Chapter 4
Corynephages: Infections of the Infectors

Vartul Sangal and Paul A. Hoskisson

Abstract *Corynebacterium diphtheriae* is the etiological agent of diphtheria; an acute toxin-mediated infection of the upper respiratory tract and skin. The toxin gene is encoded on a temperate bacteriophage, which during lysogeny is capable of toxin production. In this chapter, we will summarise current knowledge regarding corynephage, focusing on the lifecycle and biology of the toxin-carrying corynephages that are known to convert pathogenic corynebacteria, and discuss the insights recent genome corynebacterial sequencing has given us.

Keywords Bacteriophage · Corynephage · *Corynebacterium* · Diphtheria · Phage conversion · Pathogenicity · Toxigenic · Toxin

4.1 Introduction

Diphtheria is an acute, toxin-mediated disease of the upper respiratory tract, skin and occasionally other mucous membranes caused by the *Corynebacterium diphtheriae* and its relatives. Whilst there is a requirement for colonization of the host by *C. diphtheriae* (or its close relative *Corynebacterium ulcerans*), it is the infection of the bacterium by a group of closely related bacteriophages, which carry a toxin gene that causes the primary clinical symptoms in humans. It is this intimate link between the host (the bacterium in this case), the pathogen (the bacteriophage) and the human host that makes the biology of corynephages so fundamental to global human health.

In 1951, Freeman reported that following exposure of non-toxigenic *C. diphtheriae* to bacteriophage, the isolation of diphtheria toxin-producing strains was possible (Freeman 1951). Subsequently it was shown that there was a direct link

P. A. Hoskisson (✉) · V. Sangal
Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde,
161 Cathedral Street, Glasgow G4 0RE UK
e-mail: Paul.hoskisson@strath.ac.uk

between toxin production and lysogeny (Freeman and Morse 1952). Initial hypotheses suggested that the bacteriophage selected for toxin-producing mutants, however it was later shown that it was a bacteriophage-specific phenotype and that the β phage carried the genetic material for toxin production (Groman 1953; Bardsdale and Pappenheimer 1954; Groman 1955). Despite its importance, limited efforts were made to study pathobiology of corynebacterial phages over last 60 years. In this chapter we will review the current knowledge of corynephages, focusing on toxigenic phage, the role they play in corynebacterial biology, discuss the role of genomics in understanding corynephage dynamics from evolutionary point and also some of the clues to bacteriophage resistance from the multitude of corynebacterial genome sequences that are increasingly available.

4.2   Non-toxigenic Corynephages

Bacteriophage have been studied extensively for many years and were a driving force in the molecular biology revolution, in terms of their use as model systems and tools to study a wide range of biological processes. However the role of bacteriophage in the evolution of bacteria cannot be under-estimated. The extensive sequencing of bacterial and archaeal genomes that has been undertaken in the last decade has lead to a greater understanding of the role of bacteriophages in genome evolution and has lead to a resurgence in interest in bacteriophage biology (Brüssow et al. 2004).

The acquisition of beneficial DNA sequences through horizontal gene transfer (conjugation, transposition, transformation, transduction and lysogenisation—the last two in this list being bacteriophage-mediated) offers a significant selective advantage under a wide range of conditions, extending the genetic diversity of bacterial strains. These mechanisms often lead to loss as well as gain of genes (Lawrence and Ochman 1997; Jain et al. 2002), and as suggested by Brüssow et al. (2004), it is the transient selective advantage of gene loss/gain in a dynamic environment that is the real driving force of bacterial evolution.

Despite the industrial and medical importance of corynebacteria, relatively little is known about their bacteriophages. Several bacteriophages have been isolated for *Corynebacterium glutamicum* strains (some strains previously designated *Brevibacterium flavum*), an industrial producer of amino acids, due to their negative impact on industrial production (Sonnen et al. 1990) or for the development vectors for site-specific insertion and genetic manipulation (Oram et al. 2002). The corynephages against *C. glutamicum* have generally been isolated directly from production fermenters, from areas close to production facilities (Kato et al. 1984; Trautwetter et al. 1987; Sonnen et al. 1990) or from soil (Trautwetter and Blanco 1988), which, as one might expect is a good hunting ground for corynephages. The corynephages that have been isolated against these strains generally have a very narrow host range due to the close genetic relationship between the industrial bacterial strains (Trautwetter and Blanco 1988; Sonnen et al. 1990). Little is understood

of the wider ecology of corynephage infecting *C. diphtheriae* and *C. ulcerans*, such as their abundance in their natural environmental niches, their host-range and the level of genome diversity amongst corynephages.

