53 bp segment (Box1) that is identical in *attB* and *attP* and an approximately 40 bp segment (Box 2) that differs between *attB* and *attP* at several nucleotide positions. When phage β integrates into *attB2* (panel c), for example, the *attB2* and *attP* sites recombine to form the *attL* and *attR* junctions between the prophage and the bacterial chromosome (Buck and Groman 1981b). These hybrid *attL* and *attR* sites have identical Box1 regions; their Box2 regions correspond to the different Box2 regions of *attP* and *attB*, respectively; and the Arg-tRNA₂ genes associated with *attL* and *attB2* are identical (Ratti et al. 1997). Finally, integration of phage β at *attB2* does not alter *attB1*, and integration of phage β at *attB1* does not alter *attB2*.

integrated prophage β is a cyclic permutation of its vegetative map (Laird and Groman 1976; Holmes 1976). The integration of corynephage β appears to occur via site-specific recombination between a phage attachment site (*attP*) and one of two functionally equivalent bacterial attachment sites (*attB1* and *attB2*) in the chromosome of *C. diphtheriae* (Rappuoli and Ratti 1984). Within the *C. diphtheriae* C7(–) chromosome, the two *attB* sites are located within two Arg-tRNA₂ genes that are 2.25 kb apart, and they share a 93 bp core sequence with high homology to the *attP* sites of the closely related phages β , γ , and ω (Ratti et al. 1997; Buck et al. 1985). This 93 bp *attB* core sequence contains a 53 bp segment that is identical to a sequence in these phage *attP* sites (Ratti et al. 1997). When the phage genome integrates into the bacterial chromosome to form the prophage, the *attP* site recombines with the *attB* site to form hybrid *attL* and *attR* junctions between the phage genome and the bacterial chromosome (Buck and Groman 1981b). During this process, the tRNA sequence which is adjacent to *attL* is unaltered (Ratti et al. 1997). Although the crossover site between the phage and bacterial genomes that results in integration of phage β has not been precisely determined, it is suspected to be within the tRNA sequence since the first nucleotide mutation, suggesting the substitution of phage DNA for bacterial DNA, occurs only 6 bp downstream from the 3' end of the tRNA coding region (Ratti et al. 1997).

Because *C. diphtheriae* has two different *attB* sites for integration of β or related phages, monolysogens are expected to harbor a prophage integrated at either of the two *attB* sites, and double lysogens are expected to harbor either single prophages at both *attB* sites or tandemly integrated prophages at one of the two sites. Experimental studies with heteroimmune tandem double lysogens of *C. diphtheriae* showed that they are unstable and often generate monolysogenic segregants (Laird and Groman 1976). Furthermore, genetic analysis of phage progeny released after induction of heteroimmune tandem double lysogens of *C. diphtheriae* showed that they were most often excised by generalized recombination between tandem prophage genomes, which is in contrast to the site-specific recombination expected for excision of prophage from a monolysogen (Groman and Laird 1977).

The pangenomic study of *C. diphtheriae* reported in 2012 is based on the complete genome sequences of five toxinogenic isolates, including the C7(β) and PW8 isolates described previously, and eight nontoxinogenic isolates (Trost et al. 2012). Only the highly toxinogenic *C. diphtheriae* PW8 isolate has two *tox*⁺ prophages (an $\omega^{\text{tox}+}$ prophage at each *attB* site), as previously shown by mapping of restriction fragments (Rappuoli et al. 1983). Among the four isolates with a single *tox*⁺ prophage, C7(β) is the laboratory isolate carrying the prototypic β -prophage, two isolates harbor prophages closely related to β , and one (isolate 31A) has a very different *tox*⁺ prophage that is most homologous to a prophage designated CULC22IV that is integrated in a tRNA^{thr} gene in the chromosome of the nontoxinogenic BE-AD22 strain of *Corynebacterium ulcerans*.

