Chapter 8
Toxigenic Corynebacteria: Adhesion, Invasion and Host Response

Lisa Ott and Andreas Burkovski

Abstract *Corynebacterium diphtheriae*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* form a distinct group within the genus *Corynebacterium*, the toxigenic corynebacteria. The three species are able to colonize a number of hosts including humans, e.g. in case of diphtheria. However, besides diphtheria toxin action, information about molecular mechanisms of host pathogen interaction is rare. The recent availability of genome sequence information gave new impetus to the characterization of putative virulence factors of *C. ulcerans*, *C. pseudotuberculosis* and *C. diphtheriae* and the recent knowledge about these is summarized here.

Keywords Adhesion · Diphtheria toxin · Inflammatory pathways · Invasion · Phagocytosis

8.1 Introduction

Corynebacteria are morphologically similar, irregular- or clubbed-shaped microorganisms, which belong to the class of Actinobacteria (high G + C Gram-positive bacteria) (Ventura et al. 2007; Zhi et al. 2009). Almost all members of the genus are characterized by complex cell envelope architecture. In these bacteria the cytoplasmic membrane is covered by a peptidoglycan layer, a layer of arabinogalactan polymer, which is covalently linked to the peptidoglycan, and an outer lipid layer composed of mycolic acids (corynomycolates), which is functionally equivalent to the outer membrane of Gram-negatives (Hoffmann et al. 2008; Niederweis et al. 2010; Burkovski 2013).

A. Burkovski (✉) · L. Ott
Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, 91058, Erlangen, Germany
e-mail: andreas.burkovski@fau.de

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Today, the genus *Corynebacterium* comprises 88 species, including soil bacteria with biotechnological importance, commensals found on skin of humans and animals as well as pathogens. Often, members of the latter group are not highly virulent and only immune-compromised patients are infected. Sometimes, virulence is dramatically increased when bacteria are lysogenized by toxin-encoding phages, as in the case of *Corynebacterium diphtheriae* and related toxigenic corynebacteria.

C. diphtheriae is the classical etiological agent of diphtheria and the type species of the genus *Corynebacterium* (Lehmann and Neumann 1896; Barksdale 1970). Its main virulence factor, the diphtheria toxin, is encoded by a corynebacteriophage integrated into the genome of toxigenic strains. As a result of infection with *tox* gene carrying corynebacteriophages, two closely related species, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, can produce diphtheria toxin as well (Groman et al. 1984; Buck et al. 1985; Cianciotto and Groman 1996). *C. ulcerans* has been detected as a commensal in domestic and wild animals (Schuhegger et al. 2009; Dixon 2010; Sykes et al. 2010) that may serve as reservoir for zoonotic infections. Human infections usually occur in adults, who consumed raw milk (Bostock et al. 1984; Hart 1984) or had close contact with domestic animals (Wagner et al. 2010); no person to person transmission was reported up to now. *C. pseudotuberculosis* is the etiological agent of caseous lymphadenitis of sheep, goats and other farm and wild animals (Dorella et al. 2006; Baird and Fontaine 2007). Infections due to *C. pseudotuberculosis* are rare in humans; occasionally, the pathogen is isolated from cases of suppurative lymphadenitis in patients with a classical risk exposure of close contact with sheep. Despite the putative presence of a diphtheria toxin in *C. pseudotuberculosis*, diphtheria of respiratory tract or skin is not evoked by this bacterium.

8.2 *Corynebacterium diphtheriae:* Adhesion, Invasion and Host Response

C. diphtheriae causes diphtheria of the upper respiratory tract and cutaneous diphtheria, which outnumbers the classical respiratory diphtheria in tropical areas. Besides these infections, toxigenic and non-toxigenic *C. diphtheriae* strains are associated with other diseases, e.g. endocarditis, osteomyelitis and septic arthritis (Puliti et al. 2006; Hirata et al. 2008), indicating that *C. diphtheriae* is able to colonize not only epithelia but also deeper parts of the body and that the bacteria interact with various types of host cells.

8.2.1 Adhesion

Experimental data regarding host recognition and adhesion by *C. diphtheriae* are limited; however, a few adhesion factors were described, the best investigated being

pili structures on the surface of *C. diphtheriae* which are covalently attached to the cell wall peptidoglycan (Navarre and Schneewind 1999). The genome of *C. diphtheriae* NCTC 13129 encodes three distinct pili clusters (*spaABC*, *spaDEF*, *spaGHI*), which are organized together with sortase-encoding genes (*srtA*, *srtB*, *srtC*, *srtD, srtE* and *srtF*) (Ton-That and Schneewind 2003). Corynebacterial pili consist of a major pilin subunit SpaA (SpaD and SpaH, respectively), a minor subunit SpaB (SpaE and SpaG, respectively) and the tip protein SpaC (SpaF and SpaI, respectively) (Ton-That and Schneewind 2003). The major pilin proteins carry a cell wall sorting signal at the C-terminus, composed of an LPxTG motif, followed by a hydrophobic membrane-spanning domain and a positively charged tail (Schneewind et al. 1993). This motif is responsible for efficient anchoring of pili to the cell wall by housekeeping sortase SrtF. The additional sortases SrtA-E catalyze the covalent cross-linking of single pilin monomers to form the pilus. The specificity of the pilus subunits for the corresponding sortases seems to be determined by the amino acid sequence LxET, designated as E box and localized between the LPxTG motif and the pilin motif WPK (Ton-That et al. 2004). The E box is conserved in several Gram-positive species that encode sortase and pilin subunit genes with sorting signals and pilin motifs. Minor pilin subunits contain the LPxTG motif but neither the E box nor the pilin motif was detected.

Mandlik and co-workers systematically address the function of the different pili as well as their individual pilin subunits in respect to corynebacterial adherence by the use of different epithelial cell lines and a number of *C. diphtheriae* mutant strains (Mandlik et al. 2007). These analyses revealed that only the SpaA-type pili contribute to corynebacterial adherence to human pharyngeal cells (Detroit 562), while the SpaD and SpaH-type pili support adhesion to laryngeal (HEp-2) and lung cells (A549). Strains expressing only SpaA-type pili were able to adhere to pharyngeal cells properly but showed less adhesion to other cell lines. The deletion of *srtA*, which prevents crosslinking of SpaA-type pili subunits and consequently proper pili formation, impaired binding to pharyngeal cells. Mutant strains lacking all sortases showed decreased adhesion rates to different epithelial cell lines. Interestingly, it was shown that the minor pilin subunits do not only decorate the pilus shaft but are also distributed in clusters all over the bacterial surface. These minor pilins directly anchored to the cell wall are sufficient to mediate specific host cell adhesion. The deletion of either *spaB* or *spaC* resulted in significantly lower adhesion rates and the *spaBC* double mutant revealed marginal binding to Detroit 562 cells. Furthermore, the incubation of bacteria cells with antibodies directed against SpaB, SpaC blocked corynebacterial adhesion to pharyngeal cells; this was not the case after pre-incubation with an antibody directed against SpaA (Mandlik et al. 2007).

In summary, *C. diphtheriae* strain NCTC 13129 carries genes for three pili clusters, with the minor pili subunits being crucial for adhesion and the major subunits determining pili length (Gaspar and Ton-That 2006; Scott and Zahner 2006; Swierczynski and Ton-That 2006). When other isolates were investigated, strain-specific differences in pili formation and adhesion properties were observed; these are summarized in Table 8.1.