Virulent corynephage for *C. glutamicum* have largely been isolated from production facilities where they were found to significantly reduce productivity through slow growth and lysis of cultures (Halgasova et al. 2005). There have been a number of corynephage isolated, largely belonging to the B group of bacteriophages based on the Bradley classification corresponding to the *siphoviridae* (Bradley 1967; Ackermann 2009). Koptides et al. (1992) isolated the virulent phage BFK20 from *C. (Brevibacterium) flavum*, which was found to be a double-stranded DNA bacteriophage with a non-contractile tail and a 50 nm polyhedral head. There has been considerable further work on the analysis of BFK20 in terms of its genome sequence (EMBL Acc. No.: AJ278322; (Bukovska et al. 2006)), its replication (Halgasova et al. 2005) and host cell binding and entry (Gerova et al. 2011). Trautwetter et al. (1987) isolated CG33 from *C. glutamicum* that was found to be a group B bacteriophage of comparable morphology to BFK20 (Koptides et al. 1992). Similarly, Trautwetter and Blanco (1988) isolated 20 novel corynephage for *Corynebacterium* and reported that 19 of these were virulent. All appeared to be Group B (*siphoviridae*), with non-contractile tails of around 100 nm and polyhedral heads in the range of 41–57 nm.

Temperate corynephages are so far the best studied examples, primarily due to the ability to induce them through ultra-violet light treatment (Kato et al. 1984; Patek et al. 1985; Moreau et al. 1995; Frunzke et al. 2008). The temperate corynephage BK1 was isolated from *C. (Brevibacterium) ketoglutamicum* through the isolation of cloudy plaques (Trautwetter and Blanco 1988), and was further demonstrated to be inducible using mitomycin C. Patek et al. (1985) isolated three new corynephage from *C. glutamicum* CBII, each being non-contractile tailed DNA phages, yet were little characterized thereafter. Isolation of the temperature corynephage φGA1 from a *C. (Brevibacterium) flavum* ATCC14067 lysogen is particularly interesting given the relationship this corynephage has with the virulent corynephage, Cog, from *C. glutamicum* LP-6 (Sonnen et al. 1990). The temperate corynephage φGA1 was found not to cause plaques on a wide range of strains or to cause lysis following induction. The virulent phage Cog was only able to plaque on *C. glutamicum* LP-6. Cog was also found to adsorb to a range of strains, but was not able to form plaques. Interestingly, Southern hybridizations between these two corynephages indicated high levels of homology between the two, suggesting a close relationship at the genetic level. It was hypothesized that introduction of DNA from one phage, without establishing an infection, is a driver of bacteriophage evolution through recombination of homologous regions between corynephages and as such may generate novel recombinant bacteriophages (Sonnen et al. 1990). This homologous recombination between related bacteriophages, which share partial homology can result in the exchange of small functional units or 'gene modules' resulting in the emergence of new bacteriophages and forms the basis of why we see the so-called mosaic genomes of bacteriophage (Hatfull and Hendrix 2011). Given this close relationship, in terms of morphology and genetics, between the temperate and lytic corynephages

and the *Corynebacterium* strains used for industrial amino acid production, it is not surprising that the corynephages isolated to date have all appeared to be related, and with limited studies also suggesting a close relationship at the genetic level. This is an area that requires further investigation given the industrial and medical importance of corynebacteria, however it is clear that we are currently underestimating the diversity of these phages and it is an area that should receive more attention.