2.6 Phage Conversion and Toxinogenicity in Other *Corynebacterium* spp.

Phage conversion leading to the ability to produce diphtheria toxin occurs not only in *C. diphtheriae* but also in some other *Corynebacterium* spp. Diphtheria toxin-producing strains of *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* have been isolated from nature (Maximescu et al. 1974a), and nontoxinogenic isolates of *C. ulcerans* and *C. pseudotuberculosis* were converted to toxinogenicity by lysogenizing them with phages isolated from *C. diphtheriae* (Maximescu 1968; Maximescu et al. 1974a, b). Because the *attB* site of *C. diphtheriae* that is used for integration of phage β is present in numerous other *Corynebacterium* spp. (Cianciotto et al. 1986), it is not surprising that a tox^+ β -like phage can lysogenize a *Corynebacterium* species other than *C. diphtheriae* if it is able to infect that species. Thus far, however, only *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* have been shown to produce diphtheria toxin and to harbor tox^+ phages (Cianciotto et al. 1986).

In recent years, many studies have focused on diphtheria toxin-producing isolates of *C. ulcerans*, because they have assumed increasing clinical importance worldwide as human and animal pathogens and are capable of causing infections in humans that are indistinguishable on clinical grounds from classical diphtheria caused by *C. diphtheriae* (Dewinter et al. 2005; Sing et al. 2005; de Carpentier et al. 1992; Wagner et al. 2001, 2010; Kaufmann et al. 2002; Hatanaka et al. 2003; von Hunolstein et al. 2003; Komiya et al. 2010; Bonnet and Begg 1999). It is important to note that *C. ulcerans* can be transmitted from animals to humans to cause zoonotic infections (Lartigue et al. 2005), unlike *C. diphtheriae* which causes disease in humans but not in animals. Interestingly, a recent study determined the genome sequence of a *C. ulcerans* isolate from a patient in Japan who had a characteristic diphtheritic pseudomembrane and showed that it contained a novel tox^+ prophage ($\Phi\text{CULC0102-I}$) that is quite different from the β -like prophage in the genome of *C. diphtheriae* NCTC13129 (Sekizuka et al. 2012).

2.7 Biosynthesis, Structure, and Mode of Action of Diphtheria Toxin

Diphtheria toxin is synthesized by *C. diphtheriae* as a 560 amino acid pre-protein consisting of an N-terminal 25 amino acid signal sequence and a 535 amino acid (58,342 Da) mature protein (Smith et al. 1980; Greenfield et al. 1983). The pre-protein is transported across the cytoplasmic membrane by the *sec* apparatus; the signal sequence is removed by signal peptidase; and mature DT is released as a soluble, extracellular protein (Smith et al. 1980; Greenfield et al. 1983; Leong et al. 1983). Biochemical and X-ray crystallographic studies show that DT consists of three structural domains that have distinct roles in the intoxication process. The

N-terminal domain of DT is the proenzyme form of the catalytically active fragment A that mediates intracellular intoxication; the centrally positioned translocation domain (T-domain) mediates entry of the fragment A into the cytosol of the target cell; and the C-terminal receptor-binding domain (R-domain) mediates binding of DT to its cell-surface receptor (Collier and Kandel 1971; Gill and Pappenheimer 1971; Gill and Dinius 1971; Drazin et al. 1971).

Early studies showed that DT is highly toxic for humans and some other animals such as rabbits and guinea pigs, and the minimal lethal dose of DT for humans and other highly susceptible animals is approximately 0.1 µg/kg of body weight (Pappenheimer 1984). Some animal species including mice and rats are much more resistant to the toxic effects of DT. Injection of very small doses of DT into highly susceptible animals by the intradermal route causes dermonecrosis. The ability of circulating anti-DT antibodies to neutralize this dermonecrotic response to DT was the basis for the Schick test, introduced in 1913, as a means to distinguish between individuals who are susceptible to diphtheria and those with acquired immunity to DT who are resistant to diphtheria.

Studies in the 1950s showed that small amounts of DT were able to kill a variety of eukaryotic cell lines derived from susceptible animals (Lennox and Kaplan 1957; Placido Sousa and Evans 1957), and inhibition of protein synthesis was shown to be the first manifestation of toxicity in HeLa cells exposed to DT (Strauss and Hendee 1959). Diphtheria toxin also inhibited protein synthesis in cell-free extracts, and nicotinamide adenine dinucleotide (NAD) was shown to be essential for this effect (Collier and Pappenheimer 1964). Further studies with cell extracts identified elongation factor 2 (EF-2) as the biochemical target for intoxication by DT (Goor and Pappenheimer 1967; Collier 1967). The EF-2 is required for transfer of peptidyl tRNA from the A site to the P site of the ribosome (Moldave 1985), and EF-2 activity is essential for protein synthesis. Diphtheria toxin was subsequently shown to catalyze the transfer of the adenosine diphosphate ribose (ADPR) moiety of NAD to EF-2 (Honjo et al. 1969), thereby inactivating EF-2 and blocking protein synthesis (Van Ness et al. 1980; Bodley et al. 1984).