Table 8.1 Adhesion rates of different *C. diphtheriae* strains

Strain	Growth medium	Time p. i. [min]	Adhesion rate [%]	Cell line	Reference
ISS3319	HI (Heart Infusion)	90	3.55±0.28	Detroit 562 (pharyngeal carcinoma cells)	(Ott et al. 2010b)
ISS4060			3.99±0.27		
ISS4746			6.54±0.97		
ISS4749			7.34±2.33		
DSM43988			0.69±0.12		
DSM43989			0.34±0.05		
DSM44123			1.84±0.26		
NCTC 13129		60	9.00		(Mandlik et al. 2007)
ISS3319	HI	90	58.39±8.25	HeLa (cervix carcinoma cells)	(Ott et al. 2013)
ISS4060			72.38±13.74		
ISS4746			42.23±2.50		
ISS4749			53.56±5.23		
DSM43988			69.92±5.87		
DSM43989			1.25±0.14		
DSM44123			40.87±1.00		
NCTC 13129		60	25.00	HEp-2 (laryngeal carcinoma cells)	(Mandlik et al. 2007)
241	TSB (Tryptic Soy Broth)	30	61.86		(Moreira et al. 2003)
241		120	32.98		
241	TSB-Fe	30	91.75		
241		120	57.20		
CDC-E8392	TSB	30	18.29		
CDC-E8392		120	59.11		
CDC-E8392	TSB-Fe	30	15.21		
CDC-E8392		120	58.02		
CDC-E8392	TSB	30	23.40		(Hirata et al. 2004)
		60	27.60		
		120	30.10		
		180	38.30		
		360	3.80		
NCTC 13129	HI	60	20.00	A549 (lung carcinoma cells)	(Mandlik et al. 2007)

In order to address these strain-specific differences in adhesion rates, Ott and co-workers investigated the surface structures of several wild type strains by atomic force microscopy. This approach revealed significantly different macromolecular surface structures of the wild type strains tested. Interestingly, not all strains investigated by Ott and co-workers showed pili-like structures on the surface and the pili observed differed in length and number. Some isolates completely lacked pili, some presented short spike-like structures on their surface, while others showed long,

hair-like protrusions. Interestingly, adhesion and pili formation were not strictly coupled, as strain ISS4060, which lacks pili-like structures, shows comparable adhesion rates to strain ISS3319, which revealed spike-like pili (Ott et al. 2010b). RNA hybridization experiments carried out in order to investigate mRNA levels of the different *spa* genes (Ott et al. 2010b) revealed distinct differences in the expression pattern of pili subunits for the strains investigated. Transcripts detected belong to the *spaABC* or *spaGHI* cluster, mRNA of genes of the *spaDEF* cluster was not found in these strains. Subsequently carried out PCR experiments indicated corresponding strain-specific differences in the presence of the different genes. A recent pangenomic study of twelve isolates from patients with classical diphtheria, endocarditis and pneumonia provided further evidence for a high degree of genetic variability between different *C. diphtheriae* strains (Trost et al. 2012). A fourth type of pili was observed in this analysis, and despite the fact that all strains contained at least two pili gene cluster, the single clusters were differently distributed among the analyzed isolates. The reasons for strain-specific differences in respect to the pili repertoire remain unclear; probably, these are connected to the binding of different receptors on the eukaryotic surface. This strategy might also be reflected by the existence of adhesion factors besides pili.

One important adhesin in this respect might be the non-fimbrial surface protein 67–72p, named according to its initial identification as two bands with distinct apparent mass in SDS-PAGE (Colombo et al. 2001). Recent studies revealed that the 67–72p protein seems to be encoded by a single gene, DIP0733 (Sabbadini et al. 2012). Both, the 67 and 72 kDa polypeptide bind to erythrocyte receptors, leading to hemagglutination. Hirata and co-workers (Hirata et al. 2004) demonstrated that 67–72p binds not only human erythrocytes but also the human epidermoid laryngeal carcinoma cell line HEp-2 and that binding was effectively blocked by anti-67–72p IgG antibodies. A negative correlation between bacterial adhesion to HEp-2 cells and erythrocytes was found; strains with low hemagglutinating activity showed high adhesion rates to HEp-2 cells and *vice versa*.

Erythrocytes are not the only targets of components of *C. diphtheriae* within the blood; another target is fibrinogen, a major component of the human plasma. The main role of this protein lies in blood clot formation by its conversion into insoluble fibrin, which forms the matrix of the clot. This process is hijacked by many pathogens, e.g. by the majority of virulent *Staphylococcus* strains (Duthie 1954) and by *Yersinia pestis* (Beesley et al. 1967; Rivera et al. 2007), by producing an enzyme termed coagulase. Sabbadini and co-workers demonstrated that *C. diphtheriae* is also able to bind to fibrinogen and convert fibrinogen to fibrin (Sabbadini et al. 2010). Non-toxigenic and toxigenic strains formed bacterial aggregates in the presence of plasma and fibrinogen binding occurred in varying intensities. The fibrinogen polymerisation process might result in a fibrin layer on the bacterial cell wall, and the binding of fibrinogen to the surface of *C. diphtheriae* may be an efficient method to avoid phagocytosis. This was already shown for other Gram-positive pathogens (Schubert et al. 2002; Rennermalm et al. 2004; Pierno et al. 2006). In addition to that, Sabbadini and co-workers (Sabbadini et al. 2010) suggested that the conversion of fibrinogen

to fibrin may be connected to pseudomembrane formation, since differences in the abilities to bind and convert fibrinogen may partially explain differences in the extent of pseudomembrane formation during diphtheria.

Besides host factors such as fibrinogen, sugar moieties and sugar-converting enzymes might be involved in bacterial surface decoration and camouflage to mislead the host's immune system. In this respect, sialidases, also called neuraminidases, play an important role in the virulence of many pathogenic organisms. Sialidases belong to a class of glycosyl hydrolases that catalyze the removal of terminal sialic acid residues from a variety of glycoconjugates of the host surface (Vimr 1992), which can subsequently used a nutrient or for bacterial surface decoration. In fact, *C. diphtheriae* exposes sialic acids on its surface, but the genes involved in sialic acid cell wall binding have not been identified so far. Sialidase activity was first observed in a crude diphtheria toxin preparation (Blumberg and Warren 1961). Later, exo-sialidase activity was further characterized by Warren and co-workers (Warren and Spearing 1963), who showed that the exo-sialidase is induced in iron-enriched cultures. Moreira and coworkers found that the iron concentration in the culture medium directly affects sialidase production and cell surface carbohydrates of *C. diphtheriae* (Moreira et al. 2003). The work led to the identification of a putative exo-sialidase NanH (DIP0543) from *C. diphtheriae*. Biochemical studies of purified NanH heterologously expressed in *E. coli* revealed the highest cleavage rate for the α-2,6-linked sialic acids of sialyllactose, with comparable activity for the α-2,3-linked sialic acids. Furthermore, *C. diphtheriae* NanH showed trans-sialylation activity using sialyl-α2,3-lactose and sialyl-α2,6-lactose as donors. In addition to that, it could also catalyze the transfer of sialic acids from other sialoconjugates to 4-methylumbelliferyl-a-D-galactopyrano-side (MU-Gal) and the sialidase-mediated transglycosylation reaction using sialic acid-conjugated free oligosaccharides and glycoproteins as donors. *C. diphtheriae* may transport sialic acids hydrolyzed by extracellular sialidases for synthesis of sialic acid derivates. However, it has still to be clarified whether NanH is involved in sialic acids decoration or not (Kim et al. 2010).