4.3 Toxigenic Corynephages

The conversion of non-toxigenic pathogenic corynebacteria (*C. diphtheriae* and *C. ulcerans*) to toxigenic strains through lysogeny of a bacteriophage is a fascinating story of host-pathogen interaction. The colonization of human hosts by non-toxigenic *C. diphtheriae* that are subsequently converted to toxigenic strains, causing clinical diphtheria symptoms are known in the literature (Simmons et al. 1980). However, this finding proved elusive in a natural setting for many years. The early work of Freeman (1951, 1952) showed that there was a direct link between lysogeny of a bacteriophage, the formation diphtheria toxin and that the β phage carried the gene or genes required for toxin production (Groman 1953; Bardsdale and Pappenheimer 1954; Groman 1955). The majority of our knowledge regarding the conversion of strains to toxigeny has come from study of β phage, yet ω and γ phage, are also known to have converting activity, with up to nine temperate *C. diphtheriae* corynephage known (Holmes and Barksdale 1970). Holmes and Barksdale (1970) subsequently showed that six of their nine temperate corynephages are toxin-positive (α, β, δ, L, P, π) and were able convert, while a seventh, although non-converting carried the toxin gene (γ). Interestingly data investigating the relationships and evolution of these groups of converting and non-converting strains is scarce, with restriction mapping being the primary discriminatory feature. Until recently only one published, fully sequenced toxigenic corynephage genome was available (Cerdeno-Tarraga et al. 2003), however recent sequencing projects have contributed significantly to our knowledge of toxin carrying corynephage (Sangal et al. 2012a, b; Trost et al. 2012).

The plaques of α, β, δ, L, P, π, K, γ and ρ are generally uniform, turbid and reach around 2 mm in diameter, forming densely turbid peripheries upon prolonged incubation (Holmes and Barksdale 1970). The same authors performed one-step growth curves on the phages and found that β, γ and L phage were indistinguishable with a mean burst size of 37 phage per cell over a latent period of 65 min, followed by a rise period of 18 min. The β phage is the best studied of the converting corynephages and is a typical lambdoid bacteriophage, with a polyhedral head of around 55 nm in diameter and with a long (270 nm) tail (Holmes and Barksdale 1970). The genome is linear double-stranded DNA and was estimated to be 34.7 kbp by restriction endonuclease mapping and could be circularized through cohesive ends (*cos*) (Buck et al. 1978). Initial estimates suggested that there was enough DNA to code for 30–40 proteins (Groman 1984), yet it was not until the genome of *C. diphtheriae*

gravis was sequenced that the full complement of a toxin-carrying phage genome was known to be 42 genes (Cerdeno-Tarraga et al. 2003). The toxin-producing corynephages appears to be conventional in their lytic cycles and of course can form lysogenic infections resulting in the formation of toxin producing strains (Groman 1984). Interestingly, as expected most phage genes are repressed during lysogeny, yet it is of note that toxin is produced by *C. diphtheriae* during this period and expression is related to the presence of the global iron regulator DtxR and iron availability in the host (Kunkle and Schmitt 2003, 2005).

4.4   Identifying the Diversity of Corynebacterial Prophages

Phages affect bacterial genome architecture and are major drivers in bacterial evolution. The majority of corynephages identified to date have been through the analysis of genome sequences as prophages (Frunzke et al. 2008). Using bioinformatics tools such as Phage_finder (http://phage-finder.sourceforge.net/: (Fouts 2006)) or PHAST (http://phast.wishartlab.com) (Zhou et al. 2011)) it is possible to identify prophages within completely sequenced bacterial genomes. The range and number of prophages within fully sequenced *C. diphtheriae* and its potentially toxigenic relatives genomes varies widely. It is however interesting to note the diversity of prophages carried by different strains (Table 4.1). Some strains of *C. diphtheriae* carry multiple prophages, with *C. diphtheriae* C7 beta carrying four, one with homology to BFK20, two with homology to mycophages and one similar to an *Escherichia coli* bacteriophage (http://phast.wishartlab.com/Download.html). *C. diphtheriae* HCO2 carries three corynephages, one showing homology to an *Escherichia coli* phage, one to a phage from *Geobacillus* and another similar to an *Enterococcus* phage (Table 4.1). Various corynebacterial strains detailed in Table 4.1 contain single or multiple phages, with homology to bacteriophages from a diverse range of organisms such as *Burkholderia*, *Pseudomonas, E. coli*, *Brucella, Rhodococcus, Streptomyces, Mycobacterium, Ralstonia* and *Listeria*. Interestingly, the toxigenic bacteriophages in *C. diphtheriae* strains C7, CDCE8392, PW8 and NCTC13129 all show homology to the *C. glutamicum* phage BFK20 (see above). This homology is especially conserved in the late genes, encoding proteins that function in the assembly of the bacteriophage capsid and tail (Bukovska et al. 2006). Interestingly significant homology to BFK20 also is apparent in a prophage contained in the genome of *C. lipophiloflavum* (http://phast.wishartlab.com/Download.html), suggesting the ubiquity of this phage type amongst corynebacteria.