As mentioned above, DT is a proenzyme. It must undergo proteolytic cleavage and reduction before it exhibits its NAD-dependent ADP ribosyltransferase activity. Diphtheria toxin has three surface-exposed arginine residues (at positions 190, 192, and 193) that are highly susceptible as targets for proteolysis by trypsin, and it has four cysteine residues (at positions 186, 201, 461, and 471) that form intramolecular disulfide bonds between C186 and C201 and between C461 and C471. Mild treatment of DT with trypsin generates nicked DT, consisting of the N-terminal fragment A (residues 1–190/192/193) and the C-terminal fragment B (residues 194–535) linked to each other by the C186–C201 disulfide bond, and reduction of nicked DT generates the free fragments A and B (Gill and Pappenheimer 1971; Gill and Dinius 1971; Drazin et al. 1971).

Cells from highly susceptible animals were shown to have more DT receptors on their surface than cells from less susceptible animals (Dorland et al. 1979; Middlebrook et al. 1978; Middlebrook and Dorland 1977). The gene that encodes the DT receptor was cloned, and the heparin-binding epidermal growth factor

precursor (HB-EGF precursor) was identified as the functional receptor for DT (Naglich et al. 1992a, b). The receptor was purified (Mekada et al. 1991); DT was shown to bind to the EGF domain of the DT receptor (Hooper and Eidels 1995; Mitamura et al. 1995); and an interaction between the DT receptor and DRAP27/CD9 in plasma membranes was shown to cause enhanced receptor activity and increased susceptibility to DT (Mitamura et al. 1992; Iwamoto et al. 1994). Characterization of the HB-EGF precursors from DT-susceptible humans and monkeys and from DT-resistant mice showed that residue E141 in the HB-EGF precursor is essential for its binding to DT, and residues R115 and L127 in the HB-EGF precursor make additional contributions to its ability to function as the DT receptor (Hooper and Eidels 1996; Mitamura et al. 1997).

After binding to the HB-EGF precursor, DT is endocytosed via clathrin-coated vesicles and trafficks to the endosomal pathway (Morris et al. 1985). Acidification of the endosome induces a conformational change in the T-domain of DT (Draper and Simon 1980; Sandvig and Olsnes 1980), resulting in insertion of the T-domain into the membrane and subsequent pore formation that facilitates translocation of fragment A from the lumen of the endosome, across the endosomal membrane, and into the cytosol (Olsnes et al. 1988; Kagan et al. 1981; Hu and Holmes 1984; Moskaug et al. 1988). In the cytoplasm, fragment A binds to NAD before interacting with EF-2 (Chung and Collier 1977) and then catalyzes transfer of the ADP-ribose group from NAD to diphthamide (a posttranslationally modified histidine residue) in EF-2 (Van Ness et al. 1980), thereby inhibiting protein synthesis. Diphthamide is conserved in EF-2 in both eukaryotes and archaea, and DT is able to inhibit protein synthesis in cell extracts prepared from eukaryotes or archaea (Bodley et al. 1984). The diphthamide residue in EF-2 is not required for viability of eukaryotic cells, and therefore any cellular mutant that is unable to synthesize the diphthamide residue is resistant to the activity of DT (Moehring and Moehring 1979; Moehring et al. 1980; Chen et al. 1985). Because fragment A of DT is quite stable in the cytosol and exerts its toxic effect by an efficient catalytic mechanism, delivery of a single molecule of wild-type DT-A into the cytosol is sufficient to kill a eukaryotic cell (Yamaizumi et al. 1978).