Hemagglutination was found to be resistant to heating at 100°C, detergents and trypsin treatment (Colombo et al. 2001), suggesting the additional involvement of non-proteinaceous molecules in this process and probably also in adhesion to other cell types. One candidate factor in respect to adhesion is a 10 kDa lipoglycan of *C. diphtheriae*, designated CdiLAM, which shows similarity to mycobacterial LAM, and is presented on bacterial surface. Moreira and co-workers (Moreira et al. 2008) found that in contrast to LAMs of other actinomycetes, CdiLAM presents an unusual substitution at position 4 of α 1→6 mannan backbone by α-*D*-Ara*f*. Unlike the non-fimbrial adhesin 67–72p, CdiLAM does not bind to human erythrocytes but to human respiratory epithelial cells. Pre-incubation of bacterial cells with anti-CdiLAM IgG antibodies or pre-incubation of cells with purified CdiLAM blocked adhesion effectively, indicating CdiLAM as a specific adhesin to human respiratory cells.

Table 8.2 Adhesion factors of *C. diphtheriae*

Adhesion factor	Function
SpaA type pili	contribute to adherence to Detroit 562 and A549 cells
67–72p protein (DIP0733)	hemagglutination; adherence to HEp-2 cells
coagulase	binding of fibrinogen and conversion to fibrin
NanH	sialidase activity
CdiLAM	binding of HEp-2 cells

8.2.2 Invasion

Besides questions concerning host recognition and adhesion, there are still open questions with respect to the infection process and spread of bacteria. *C. diphtheriae* was generally considered as an extracellular colonizer of the upper respiratory tract; however, failure of penicillin treatment in cases of severe pharyngitis and tonsillitis could only partially be explained by penicillin tolerance (von Hunolstein et al. 2002). Furthermore, the occurrence of diphtheria among vaccinated adults in Rio de Janeiro, as well as an increasing number of cases of endocarditis caused by non-toxigenic strains, indicates the possibility of deeper colonization of the human host (Mattos-Guaraldi et al. 2000; Mattos-Guaraldi et al. 2001). Cases of invasive disease with non-toxigenic strains have been reported for injection drug users in Switzerland (Gubler et al. 1998), Aborigines in Australia (Hogg et al. 1996; Holthouse et al. 1998), homeless people in France (Patey et al. 1997), patients with bone and joint infections in Germany (Funke et al. 1999) and patients with invasive bloodstream infections in North America (Romney et al. 2006). Taken together, these observations lead to the assumption that the bacteria are not only able to adhere to host cells of the respiratory tract but are also able to gain access into deeper tissues.

Hirata and co-workers (Hirata et al. 2002) studied *C. diphtheriae* with respect to its ability to enter and survive within HEp-2 monolayers, using gentamicin protection assays. Gentamicin is an antibiotic which is not able to pass eukaryotic membranes and kills extracellular adherent bacteria, while intracellular bacteria are protected. Strain-specific differences in respect to internalization into HEp-2 cells were found, whereas no correlation between adhesion and invasion rates was observed. Thin-section electron micrograph of *C. diphtheriae* revealed the presence of bacteria inside a membrane-bound vacuole in close proximity to the nuclear membrane. Since it was assumed that actin polymerization is triggered by *C. diphtheriae* to induce uptake in HEp-2 cells (Mattos-Guaraldi et al. 2002), the effect of cytochalasin D, a known inhibitor of eukaryotic microfilament formation, on the adhesion and invasion process was investigated. In fact, internalization of various strains was completely blocked by cytochalasin E, supporting the assumption that cytoskeletal rearrangements are required for bacterial entry. Furthermore, phosphotyrosine-signaling seems to be a relevant mechanism during the invasion process in cultured respiratory cells.

Similar experiments were carried out with the pharyngeal carcinoma cell line Detroit 562 (Bertuccini et al. 2004). Ultrastructural analysis of the internalization process revealed a localized pattern of adherence on colonized Detroit 562 cells, which seemed to be mediated by cellular microvilli, establishing a close contact to the bacterial cell wall. Bertuccini and co-workers observed a ring-like structure, with a ruffle-like appearance, which is part of the host cell surface during the internalization of a single bacteria cell. Cytochalasin D showed a dose-dependent inhibitory effect on the internalization process, pointing to the involvement of F-actin in the bacterial entry. Bacterial binding seems to induce intracellular signal transduction responsible for actin microfilament rearrangements (Rosenshine et al. 1992). In contrast, colchicine had no effect on the number of internalized bacteria, indicating that microtubuli are not involved in bacterial uptake. Staurosporine, an inhibitor of different classes of phosphokinases, positively affected *C. diphtheriae* internalization, indicating that the activation of phosphokinases negatively controlled bacterial uptake. In summary, the possibility to enter the host cell, to hide in epithelial cells even at low numbers and to persist at least for 48 h, gives the bacteria the opportunity to escape from the hosts immune response and antibiotic treatment. The fact that *C. diphtheriae* is able to survive significantly longer within Detroit 562 cells than in HEp-2 cells might indicate better adaption of the bacteria to upper respiratory tract epithelia.

Using an immune-fluorescence microscopy approach, Ott and co-workers (Ott et al. 2010b) observed V-shaped *C. diphtheriae* dimers within the host cell. V-forms are *Corynebacterium*-specific snapping division stages and indicate proliferating bacteria. Consequently, it can be assumed that *C. diphtheriae* is able to replicate within the host cells, and growth and elimination are parallel processes.

The molecular mechanism of invasion is more or less unclear. Ott and co-workers (Ott et al. 2010a) focused their work on the surface-associated protein DIP1281, a member of the NlpC/P60 family (Anantharaman and Aravind 2003), which was annotated as hypothetical invasion-associated protein. NlpC/P60 proteins define a large superfamily of proteins found in bacteria, bacteriophages, RNA viruses and eukaryotes, and several members are conserved among corynebacteria. DIP1281 mutant strains almost completely lacked the ability to adhere to host cells and, in contrast to the wild type, no intracellular bacteria were detectable in gentamicin protection assays. Further characterization of the mutant strains by immuno-fluorescence microscopy, using an antibody specific for the *C. diphtheriae* surface proteome, revealed an increased size of the single bacteria, an altered less club-like shape and formation of chains of bacteria rather than the typical V-like cell division forms or palisades of clustered *C. diphtheriae*. LIVE/DEAD staining showed that all bacteria within the observed chains were still viable and no differences were detectable between wild type and mutants. Since the mutants showed an altered antibody binding in immune-fluorescence microscopy approaches, Ott and co-workers (Ott et al. 2010a) performed a more detailed investigation of the surface proteomes of wild types and mutant strains by two-dimensional gel electrophoresis. The mutants showed a decreased number of spots in the upper molecular weight range and an increased number of spots in the lower molecular weight range. Clear differences between wild type and mutant protein patterns were also found by atomic

force microscopy. Wild type cells showed round elevations with a lateral diameter of 10–40 nm at a height of 1–4 nm at their surface, while DIP1281 mutants lost this fine structure and elongated elevation with a width of 50–100 nm was observed. In summary, DIP1281 seems to be crucial for the organization of the outer surface layer of *C. diphtheriae*. Tsuge and co-workers reported similar observations for *C. glutamicum* R, when the DIP1281 homolog cgR_1596 and other members of the NlpC/P60 protein familiy cgR_2070 were mutated (Tsuge et al. 2008). Taken together, DIP1281 and its homologs Ce1659, Cg1735 and Jk0967 in *Corynebacterium efficiens*, *C. glutamicum* and *C. jeikeium*, which were previously annotated as hypothetical invasion-associated proteins, seem to be predominantly involved in the organization of the outer layer of corynebacteria rather than in the separation of the peptidoglycan of dividing bacteria. Mutations induce pleiotropic effects which also influence adhesion and invasion.