Table 4.1 Distribution of prophages in sequenced coynebacterial genomes using the pre-computed database from PHAST (Zhou et al. 2011). Data was correct as of 1st May 2012

Species and strain	PHAST Accession number	Number of predicted prophage
Corynebacterium jeikeium ATCC 43734	ACYW00000000.1	2
Corynebacterium pseudotuber-culosis 1002	CP001809.2	0
Corynebacterium ulcerans 809	CP002790.1	1
Corynebacterium diphtheriae 31A	CP003206.1	1
Corynebacterium diphtheriae 241	CP003207.1	1
Corynebacterium diphtheriae INCA 402	CP003208.1	1
Corynebacterium diphtheriae BH8	CP003209.1	1
Corynebacterium diphtheriae C7 (beta)	CP003210.1	4
Corynebacterium diphtheriae CDCE 8392	CP003211.1	2
Corynebacterium diphtheriae HC01	CP003212.1	1
Corynebacterium diphtheriae HC02	CP003213.1	3
Corynebacterium diphtheriae HC03	CP003214.1	1
Corynebacterium diphtheriae HC04	CP003215.1	0
Corynebacterium diphtheriae PW8	CP003216.1	2
Corynebacterium diphtheriae VA01	CP003217.1	0
Corynebacterium diphtheriae NCTC 13129	NC_002935.2	1
Corynebacterium glutamicum ATCC 13032	NC_006958.1	1
Corynebacterium ulcerans BR-AD22	NC_015683.1	3
Corynebacterium lipophilofla-vum DSM 44291	ACHJ00000000.1	3

4.5   Comparative Genomics of Toxigenic Corynephage

Prophages containing *tox* genes were recently identified in multiple strains of *C. diphtheriae* C7, CDCE8392, PW8 and NCTC13129 (Trost et al. 2012) in addition Sangal et al. (2012a, b) also identified homologous *tox* gene-containing corynephages in two strains, one a *C. diphtheriae mitis* strain (NCTC03529) and a *C. diphtheriae intermedius* strain (NCTC05011) indicating the prominence of this corynephage lineage within the species (Fig. 4.1). One point of note is the presence of two copies of corynephage ω in *C. diphtheriae* PW8, a strain which is used widely
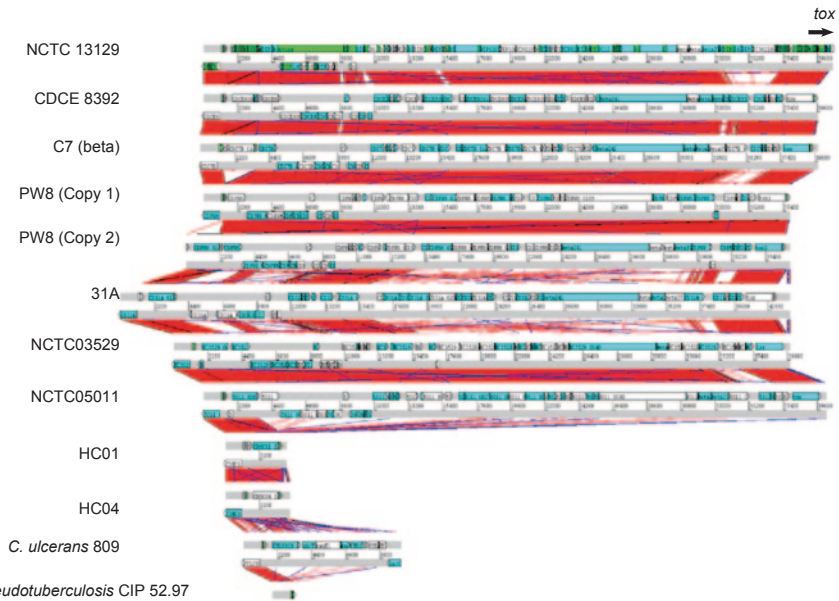
Fig. 4.1 Linear comparison of corynephage sequences from *C. diphtheriae* strains NCTC13129, CDCE8392, C7(beta), PW8, 31A, NCTC03529 and NCTC05011 (GenBank Accession Nos.: BX248353.1, CP003211.1, CP003210.1, CP003216.1, CP003206.1, AJGI00000000.1 and AJVH00000000.1, respectively) using Artemis Comparison Tool (ACT). These sequences were extracted from genomes after a nucleotide blast search of sequences from DIP0178-DIP0224 of *C. diphtheriae* NCTC13129. Homologous sequences were also extracted from non-toxigenic *C. diphtheriae* strains HC1 (Acc. No.: CP003212.1) and HC4 (Acc. No.: CP003215.1), *C. ulcerans* strain 809 (Acc. No.: CP002790.1) and *C. pseudotuberculosis* strain CIP52.97 (Acc. No.: CP003061.1). The location of the *tox* gene is indicated on the uppermost sequence for orientation