Only one serotype of DT has been identified. Sequencing of *tox* alleles from clinical isolates of *C. diphtheriae* collected from patients in Russia and Ukraine during the diphtheria epidemic of the 1990s identified one silent mutation in the coding region for fragment A and three silent mutations in the coding region for fragment B (Nakao et al. 1996; Popovic et al. 1996). These genetic polymorphisms were useful for epidemiological typing of the *C. diphtheriae* clinical isolates, but each of the isolates produced DT with the same deduced amino acid sequence. In contrast, sequencing of *tox*⁺ alleles from three clinical isolates of *C. ulcerans* (obtained from two patients with non-pharyngeal infections and one patient with a pharyngeal infection) indicated that each isolate produced a different variant of DT, each of which had several amino acid substitutions (mostly in the T- and R-domains) that differed from the corresponding residues in the reference DT from *C. diphtheriae* (Sing et al. 2003, 2005). Based on these preliminary observations, it is tempting to speculate that the *tox* gene is subjected to different selective pressures

when it is present in *C. diphtheriae* vs. *C. ulcerans*. Under laboratory conditions, mutant forms of DT can be produced easily by genetic manipulation of *tox*⁺ phages or the cloned *tox* gene, and variant forms of DT produced by such methods have been widely used in studies on structure-function relationships of DT (Uchida et al. 1971; Holmes 1976; Laird and Groman 1976).

2.8 Regulation of Diphtheria Toxin Production

In 1936, Pappenheimer and Johnson reported that *C. diphtheriae* produces large amounts of DT when it is grown under low-iron conditions, but very little DT when it is grown under high-iron conditions (Pappenheimer and Johnson 1936). During the next 35 years, many studies identified significant differences in the biochemical and physiological properties of *C. diphtheriae* grown under high-iron vs. low-iron conditions, but they did not reveal how diphtheria toxin production is regulated by iron (reviewed in Barksdale 1970). The discovery in 1971 that the *tox* gene of phage β encodes DT (Uchida et al. 1971) provided new tools to investigate this question at the molecular level.

In 1974, Murphy et al. showed that DT could be synthesized in an *E. coli* in vitro transcription/translation system using purified DNA from phage β as the template (Murphy et al. 1974). Furthermore, adding iron to this *E. coli* in vitro system did not inhibit synthesis of DT, but adding an extract prepared from nonlysogenic *C. diphtheriae* C7(–) did inhibit production of DT but not other β -phage proteins (Murphy et al. 1974). These studies provided strong preliminary evidence that a bacterial factor from *C. diphtheriae*, in addition to iron, was required for specific inhibition of DT production from the genome of phage β . This conclusion was supported by isolating *C. diphtheriae* C7(β) mutants that produced DT under both high- and low-iron conditions and demonstrating that production of DT by newly constructed C7(β) lysogens harboring the β -phages from such mutants was inhibited under high-iron growth conditions (Kanei et al. 1977). Conversely, other studies identified phage β -mutants that conferred resistance to iron-dependent inhibition of DT production when they were present as prophages in wild-type *C. diphtheriae* and led to identification of the cis-dominant *tox* regulatory locus, immediately upstream from the *tox* structural gene, that is also required for inhibition of DT production by iron (Welkos and Holmes 1981a, b; Murphy et al. 1976, 1978). Taken together, these early genetic and biochemical studies led to the hypothesis that regulation of DT production is mediated by a repressor (produced by *C. diphtheriae*), which uses iron as a corepressor and, in its activated form, interacts with the *tox* regulatory locus to prevent transcription of the phage-encoded *tox* gene and production of DT under high-iron growth conditions.

In 1989, Fourel et al. reported results of in vitro DNase I protection assays showing that crude extracts from *C. diphtheriae* grown under high-iron conditions, but not under low-iron conditions, were able to protect a specific nucleotide sequence in the *tox* operator region of phage β DNA (Fourel et al. 1989). Shortly

thereafter, two groups independently identified the diphtheria toxin repressor (*dtxR*) gene by screening chromosomal libraries of *C. diphtheriae* C7 genes in *E. coli* reporter systems for their ability to repress *tox* gene expression in an iron-dependent manner (Boyd et al. 1990; Schmitt and Holmes 1991b). Transcription of *dtxR* by *C. diphtheriae* was shown to occur constitutively under both low- and high-iron growth conditions (Schmitt and Holmes 1991b).