Sabbadini and co-workers (Sabbadini et al. 2012) studied the role of surface-exposed non-fimbrial 67–72p protein (DIP0733), previously described as adhesin/hemagglutinin (Colombo et al. 2001; Hirata et al. 2004), in respect to internalization of *C. diphtheriae* in HEp-2 cells. Investigations of the interaction of HEp-2 cells with 67–72p-coated beads by optical and transmission electron microscopy demonstrated the autoaggregative and hydrophobic properties of 67–72p as well as the internalization of 67–72p-adsorbed microspheres by HEp-2 cells. Pretreatment of bacterial suspensions or HEp-2 cells with 67–72p led to an increased number of bacteria in the supernatants of infection assays, while the number of internalized bacteria was decreased (Sabbadini et al. 2012), indicating a shielding of receptors or a jamming of the internalization machinery by 67–72p.

8.2.3 Host Immune Response

The mammalian immune system includes innate and adaptive components, with the innate immunity providing the first line of defense against infectious diseases, while the adaptive immune system is activated only due to exposition to an antigenic challenge. Most bacteria infecting healthy vertebrates are readily cleared within a few days after infection, before activating the adaptive immune response. In this case, microorganisms are recognized by the innate immune system through a limited number of pattern-recognition receptors (PRRs) (Akira et al. 2006). These receptors, which recognize pathogen-associated molecular patterns (PAMPs), are constitutively expressed on all cells of a given type and detect pathogens in any life-cycle stage. The interaction of PRRs with specific PAMPs leads to activation of specific inflammatory signaling pathways, resulting in specific antimicrobial response (Akira et al. 2006). A prominent group of PRRs are Toll-like receptors (TLRs) which are conserved from *Caenorhabditis elegans* to mammals (Medzhitov and Janeway 2002; Hoffmann 2003; Akira and Takeda 2004; Beutler 2004). To date, twelve members of the TLR family have been identified, described as type I integral membrane glycoproteins with extracellular domains containing varying numbers of leucins-rich-repeats (LRRs) and a cytoplasmatic signaling domain, named the Toll/

IL-1 R (TIR) homology domain, due to its homology to interleukin 1 receptor (IL-1 R) (Bowie and O'Neill 2000). Members of the TLR family are able to recognize lipopolysaccharide (LPS) from Gram-negative bacteria, whereas in Gram-positives, which lack LPS, lipoteichoic acids (LTA), mycolic acids and arabinogalactan (in case of mycobacteria, nocardia and corynebacteria) are recognized (Akira et al. 2006). The activation of TRLs leads to activation and recruitment of phagocytic cells, such as macrophages and neutrophils, to the site of infection. Furthermore, TLR signaling through conserved signaling elements leads to transcription of distinct proinflammatory genes and consequently to the production of cytokines, interferons, chemokines, cell surface molecules and chemokine receptors (Sioud 2005).

8.2.4 Cytokine Production During Infection with C. diphtheriae

As mentioned above, besides evoking diphtheria, *C. diphtheriae* is associated with invasive infections, such as endocarditis, septic arthritis, splenic abscesses, bacteraemia and osteomyelitis, which are usually caused by non-toxigenic strains (Alexander 1984; Tiley et al. 1993; Poilane et al. 1995; Funke et al. 1999; Belko et al. 2000; Mattos-Guaraldi et al. 2001). The clinical disease pattern of human diphtheroid arthritis can occur as an acute, purulent process or as a chronic arthritis with plasmacytic or histiocytic inflammation. Puliti and coworkers introduced mice as a model system for septic arthritis (Puliti et al. 2006). Depending on the number of bacteria injected, mice died or developed clinical signs of arthritis, most frequently in ankles and wrists. By quantitative monitoring of *C. diphtheriae,* Puliti and coworkers (Puliti et al. 2006) also demonstrated that *C. diphtheriae* bacteria were cleared from the bloodstream within five days after infection, but the bacteria were able to persist in kidneys and spleens for more than two weeks after infection. Similar to cases reported of osteoarthritis in humans, histopathological examinations of mice inoculated with *C. diphtheriae* revealed a mild to moderate arthritis including the presence of inflammatory cells. Cytokine levels of serum and joint samples of infected mice were determined by ELISA. The investigated non-toxigenic strains induced systemic and local IL-6, IL-1β and TNFα secretion, which might contribute to articular damage in cases of septic arthritis based on the fact that some strains evoked severe arthritis symptoms and high levels of IL-6 and IL-1β, while infection with other strains led to low IL-6 and IL-1β synthesis together with a low incidence and severity of arthritis. Interestingly, induction of high and early IFN-γ production was only observed in mice infected with a distinct isolate, indicating that IFN-γ production counterbalances the proinflammatory response.

Induction of host response was investigated *in vitro* at a molecular level by Ott and co-workers, using luciferase-expressing reporter cell lines to monitor the induction of various host signaling pathways. Strain- and cell line-specific differences were found in respect to induction of N-FAT, NFκB, STAT5RE and AP-1 (unpublished observations). Furthermore, Ott and co-workers demonstrated that induction of the NFκB transduction pathway in epithelial cells only occurred in response to

incubation with living bacteria and required invasion of the bacteria into the epithelial cells (Ott et al. 2013).

8.2.5 Phagocytosis and Induction of Apoptosis

Antibodies are present in plasma and extracellular liquids and protect against pathogens or toxigenic products by binding of antigens to inhibit their interaction with the host cells and thus avoiding their damage, a process termed neutralization. In case of bacteria proliferating in the extracellular space, antibodies recruit phagocytes that internalize bacteria for degradation. This mechanism is specific for bacteria that are able to escape direct recognition of phagocytes. In this case, the bacteria are labeled with antibodies for ingestion by phagocytes, a process called opsonization. Additionally, antibodies activate the complement system, a system of plasma proteins, which is enabling phagocytes to ingest and degrade bacterial cells that are otherwise undetectable for the cells.

Non-opsonic phagocytosis, mediated by phagocyte receptors that recognize structures on microbial surfaces, have become of greater interest as a potential host defense mechanism against intracellular pathogens (Ofek et al. 1995). Dos Santos and co-workers (dos Santos et al. 2010) investigated the ability of homologous non-toxigenic and toxigenic *C. diphtheriae* strains to be phagocytozed by the human macrophage cell line U937 in the absence of immune serum (opsonins). The fact, human macrophages were able to ingest *C. diphtheriae* bacilli by an antibody-independent mechanism. The highest number of cell-associated bacteria of the non-toxigenic strain was observed 3 h post infection and non-toxigenic *C. diphtheriae* able to survive within U937 cells for a time period of 24 h. The observation that the internalized non-toxigenic bacteria were able to survive longer periods within the macrophages might indicate that human macrophages may be not effective in killing *C. diphtheriae* in the absence of opsonins (dos Santos et al. 2010). The tox^+ strain showed significant higher numbers of associated bacteria after 1 to 2 h post infection, but was not able to survive within the cells over a time period of 24 h. Non-opsonic phagocytosis of both toxigenic and non-toxigenic strains induced cytopathogenicity in U937 cells. More than 42% of U937 cells were killed during infection with the tox^+ strain (3h post infection), while roughly 18% were killed during infection with the non-toxigenic strain. Changes of nuclear morphology upon *C. diphtheriae* infection were indicating that *C. diphtheriae* induces apoptosis and necrosis in human macrophages, independent of the presence of the diphtheria toxin.