as a toxoid producer (Trost et al. 2012). The two copies of the phage differ only by 5 nucleotides in a genome of 36 kbp. Trost et al. (2012) identified the genomes of the toxin-producing β and ω corynephages from the published restriction maps of the prophages (Michel et al. 1982), with ω being found in *C. diphtheriae* PW8 and β corynephage being found in *C. diphtheriae* C7, CDCE8392 and a divergent version of the β phage (differing in sequence and gene content) in *C. diphtheriae* NCTC 13129. Remarkably, these strains are related to the *tox⁻* corynephage BFK20, known from the literature and can explain some of the converting activity and recombination observed in the early studies given the high degree of homology. Comparison of the *tox⁺* corynephages showed high levels of homology, with the remarkable exception of the *tox⁺* coryneprophage in *C. diphtheriae* 31A, a strain isolated from a vaccinated adult in Rio de Janeiro, Brasil (Trost et al. 2012). This coryneprophage shows homology to the β-like phages predominantly in the *tox* containing, right-hand end of the prophage genome. Interestingly this prophage shows homology to some of the genes found in the prophage CULC22IV from *C. ulcerans* BR-AD22 (Trost et al. 2011) and similarity to a *Streptomyces* phage φSASD1 (http://phast.
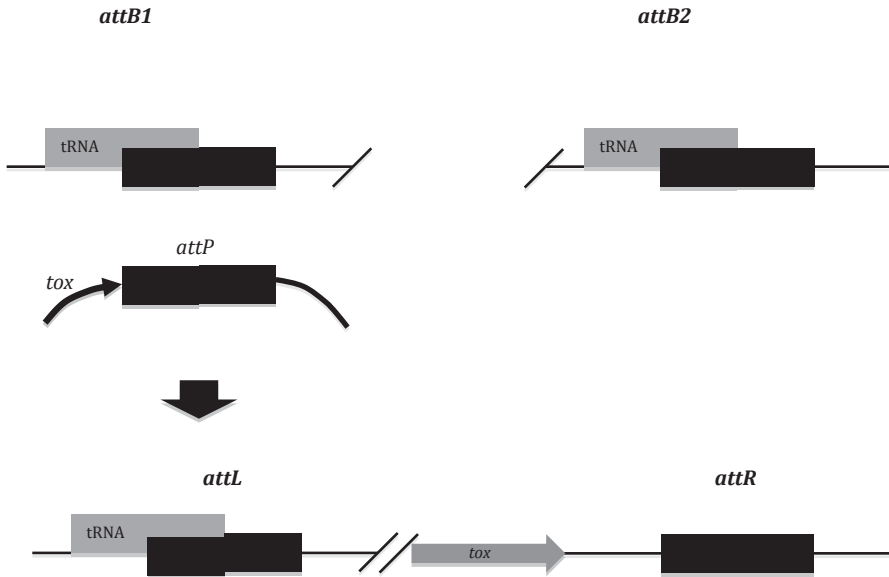
Fig. 4.2 A schematic representation of the tandem *attB* sites in the *Corynebacterium diphtheriae* genome, the formation of *attL* and *attR* following integration of the *tox*+corynephage and the proximity of *tox* to the right-hand end of the corynephage genome. Note the difference in the two half-sites for *attB* representing the functional constraint on the reconstitution of a functional tRNA at *attL* upon excision

wishartlab.com/Download.html). These data suggest that *tox*+ corynephages have evolved on at least two occasions in toxigenic corynebacteria or that gene shuffling occurs frequently in corynephage (Trost et al. 2012).