Studies with purified diphtheria toxin repressor protein (DtxR) using electrophoretic mobility shift, DNase I protection, and other assays showed that binding of DtxR to the *tox* promoter/operator sequence in DNA requires specific divalent cations (Cd^{2+} , Co^{2+} , Fe^{2+} , Mn^{2+} , Ni^{2+} , or Zn^{2+}) (Schmitt et al. 1992; Tao et al. 1992; Tao and Murphy 1992; Schmitt and Holmes 1993), although Fe^{2+} is primarily responsible for activating DtxR in the cytoplasm of *C. diphtheriae*. In DNase I protection assays, activated DtxR protects an ~31 bp footprint in *tox* or other DtxR-regulated promoter/operator sequences (see below) and recognizes a pseudo-palindromic 19 bp core region (the “dtxR-box”) with a TTAGGTTAGCC-TAACCTAA consensus sequence (Schmitt and Holmes 1994; Tao and Murphy 1994; Lee et al. 1997). Purified apo-DtxR exists in solution as monomers in equilibrium with homodimers, and conversion to holo-DtxR by binding of one divalent cation per monomer induces conformational changes that increase affinity of the homodimer for cognate DtxR-regulated promoter/operator sequences (Boyd et al. 1990; Schmitt and Holmes 1991b). Each DtxR monomer consists of a 226 amino acid polypeptide organized into three domains: domain 1 (residues 1–73) at the N-terminus is the DNA recognition/binding unit; domain 2 (residues 74–144) mediates homodimerization and contains the metal-ion-binding motif that triggers activation of DtxR; and domain 3 (residues 145–226) at the C-terminus contains an SH3-like domain whose role in function of DtxR is not well defined (Qiu et al. 1995, 1996; Schiering et al. 1994, 1995; Ding et al. 1996). Domain 3 is not needed for DNA-binding activity of DtxR in vitro but is needed for full repressor activity in vivo (Oram et al. 2005). Structures have been determined by X-ray crystallography for apo-DtxR, holo-DtxR, and holo-IdeR (a homolog of DtxR from *M. tuberculosis*) in complex with a cognate operator sequence in double-stranded DNA, and the IdeR-DNA complex was shown to consist of two activated IdeR homodimers bound to opposite faces on its DNA target (Qiu et al. 1996; Pohl et al. 1997, 1998, 1999, 2001; Goranson-Siekierke et al. 1999).

The diphtheria toxin repressor functions as a global regulator of iron-dependent gene expression in *C. diphtheriae* (Boyd et al. 1990; Schmitt and Holmes 1991b). Iron is essential for the growth of most bacteria; however, the bioavailability of iron in the host is limited because it is complexed with iron-binding proteins such as transferrin, lactoferrin, and ferritin, or it is incorporated into compounds such as heme. To overcome this challenge, many pathogenic bacteria, including *C. diphtheriae*, use multiple kinds of iron-acquisition systems to assimilate iron from the host (Albrecht-Gary and Crumbliss 1998; Boukhalfa and Crumbliss 2002; Stintzi and Raymond 2002; Winkelmann 2002). While iron is essential for most bacteria and other organisms, excessive amounts of intracellular iron can be highly toxic. Therefore, the uptake of iron is highly regulated, and in *C. diphtheriae* DtxR

has a central role in maintaining iron homeostasis. In the chromosome of *C. diphtheriae*, more than 20 functional DtxR-binding sites have been identified by several methods, including functional cloning and in vivo DtxR competition assays (Kunkle and Schmitt 2003, 2005; Schmitt and Holmes 1991a, 1993, 1994; Lee et al. 1997; Trost et al. 2012). To date, all DtxR-regulated promoters characterized in *C. diphtheriae* are repressed by activated DtxR under high-iron growth conditions. The proteins encoded by these DtxR-regulated genes of *C. diphtheriae* that have known functions include DT (Boyd et al. 1990; Schmitt and Holmes 1991b), protein components required for the biosynthesis and export of the primary siderophore (corynebactin) and for corynebactin-mediated iron uptake (Kunkle and Schmitt 2003, 2005), protein components of other putative iron uptake systems (Lee et al. 1997; Qian et al. 2002; Schmitt et al. 1997; Schmitt and Holmes 1994), and proteins required for the acquisition of iron from heme (Schmitt 1997a, b; Wilks and Schmitt 1998; Drazek et al. 2000). Interestingly, the physiological function of DtxR as a global regulator of iron-dependent gene expression in *C. diphtheriae* is comparable to that of the ferric uptake regulator (Fur) protein as a global regulator of iron-dependent gene expression in *E. coli* (Hantke 1981), but DtxR and Fur exhibit different specificities and bind to different target sequences in the promoters that they regulate. Figure 2.2 illustrates representative interactions in *C. diphtheriae* between iron uptake systems, extracellular and intracellular iron concentrations, DtxR activity, and synthesis of DT.