Sabbadini and co-workers (Sabbadini et al. 2012) made similar observations concerning apoptosis induction during the infection of non-phagocytic cell line HEp-2 with *C. diphtheriae*. Blebs and "budding" cells were found within 3 to 6 h of treatment with 67–72p. The assessment of apoptosis by double staining propidium iodide (PI)/Annexin V (AV) revealed significant numbers of early (PI^-/AV^+) and late (PI^+/AV^+) apoptotic HEp-2 cells treated for 6 h with 67–72p. Using the trypan blue exclusion method Sabbadini and co-workers (Sabbadini et al. 2012) showed

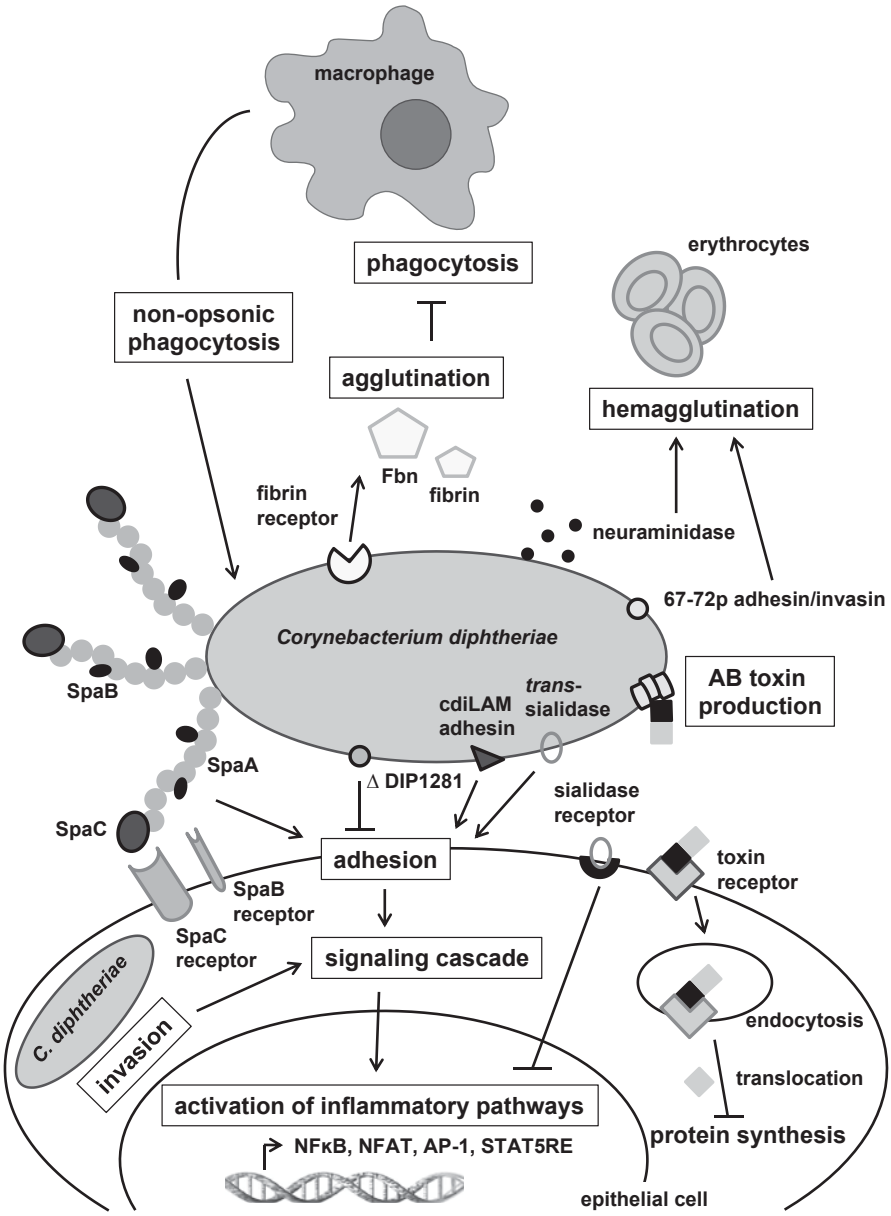

Fig. 8.1 Infection properties of *C. diphtheriae.* The most prominent virulence factor of *C. diphtheriae* is the diphtheria toxin which is encoded by a β-corynephage integrated in the bacterial chromosome. This AB toxin is released by the bacterial cell and binds the toxin receptor (HB-EGF) on the host's surface. The toxin gets internalized by endocytosis which leads to translocation of the A-fragment and inhibition of the protein biosynthesis by binding of the elongation factor 2. Best investigated adhesion factors of *C. diphtheriae* might be the pili structures. Strain NCTC 13129 exhibits three different pilus types, the SpaABC type pilus is depicted here exemplarily. SpaA forms the pilus shaft, SpaB is linked to SpaA via transpeptidation, and SpaC forms the tip

that the plasma membrane of HEp-2 cells treated with 67–72p suffered permeability changes, vacuolization and DNA fragmentation after 12 h. A high occurrence of apoptotic bodies was observed after 24 h, as well as a detachment of HEp-2 cells post treatment with 67–72p. DNA condensation and aggregation was observed by DAPI staining of 67–72p treated cells. Furthermore, the incubation of HEp-2 cells with 67–72p caused a reduction in cell volume which is a distinct marker of apoptosis, termed apoptotic volume decrease (Bortner and Cidlowski 2002).

The ability of *C. diphtheriae* to kill non-phagocytic cells might be beneficial for the survival and the invasion of deeper tissue of this organism and thus to disperse in the whole body. In summary, the interaction of the Gram-positive pathogen *C. diphtheriae* with its host is much more complex than initially expected. Up to now, several different interaction mechanisms were elucidated (summarized in Fig. 8.1), but there is still room for research, especially regarding interactions at the molecular level. Based on the already described results and ongoing research, *C. diphtheriae* might work as a model for other pathogenic but molecular biologically less addressable corynebacteria such as *C. jeikeium*.

8.3 Pathogenicity of *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*

Besides *C. diphtheriae*, the most prominent member of toxigenic corynebacteria, two further species might carry toxin-encoding corynebacteriophages and might have impact on human health, *C. ulcerans* and *C. pseudotuberculosis*.