4.6   Integration of the Toxigenic Corynephage DNA

The integration of toxigenic corynephage in to the *C. diphtheriae* genome, as in many bacteriophages, insertion sequences and pathogenicity islands, occurs in tRNA genes, the most obvious evolutionary reason for this is the conservation and redundancy of tRNA genes in bacterial genomes. There are two distinct attachment sites found within the *C. diphtheriae* genome, *attB1* and *attB2* (Rappuoli et al. 1983). These two sites overlap with a putative $tRNA_2^{Arg}$ gene that flank a putative membrane protein and is conserved in all *C. diphtheriae* genomes (Ratti et al. 1997; Trost et al. 2012). The *attB* sites can be divided in to two segments (half-sites), the first half-site has perfect identity in both *att* sites found in the genome. The second half-site however contains several point mutations. This is likely the result of the overlap of the first half-site with the tRNA, and the constraint to resolve *attL* completely to reconstitute functionality (Ratti et al. 1997, Fig. 4.2). Earlier studies had

also shown that *C. diphtheriae* could form stable single, double or triple lysogens (Rappuoli et al. 1983) with a concomitant increase in the production of toxin. Interestingly, the recent pan-genome study of Trost et al (2012) demonstrated the stable insertion of two ω^{tox+} phages in to the *attB1* and *attB2* sites in the *C. diphtheriae* PW8 genome, this indicating why this strain was probably selected as one of the main toxoid producing strains for vaccine.

Integration of the corynephage is mediated by a tyrosine-like recombinase/integrase, encoded by DIP0182 in *C. diphtheriae* NCTC 13129 (Oram et al. 2007). The mechanism of tyrosine recombinases is well studied based primarily on the similarity to the bacteriophage lambda integrase (for a detailed explanation of the integration mechanism see (Van Duyne 2005).

4.7 Evolution of Toxin Regulation by a Host Transcription Factor

The intriguing link between regulation of toxin production and iron-responsive genes suggests an intimate link between the DtxR regulon in the host bacterium and the infecting corynephage. Iron is an essential nutrient for cellular processes such as respiration and is often limited in mammalian hosts, suppressing bacterial growth; therefore pathogenic microorganisms invest heavily in acquisition and regulation of iron-scavenging and iron-responsive genes such as siderophores and proteins containing iron centres. The production of toxin in *tox+* strains of *C. diphtheriae* is regulated in an iron-dependent manner by DtxR, the diphtheria toxin repressor (De Zoysa et al. 2005). DtxR controls a complex network of genes involved in iron homeostasis and is highly conserved throughout the mycolata and is physiologically similar to the Fur (Ferric Uptake Regulator) protein of Gram-negative bacteria (De Zoysa et al. 2005; Wennerhold and Bott 2006). In low iron concentrations, DtxR is de-repressed from multiple promoters resulting in gene expression, including the transcription of the *tox* gene in lysogenized strains. There is also evidence of gene activation by DtxR in *C. glutamicum* (Wennerhold and Bott 2006), and given the high degree of regulon conservation it is likely that DtxR may function as an activator in other corynebacteria too. Recent comparative analysis of the pan-genome of *C. diphtheriae* revealed a high degree of conservation in the DtxR regulon across 13 strains of *C. diphtheriae* (Trost et al. 2012), with the 19 bp operator sequence being found upstream of 36 genes, including 26 that highly conserved across all strains. Interestingly the *tox* gene when carried on a corynephage is also subject to regulation by DtxR. This link between toxin production encoded by a bacteriophage and the evolution of its regulation by a host strain transcription factor is currently not well understood.