The diphtheria toxin repressor (DtxR) is the prototype for a novel and rapidly growing family of bacterial metalloregulatory proteins. Close homologs of DtxR are widely distributed among *Corynebacterium* spp. (Oram et al. 2004; Brune et al. 2006), and more distantly related homologs of DtxR have been identified in numerous bacterial genera (particularly among Gram-positive and acid-fast bacteria), including but not limited to: *Brevibacterium lactofermentum* (Oguiza et al. 1995), *Chlamydia trachomatis* (Thompson et al. 2012), *Mycobacterium smegmatis* (Dussurget et al. 1996), *Mycobacterium tuberculosis* (Schmitt et al. 1995), *Staphylococcus aureus* (Hill et al. 1998), *Staphylococcus epidermidis* (Hill et al. 1998), *Streptomyces lividans* (Gunter-Seeboth and Schupp 1995), *Streptomyces pilosus* (Gunter-Seeboth and Schupp 1995), and *Treponema pallidum* (Hardham et al. 1997). The iron-dependent regulator (IdeR) from *M. tuberculosis* was the first member of the DtxR family shown to be a dual regulator that can repress transcription at some promoters that it regulates and activate transcription at other promoters that it regulates (Gold et al. 2001). The DtxR homologs in several other bacterial species including *C. glutamicum* have also been shown to function as transcriptional dual regulators (Brune et al. 2006; Wennerhold and Bott 2006), and it seems likely all members of the DtxR family will be shown to function as transcriptional dual regulators when they are better characterized. The DtxR family of proteins is related more distantly to the MntR family of manganese-activated regulatory proteins in several bacterial species, and together they constitute a superfamily of related metalloregulatory proteins in bacteria (Guedon and Helmann 2003; McGuire et al. 2013; Osman and Cavet 2010; Pennella and Giedroc 2005; Que and Helmann 2000; Schmitt 2002).