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of the pilus. SpaB and SpaC are able to bind receptors on the surface of the host cell thus enabling colonization of the cell. Besides pili formation the non-fimbrial protein 67–72p (DIP0733) might be an important adhesin. Via this protein *C. diphtheriae* is able to bind erythrocytes, a process called hemagglutination which facilitates *C. diphtheriae* to disseminate throughout the body via the blood. Furthermore, the 67–72p protein seems to be involved in the internalization process and might contribute to apoptosis and necrosis of the host cell. Neuraminidase-secreting strains were also found to induce agglutinating human erythrocytes effectively. In the case of the *C. diphtheriae* sialidase NanH, it is still unclear whether it is involved in sialic acids decoration or not. Besides proteins as adhesion factors, *C. diphtheriae* exhibits a 10 kDa lipoglycan on its surface, the CdiLAM, which binds HEp-2 cells. Additionally, *C. diphtheriae* is able to bind fibrinogen and to convert it to fibrin, resulting in a fibrin layer on the bacterial surface that may be an efficient method to avoid phagocytosis. In addition to adhesion, *C. diphtheriae* is able to internalize into non-phagocytic cells in a strain-specific manner. The invasion process seems to involve actin polymerization along with a phosphotyrosine signaling event in cultured respiratory cells. Investigations of DIP1281 (annotated as invasion-associated protein) revealed the involvement in the organization of the outer protein layer which resulted in an adhesion- and invasion-negative phenotype of the corresponding mutant strains. Furthermore, infection of epithelial cells leads to inflammatory host response, especially the activation of the NFκB signal transduction pathway. NFκB induction is strictly dependent on intracellular bacteria, since bacterial adhesion was not sufficient to activate inflammatory pathways. *C. diphtheriae* taken up by human macrophages via non-opsonic phagocytosis are able to persist inside the macrophages and to induce apoptosis and necrosis

C. ulcerans was first isolated in 1926 from a patient with throat lesions and symptoms of a diphtheria-like illness (Gilbert and Stewart 1926). Infections of humans have been classically associated with consumption of raw milk products or contact with cattle, but the bacterium was increasingly found in domestic animals, such as dogs and cats, suggesting that clinical cases in humans are more likely devolved from domestic pets (Taylor et al. 2002; De Zoysa et al. 2005; Lartigue et al. 2005). In the last decade the majority of diphtheria cases reported in Europe were caused by *C. ulcerans* (Wagner et al. 2010), indicating that this organism plays an increasing role as a zoonotic pathogen.

C. pseudotuberculosis biovar *ovis* and *equi* cause caseous lymphadentitis in sheep and goats (Batey 1986) and ulcerative lymphangitis in horses (Brumbaugh and Ekman 1981). Interestingly, *C. pseudotuberculosis* is a facultative intracellular pathogen that is able to survive and grow in macrophages, thus escaping the immune response of the host (McKean et al. 2005; Dorella et al. 2006).

8.3.1 *Gene Regions Encoding Adhesive Pili Subunits in* C. ulcerans *and* C. pseudotuberculosis

As in case of *C. diphtheriae*, also *C. ulcerans* and *C. pseudotuberculosis* genome sequences encode different putative pili. In *C. ulcerans* one gene cluster is identically organized compared to *spaDEF* from *C. diphtheriae* NCTC 13129, while the second pili cluster, *spaBC*, lacks the gene encoding major pili subunit SpaA (Trost et al. 2011). It is very likely that homodimeric/heterodimeric SpaB/SpaC proteins in *C. ulcerans*, anchored covalently to the cell wall, can provide close contact between the bacterial surface and host cell tissues even without the shaft protein as already shown for the minor pilin SpaB in *C. diphtheriae* NCTC 13129 (Mandlik et al. 2007; Chang et al. 2011). The genes in *C. pseudotuberculosis* are organized in two clusters (*spaABC* and *spaDEF*) together with their corresponding sortase-encoding genes (*srtB* and *srtC*) (Trost et al. 2010). The pili of *C. pseudotuberculosis* FRC41 are composed of major pilin subunits (SpaA and SpaD), minor pilin subunits (SpaB and SpaE) and tip proteins (SpaC and SpaF). Interestingly, there are two hypothetical proteins, SpaX (encoded in cluster *spaABC*) and SpaY (encoded in cluster *spaDEF*), with unknown function (Trost et al. 2010).

8.3.2 *Phospholipase D Activity in* C. ulcerans *and* C. pseudotuberculosis

C. ulcerans and *C. pseudotuberculosis* carry the gene for phospholipase D (PLD), the so called “ovis toxin”, a sphingomylinase (phosphatidylcholine phosphohydrolase, EC 3.1.4.4). Phospholipase D activity has been found in no other species of the genus *Corynebacterium* which makes PLD a distinctive taxonomic marker (Barksdale et al. 1981).

Although the role of phospholipases in disease and pathogenesis must often be interpreted with caution, phospholipases may be directly toxic, with their interaction with the cell's plasma membrane being the main factor. Additionally, a metabolic function was discussed as well (Ostroff et al. 1989; Shortridge et al. 1992). An illustrative example for cell damage is the formation of membrane vesicles in erythrocytes infected with *C. pseudotuberculosis* (Brogden et al. 1990), as consequence of sphingomyelin depletion of the outer leaf and invagination of the inner sheet of the membrane (Low et al. 1974). Detrimental effects of *C. pseudotuberculosis* PLD were also shown for ovine neutrophils (Jozwiak et al. 1993). Furthermore, Muckle and co-workers (Muckle and Gyles 1983) found that *C. pseudotuberculosis* PLD increases vascular permeability which might be advantageous for the bacteria to spread from the initial site of infection to regional lymph nodes (McNamara et al. 1994).

8.3.3 *Further Putative Virulence Factors of* C. ulcerans *and* C. pseudotuberculosis

Trost and co-workers identified a gene encoded secreted protease CP40, also designated Cpp, in *C. ulcerans* (Trost et al. 2011) as well as in *C. pseudotuberculosis* (Trost et al. 2010). Since the extracellular CP40 enzyme has proteolytic activity, it was assumed to play a role in virulence of *C. pseudotuberculosis*, but an enzymatic activity of CP40 was not detectable in the supernatant (Wilson et al. 1995). Nevertheless, vaccination of sheep with this antigen protects against caseous lymphadenitis, supporting the idea that the protein plays a major role in this disease (Walker et al. 1994). The amino acid sequences of CP40 from *C. ulcerans* 809 and *C. pseudotuberculosis* FRC41 showed peculiar similarities to the α-domain of the extracellular endoglycosidase EndoE from *Enterococcus faecalis* which is involved in the degradation of N-linked glycans from ribonuclease B and the hydrolysis if the conserved glycans on IgG (Collin and Fischetti 2004). Hence, both *C. ulcerans* strains 809 and BR-AD22 might be able to interact with the mammalian host cell by modulation of the host glycoproteins.

In addition to CP40, the extracellular neuraminidase NanH poses a putative virulence factor in *C. ulcerans* and *C. pseudotuberculosis* (Trost et al. 2010; Trost et al. 2011). Neuraminidases and sialidases are glycoside hydrolase enzymes that cleave the glycosidic linkages of neuraminic acids and can contribute to the recognition of sialic acids on host cell surfaces (Mattos-Guaraldi et al. 2000; Vimr et al. 2004). NanH of *C. diphtheriae* KCTC3075 was identified as an active extracellular sialidase that could transfer a sialic acid from sialylconjugates to asialoglycans via transglycosylation (Kim et al. 2010). Furthermore, Trost and co-workers could detect a GlxR-binding site upstream of *nanH* in *C. pseudotuberculosis* FRC41, indicating that this virulence factor is under control of the cAMP-sensing transcription regulator GlxR (Trost et al. 2010).