It is tempting to speculate on the evolution of this co-regulation, based on the evidence of the poorly defined non-toxigenic *C. diphtheriae* strains that carry a cryptic copy of the *tox* gene (Cianciotto and Groman 1997). It was speculated that these

strains might represent a source for *tox* sequence that could be activated through re-combination or mutation due to selection. Non-converting corynephage from non-toxigenic *C. diphtheriae* strains have been known since the 1950s, where *tox* was not expressed due to insertional inactivation (Groman 1955; Michel et al. 1982). Cianciotto and Groman (1997) characterised these strains and showed, through complementation tests using double lysogen strains, that the non-toxigenic pheno-type was the result of the phages being *cis* dominant. This lead the authors to hy-pothesize that toxin-positive strains may arise by either homologous recombination between two distinct *tox⁻* phages or spontaneous reversion within a single mutant allele. Given the *tox* gene lies at the right-hand end of the integrated corynephages, it has been suggested that it was acquired by the corynephage by recombination (Cerdeno-Tarraga et al. 2003). This may have resulted in the gene lying in proxim-ity to an inverted repeat (potentially homologous to the *dtxR* operator) towards the end of the corynephage genome, or that the *tox* gene was acquired with its upstream regulatory region from another strain. Recently there has been significant interest in the evolution of regulatory networks in horizontally acquired genes in closely related strains (Perez and Groisman 2009; Chen et al. 2011). The reliance on or-thologous regulators (in this case DtxR) to orchestrate responses (in this case to iron limitation) conferring changes in gene expression in horizontally acquired genes (in the case *tox*) to survive deleterious conditions is an attractive scenario, especially given that diphtheria toxin causes host cell lysis, releasing a potential source of iron (Chang et al. 1989). Thus the bacteriophage-encoded promoter for a specific nutrient acquisition factor is under bacterial control (Ratti et al. 1997) offering a significant advantage to cells that have colonized a mammalian host.

4.8 Corynephage of *Corynebacterium ulcerans*

Recent genome sequencing and interest in corynebacteria has lead to the study of genomes of related organisms. Two genomes of the animal commensal and increas-ingly recognized zoonotic infection reservoir *C. ulcerans* was recently sequenced and have provided significant insight in to the biology of another corynebacteri-al strain capable of conversion by β phages (Trost et al. 2011). Both sequenced strains differ significantly in their prophage complement, with *C. ulcerans* 809 carrying a single prophage and *C. ulcerans* BR-AD22 carrying four prophage-like regions. Previous work had suggested that there was potential for *C. ulcerans* to carry β-corynephage, based on PCR assays of the *tox* gene (Mattos-Guaraldi et al. 2008), however no *tox* gene or β-corynephage sequences could be identified in the *C. ulcerans* 809 or *C. ulcerans* BR-AD22 genomes. Analysis showed that there was little synteny between the integration regions of the *C. diphtheriae* and *C. ul-cerans* genomes, however remnants of a putative corynephage are present close to the tRNA[Arg] gene and the presence of a tyrosine recombinase, homologous to that found in the β-corynephages, suggests that the *C. ulcerans* genome may have pre-viously accommodated a β-corynephage. These data also link to the homologous

genes found in the novel toxin-producing corynephage present in the genome of *C. diphtheriae* C31A (Trost et al. 2012), providing further opportunity for recombination amongst homologous phage regions.

4.9 Corynephage Resistance Mechanisms

To bacteria the constant threat of bacteriophage infection has driven the emergence and acquisition of defense genes to prevent the deleterious effects of bacteriophage infection, however it is possible that bacteriophage infection permits the acquisition of novel, possibly beneficial, genes (Hoskisson and Smith 2007). The diversity of the bacteriophage resistome in bacteria is enormous and the opportunity to acquire novel genes (such as toxin production in the case of *C. diphtheriae*) has resulted in the evolution of hypervariable resistance mechanisms.