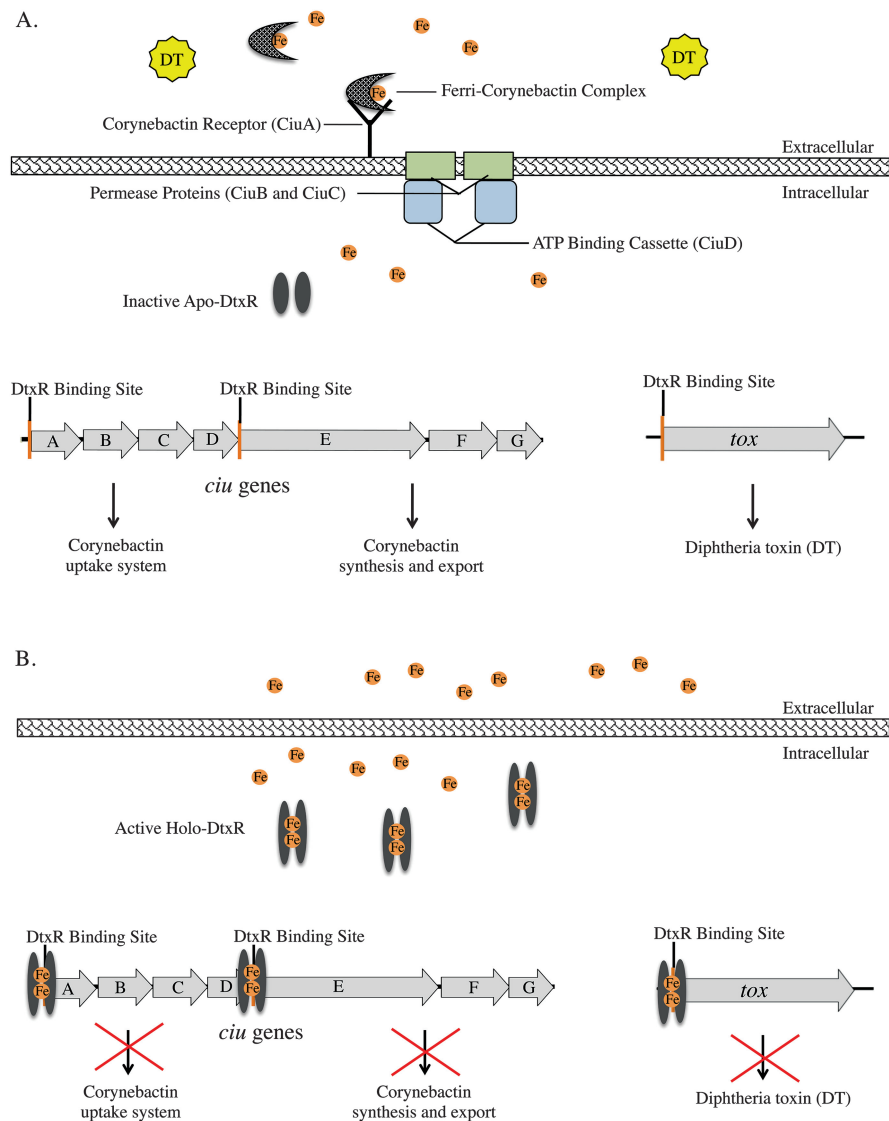


Fig. 2.2 Iron regulation of diphtheria toxin production in *C. diphtheriae*. The production of diphtheria toxin is regulated at the transcriptional level by activity of the diphtheria toxin repressor (DtxR), an iron-activated regulatory protein. The partially characterized set of genes in *C. diphtheriae* regulated by DtxR and iron (the DtxR regulon) includes the *tox* gene for diphtheria toxin (DT), the *ciuEFG* operon for corynebactin biosynthesis and export, the *ciuABCD* operon for the corynebactin-dependent iron uptake system, and the *hmuO* gene and *hmuTUV* operon for acquisition of iron from heme, plus multiple genes for other functions (Schmitt and Holmes 1991b, 1993, 1994; Lee et al. 1997; Schmitt 1997a, b; Schmitt et al. 1997; Kunkle and Schmitt 2003, 2005; Trost et al. 2012; Wilks and Schmitt 1998). Iron is essential for bacterial growth; bioavailability of iron within the human body is low; and excess intracellular iron is potentially very toxic. Therefore, *C. diphtheriae* tightly regulates iron homeostasis by modulating DtxR activity in response to changes in intracellular Fe^{2+} concentration. Panel (a) illustrates *C. diphtheriae* during growth under low-iron conditions, as declining intracellular Fe^{2+} concentrations become growth

2.9 Recent Advancements in the Development of Genetic Tools for *Corynebacterium* spp.

Genetic research in *C. diphtheriae*, as well as many other corynebacteria, has been hindered by a paucity of effective genetic tools. Historically, *Corynebacterium glutamicum* has been best studied because of its importance for biotechnology. In 2003, Ton-That and Schneewind showed that the pK19mobsac allelic exchange vector, originally developed for use in *C. glutamicum*, can also be used in *C. diphtheriae*; consequently, constructing in-frame deletions and introducing other defined mutant alleles into target genes of *C. diphtheriae* can now be done routinely (Ton-That and Schneewind 2003). In 2007, Oram et al. developed phage-based vectors with an integrase gene and an *attP* site from β -family corynephages (Oram et al. 2007). These vectors can replicate in *E. coli*, can be mobilized by conjugation into *Corynebacterium* spp., and cannot replicate but can integrate by site-specific recombination into the *attB* site of appropriate *Corynebacterium* spp., including *C. diphtheriae*, *C. glutamicum*, and *C. ulcerans*. These site-specific integration vectors permit a single copy of a cloned gene to be introduced into the chromosome of *C. diphtheriae* for complementation tests or other purposes (Oram et al. 2007). In 2009, Spinler et al. developed a broad host-range reporter transposon with a selectable but promoterless *aphA* gene that is useful as a tool to select for and identify environmentally regulated promoters in bacteria, including iron-regulated promoters in *C. diphtheriae* (Spinler et al. 2009). During the last decade, therefore, techniques for performing genetic manipulations that have been available for many years in model bacterial systems like *E. coli* or *Bacillus subtilis* have become available for routine use in *C. diphtheriae*. The availability of such techniques dramatically expands the range of molecular genetic manipulations that can now be used for basic biomedical studies in *C. diphtheriae*, and hopefully such methods can also be adapted successfully for use in other pathogenic corynebacteria of interest for human and veterinary medicine.