Table 8.3 Prophage like elements in *C. ulcerans* genomes

Prophage	Size	G + C content	Integration site	Attachment site
ΦCULC809I	41.4 kb	53%	CULC809_01141	not detected
ΦCULC22I	42 kb	53%	CULC22_01157	not detected
ΦCULC22II	44.9 kb	55%	between CULC22_01663 and CULC22_01724	TTAGATAC
ΦCULC22III	14 kb	57%	tRNALys	TTCAAGTCCCTGATGGCGCAC
ΦCULC22IV	41 kb	54%	tRNALys	TTGAGCTGGAGATGGGACTT-GAACCC

8.3.4 Further Putative Virulence Factors of C. ulcerans: *Prophage-like Sequences and Toxins*

Since the important corynebacterial virulence factor diphtheria toxin is encoded by corynephages integrated in the genome of toxigenic strains, the genome sequences of the *C. ulcerans* 809 and BR-AD22 were screened for prophage-like elements. These analyses revealed one putative prophage in the genome of *C. ulcerans* 809 and four prophage-like regions in the genome sequence of BR-AD22 (Trost et al. 2011) (Table 8.3, modified after (Trost et al. 2011).

Further characterization of the prophage-like regions revealed that ΦCULC809I from *C. ulcerans* 809 and ΦCULC22I from *C. ulcerans* BR-AD22 are closely related genetic elements. The two prophages share 36 genes which encode proteins comprising approximately 98% sequence identity. The previously suggested presence of a diphtheria toxin-encoding β-corynephage in strain 809 (Mattos-Guaraldi et al. 2008) was not confirmed. However, sequence analysis of a tyrosine recombinase of *C. ulcerans* and the integrase of the β-corynephage integrated in the genome of *C. diphtheriae* NCTC 13129 showed 92% sequence identity, supporting the assumption that a β-corynephage-like element had been integrated in *C. ulcerans* 809 and BR-AD22 genomes in former times.

While a diphtheria toxin-encoding gene is absent in the genome, *C. ulcerans* 809 carries a gene coding for a Shiga toxin-like ribosome-binding protein, Rbp. The deduced amino acid sequence of this protein shares only 24% identity with the A chains of the Shiga-like toxins SLT-1 and SLT-2 of *E. coli*, but comprises all highly conserved amino acid residues needed for the catalytic N-glycosidase activity (Trost et al. 2011). SLT-1 usually consists of a catalytic A domain that is non-covalently linked with a pentamer of chains B which are essential for binding to the specific glycolipid receptor and the translocation of the toxin into the endoplasmatic reticulum (ER) of the host cell. This leads to the retranslocation of the catalytic chain A into the cytosol, followed by the abortion of the protein biosynthesis by depurination of a single adenosine residue in the 28S rRNA of the eukaryotic ribosome (O'Loughlin and Robins-Browne 2001). Interestingly, the amino acid sequence of Rbp from *C. ulcerans* 809 lacks the ER-targeting sequence at the C-terminal end of

the protein. Instead the secretion of the putative toxin into the cytosol is supported by a typical signal sequence at the N-terminus of the protein (Trost et al. 2011).

8.3.5 *Inflammatory Response to* C. ulcerans *Infection in Mice*

When mice were intravenously infected with different doses of *C. ulcerans* strain 809, a human respiratory tract isolate, BR-AD22, an isolate from an asymptomatic dog, and CDC-KC279, an animal clinical isolate (Dias et al. 2011), all three strains had a lethal effect on mice, but at different levels. Strain 809 revealed the highest lethality rate, followed by CDC-KC279 and BR-AD22 which led to the lowest mortality. When *C. ulcerans* was recovered from blood, kidney, liver, spleen, heart, lung, joints and brain, viable bacteria of strain 809 and CDC-KC279 were detectable until day 3 post infection and of BR-AD22 until day 2 post infection. Viable bacteria were also found in kidneys, livers and spleens, independent of the strains tested, whereas colonization of lung, heart and brain was not observed. The three strains were able to persist in joints until day 20 post infection, but only mice infected with strain 809 and strain CDC-KC279 showed clinical symptoms of arthritis. In contrast, *C. ulcerans* BR-AD22 and the toxigenic *C. diphtheriae* strain ATCC 27012 were not able to induce signs of arthritis in mice. To investigate the arthritis induction during *C. ulcerans* and *C. diphtheriae* infection in more detail, Dias and co-workers (Dias et al. 2011) measured the systemic production levels of IL-6 and TNF-α at different time points of infection with different *C. ulcerans* and *C. diphtheriae*. The data indicated that TNF-α and IL-6 levels were always higher than the negative control, with the exception of TNF-α mice group infected with the toxigenic *C. diphtheriae* ATCC27012, which is in accordance with the observation that this strain is not able to induce clinical symptoms of arthritis. *C. ulcerans* BR-AD22, which was also not able to induce clinical signs of arthritis, reached the peak of TNF-α production on day 1 post infection while 809 and CDC-KC279 reached its peaks on day 3 post infection. The results of Dias and co-workers show a strong correlation between the arthritis index and *in vivo* pro-inflammatory cytokine production, demonstrating that *C. ulcerans* strains have the capacity to induce arthritis in conventional Swiss Webster mice and furthermore demonstrating that some *C. ulcerans* strains are more virulent than *C. diphtheriae*, independent of the presence of the diphtheria toxin.

8.3.6 *C. pseudotuberculosis Regulons Involved in Iron Metabolism, Oxidative Stress Response and Detoxification of Nitric Oxide*

Iron is an essential element of proteins containing heme, iron-sulfur clusters or mono- and binuclear iron species. Since many of these genes are play important

roles in pathogen-host interaction, iron uptake and metabolism are closely connected to virulence.

It was already known that *C. pseudotuberculosis* encodes an iron uptake system, *fagCBA-fagD* that is regulated by iron *in vitro* (Billington et al. 2002). Additionally, the complete genome of *C. pseudotuberculosis* FRC41 was screened for other iron uptake systems and the corresponding transcriptional regulators. Trost and co-workers (Trost et al. 2010) were able to assign a number of genes to the DtxR regulon of *C. pseudotuberculosis* FRC41 that are involved in the utilization of various host compounds as iron source. In addition to that, *htaA*-like genes (*htaD* and *htaF*) that are associated with genes encoding membrane proteins (*htaE* and *htaG*) were detected, which might be responsible for the acquisition of iron from the host. The *hmuO* gene of the DtxR regulon, a heme oxygenase, is able to release iron from the protoporphyrin ring of heme and enables the acquisition of iron from heme and hemoglobin (Schmitt 1997b, a). Trost and co-workers (Trost et al. 2010) identified two response regulators that are similar to ChrA and HrrA within the *C. pseudotuberculosis* FRC41 genome, supporting the theory that a complex hierarchical control of *hmuO* gene expression might be established in this organism.

The genome of *C. pseudotuberculosis* FRC41 contains two DtxR-regulated gene clusters that might be involved in siderophore biosynthesis, independent of non-ribosomal peptide synthetases (Trost et al. 2010). The first one is the *ciu* locus which consists of the *ciuABCD* gene cluster, encoding an ABC-type transporter system, together with *ciuE*, encoding a siderophore biosynthesis-related protein, and *ciuF*, encoding a putative efflux protein. The *ciu* gene region is homologous to the *C. diphtheriae* NCTC 13129, which additionally carries the *ciuG* gene, encoding a protein of unknown function. The second gene cluster consists of four genes, which might be involved in siderophore biosynthesis pathway (*ogs, ocd, odc* and *tsb*), one gene encoding an efflux protein (*mdtK*) and four genes encoding an ABC-type transporter (*stsABCD*) (Trost et al. 2010).