Analysis of recently sequenced *C. diphtheriae* genomes and re-analysis of existing sequences have demonstrated that *C. diphtheriae* is a much more diverse bacterium than previously thought. While the genome of *C. diphtheriae* has a highly conserved genomic backbone there is considerable variation outside of the syntenous regions, especially around the origin of replication (Trost et al. 2012). Much of this variation across strains is due to insertion sequences (Trost et al. 2012), however it is also clear that there is a lot of variation in sequences associated with resistance to horizontal gene transfer. Mokrousov (2009) completed a detailed *in silico* analysis of the so-called CRISPR (clustered regularly interspaced short palindromic repeats) regions of the *C. diphtheriae* NCTC 13129 genome, identifying two CRISPR loci, and it is this diversity that also allowed the development of a spoligotyping method to determine the phylogenetic relationship between strains (Mokrousov et al. 2007; Mokrousov 2009). CRISPRs are a platform for acquiring resistance against phage infection, where short sequences of DNA from an infecting bacteriophage are inserted between a conserved repeated sequence within a CRISPR and thereafter confers resistance to that bacteriophage, through a mechanism similar to RNA interference, along with their associated *cas* genes which process the incoming foreign DNA (Marraffini and Sontheimer 2010). In *C. diphtheriae* NCTC 13129 four of the seven CRISPR spacer regions showed homology with hypothetical proteins from bacteriophage or plasmid/transposon encoded genes involved in integration and/or recombination (Mokrousov 2009). More recently, extensive further analysis of the CRISPR loci demonstrated the presence of three different arrays across 13 strains (Trost et al. 2012). Type I CRISPR locus is composed of three *cas* genes with between one and 28 spacers, and is the most widespread of the CRISPR arrays found in eight of the 13 strains analysed by Trost et al. (2012). Type II arrays were only found in two of the analysed strains and consist of eight *cas* genes and between four and 26 repeats. The type III array was found in five of the 13 strains and again consists of eight *cas* genes with 12–42 arrays. In addition Sangal et al. (2012a, b) indicated the presence of extensive *cas*/CRISPR arrays in *C. diphtheriae mitis* (NCTC03529) and *C. diphtheriae intermedius* (NCTC05011).

Traditionally it was thought that restriction-modification systems provided the main basis for bacteriophage resistance. Type II restriction-modification (R-M) systems have been characterised in *C. glutamicum* (Schafer et al. 1997) but few studies have been carried out other than *in silico* on the R-M systems of *C. diphtheriae.* The recent study of D'Afonseca et al. (2012) indicated that the Type I R-M system in *C. diphtheriae* NCTC 13129 maybe at least partially defective, due to the insertional inactivation of an potentially alternative modification subunit, *hsdM* (putative methylase; DIP2081), which is distant to the main genes associated with the R-M system (*hsdRSM;* DIP2312, DIP2313, DIP2314). It is possible that the inactivated methylase may play an important role in diversification of the R-M system and may result in an increase in plasticity of the genome in response to stress. Similar duplicated methylases are known in related actinomycetes such as the Phage Growth Limitation system in *Streptomyces* (Sumby and Smith 2003; Hoskisson and Smith 2007). Sangal et al. (2012b) also noted the presence of putative Type III R-M systems in the *C. diphtheriae intermedius* (NCTC05011) genome, adding to the diversity of observed R-M systems providing resistance to bacteriophages.

These data suggest that there is significant investment of genomic capacity in corynebacteria to prevent the deleterious effects of bacteriophage infection, suggesting that without these mechanisms corynebacterial genomes may be more plastic (D'Afonseca et al. 2012).

4.10   Summary

There is an intimate link between the ability to cause classical diphtheria disease symptoms by *C. diphtheriae* and the integration of a toxin carrying bacteriophage. The investment by the bacterium in bacteriophage resistance mechanisms has helped to shape the genome, but it is clear that there is a reliance on incoming genetic diversity to provide a selective advantage within a host, none more so that the provision of the diphtheria toxin to facilitate access to iron under limiting conditions. The control of a bacteriophage-encoded toxin gene by a host encoded transcription factor exemplifies the intimacy of the relationship between bacteria and their viruses and the genetic diversity that they can provide. There are still many questions to be answered in this system that will be applicable in a wider context to the evolution of bacterial pathogens. The *C. diphtheriae tox*[+] bacteriophage model system provides an ideal platform for answering questions regarding the acquisition and maintenance of horizontally transferred DNA, the regulation of horizontally transferred DNA and how this shapes the evolution of regulatory networks in pathogens. The next-generation sequencing revolution has significantly enhanced our understanding of genome structure—now is the time to begin to explore the dynamics of this structure.

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