Fig. 2.2 (continued) limiting and are too low to convert inactive apo-DtxR to active holo-DtxR. Under these conditions, DtxR-repressible genes/operons are transcribed (including *ciuABCD*, *ciuEFG*, and *tox*, which are shown), and large amounts of DT are produced and secreted into the extracellular space. Panel (b) shows *C. diphtheriae* during growth under iron-replete conditions, when the concentration of intracellular Fe^{2+} is high enough to convert inactive apo-DtxR to active holo-DtxR, which binds to DtxR boxes associated with the promoter/operator regions of DtxR-repressible genes and operons, prevents them from being transcribed, and inhibits production of their gene products. Inhibiting or preventing production of iron uptake systems under high-iron growth conditions should help to protect *C. diphtheriae* from assimilating potentially toxic amounts of intracellular iron. Conversely, having small (basal) amounts of iron uptake systems under high-iron growth conditions (while not shown in the Panel b) may be necessary for *C. diphtheriae* to maintain sufficiently high concentrations of intracellular iron to keep DtxR in its active holo-DtxR state

2.10 Summary

The recognition that phage conversion determines toxinogenicity in *C. diphtheriae* was a significant historical milestone in understanding fundamental mechanisms of bacterial pathogenesis. This chapter emphasizes the role of phages in bacterial evolution, gives examples of phage conversion in several medically important bacteria, reviews the history of *C. diphtheriae* and phage conversion of DT production, and summarizes the biology of temperate corynephages. It discusses the structure and function of DT and describes the role of DtxR as the primary global regulator of iron-dependent gene expression in *C. diphtheriae*. Although *tox* is a bacteriophage gene, its evolution as a DtxR-repressible gene within the DtxR regulon couples its expression to scarcity of intracellular iron and the presence of DtxR in its inactive apo-DtxR form. Because *C. diphtheriae* will predictably encounter limited bioavailability of iron in its human host, this regulatory circuit assures that DT, a critically important virulence factor, will be produced by *C. diphtheriae* during the course of infection. Finally, recent developments of genetic tools that permit molecular genetic studies to be done with *C. diphtheriae* are briefly discussed.

An evaluation of the pangenome of *C. diphtheriae* (based on the complete genome sequences of 13 isolates, including some from recent diphtheria cases) identified a novel *tox*⁺ corynephage, suggested greater diversity than previously recognized in the genome architecture of *tox*⁺ phages in *C. diphtheriae*, and showed variations among DtxR regulons of the individual *C. diphtheriae* isolates that might reflect differences in iron assimilation, DT production, or virulence (Trost et al. 2012). Recently, diphtheria-like diseases caused by toxinogenic isolates of *C. ulcerans* are being diagnosed more frequently, and these toxinogenic *C. ulcerans* isolates sometimes harbor novel *tox*⁺ corynephages. Additional studies are warranted to investigate further the diversity and evolution of *tox*⁺ corynephages among *Corynebacterium* spp. that are pathogenic for humans, animals, or both, how the corynephages in these toxinogenic *Corynebacterium* spp. acquire or exchange *tox*⁺ determinants, and whether humans may have increased or decreased susceptibility to the diphtheria-like diseases caused by some of these novel toxinogenic *Corynebacterium* spp. (Wagner et al. 2001, 2010; Kaufmann et al. 2002; Hatanaka et al. 2003; Lartigue et al. 2005; Sing et al. 2005; Sekizuka et al. 2012).

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