In addition to that, genes encoding Dps-like proteins were detected in *C. pseudotuberculosis* (Trost et al. 2010). Dps proteins mainly protect DNA from redox stress; they also can act as iron-binding and storage proteins, but prefer H_2O_2 as oxidant instead of O_2, indicating that its primary function is in protecting DNA against the combined action of ferrous iron and H_2O_2 and rather in iron storage (Andrews et al. 2003). The protein that shares a number of physiological properties with Dps-like proteins in *C. pseudotuberculosis* FRC14 is the multifunctional histone-like protein and transcription regulator Lsr2. Lsr2 proteins protect the DNA against reactive oxygen intermediates, as it was shown for mycobacteria, and thus Lsr2 may also protect corynebacterial DNA (Colangeli et al. 2009). RipA-binding sites were detected in front of *dps-fpg1* in *C. pseudotuberculosis* (Trost et al. 2010). RipA, a DNA-binding transcription regulator is under direct control of DtxR in *C. glutamicum.* When under conditions of iron-restriction, RipA binds the DNA and acts as a repressor for genes encoding iron proteins in *C. glutamicum* (Wennerhold et al. 2005). Another gene (*fpg2*), encoding a formamidopyrimidine-DNA glycosylase, was detected by Trost and co-workers in the genome of *C. pseudotuberculosis*. These enzymes function mainly in the repair of DNA lesions caused by oxidative damage, but *fpg2* in *C. pseudotuberculosis* FRC41 lacks the RipA-binding site.

Nevertheless, the interaction of the DtxR regulon and RipA shows a complex regulatory network of iron metabolism and oxidative stress response in corynebacteria, with regard to virulence of *C. pseudotuberculosis*.

Besides DNA protection, many pathogenic bacteria developed mechanisms for the detoxification of reactive oxygen and nitrogen species produced from macrophages as part of their antimicrobial response (Nathan and Shiloh 2000; Zahrt and Deretic 2002). Examples for such detoxification proteins in important pathogens are the SodC protein of *Mycobacterium tuberculosis*, which is involved in the resistance against oxidative burst species produced by macrophages (Dussurget et al. 2001; Piddington et al. 2001), and the Cu, Zn-dependent superoxide dismutases, which have been reported in *Neisseria meningitidis* and *Hemophilus ducreyi* (Wilks et al. 1998; San Mateo et al. 1999).

In the *C. pseudotuberculosis* FRC41 genome four genes, which are probably involved in the detoxification process of reactive oxygen species, are found (Trost et al. 2010): *ahpCD* (alcyl hydroperoxide reductase), *sodA* (manganese-dependent superoxide dismutase) and *sodC* (copper, zinc-dependent superoxide dismutase). However, the function in virulence of these genes in *C. pseudotuberculosis* FRC41 has to be proven.

A further potential virulence factor of *C. pseudotuberculosis* FRC41detected by Trost and co-workers might be the *nor* gene, coding for nitric oxide reductase, which is generally involved in the detoxification of nitric oxide and therefore important for pathogens to persist within macrophages (Luthra et al. 2008). Interestingly, McKean and co-workers (McKean et al. 2005) did not find induction of the expression of the *nor* gene upon the infection of macrophages by animal pathogenic *C. pseudotuberculosis*. Thus, the regulation pattern of *nor* transcription and its part in protection against nitric oxide, is still unclear.

8.4 Conclusions

The development of new molecular tools allowed major progress in the analysis of virulence factors of toxigenic corynebacteria. Especially the availability of genome sequence information gave new insights in the presence of putative virulence factors of *C. ulcerans*, *C. pseudotuberculosis* and *C. diphtheriae* (summarized in Table 8.4). However, there are still a number of open questions concerning strain-specific differences interaction with epithelial cells and macrophages as well as induction of host pathways, which might have implication for disease development and will hopefully elucidated in future.

Table 8.4 Putative virulence factors of *C. Diphtheriae* NCTC 13129, *C. Ulcerans* 809, BR-AD22 and *C. Pseudotuberculosis* FRC41

ID *C. diphtheriae* NCTC 13129	ID *C. ulcerans* 809	ID *C. ulcerans* BR-AD22	ID *C. pseudotuberculosis* FRC41	Gene name	Annotated function of deduced protein
DIP0222	–	–	–	*tox*	Diphtheria toxin precursor
DIP2013	–	–	cpfrc_01874	*spaA*	Surface-anchored protein (pilus subunit)
DIP2011	–	CULC22_02131	cpfrc_01872	*spaB*	Surface-anchored protein (pilus subunit)
DIP2010	CULC809_01980	CULC22_02130	cpfrc_01870	*spaC*	Surface-anchored protein (pilus subunit)
DIP0235	CULC809_01979	CULC22_02106	cpfrc_01904	*spaD*	Surface-anchored protein (pilus subunit)
DIP0237	CULC809_01952	CULC22_02104	cpfrc_01902	*spaE*	Surface-anchored protein (pilus subunit)
DIP0238	CULC809_01950	CULC22_02103	cpfrc_01901	*spaF*	Surface-anchored protein (pilus subunit)
DIP2227	CULC809_01949	–	–	*spaG*	Surface-anchored protein (pilus subunit)
DIP2226	–	–	-	*spaH*	Surface-anchored protein (pilus subunit)
DIP2223	–	–	–	*spaI*	Surface-anchored protein (pilus subunit)
DIP0733	CULC809_00602	CULC22_00609	cpfrc_00553	-	67–72p hemagglutinin
DIP0543	CULC809_00434	CULC22_00437	cpfrc_00386	*nanH*	Sialidase precursor (neuraminidase H)
DIP1281	CULC809_01133	CULC22_01148	cpfrc_01079	*rpfl*	Resuscitation-promoting factor interacting protein (D, L-endopeptidase)
–	CULC809_00040	CULC22_00038	cpfrc_00029	*pld*	Phospholipase D
–	CULC809_01974	CULC22_02125	cpfrc_01895	*cpp*[b]	Corynebacterial protease CP40 (Endoglycosidase Endo E)
–	CULC809_00177	–	–	*rbp*	(Shiga toxin-like) ribosome-binding protein
–	Integration site CULC809_01141	–	–	–	Prophage ΦCULC809I
–	–	Integration site CULC22_01157	–	–	Prophage ΦCULC22I
–	–	Integration site between CULC22_01663 and CULC22_01724	–	–	Prophage ΦCULC22II

Table 8.4 (continued)

ID *C. diphtheriae* NCTC 13129	ID *C. ulcerans* 809	ID *C. ulcerans* BR-AD22	ID *C. pseudotu-berculosis* FRC41	Gene name	Annotated function of deduced protein
–	–	Integration site tRNALys	–	–	Prophage ΦCULC22III
–	–	Integration site tRNALys	–	–	Prophage ΦCULC22IV
DIP1621	CULC809_01521	CULC22_01537	–	*cwlH*	Cell wall-associated hydrolase
DIP1414	–	CULC22_01326	cpfrc_01219	*dtxR*	Diphtheria toxin repressor
–	CULC809_02066	CULC22_02219	cpfrc_01988	*htaD*	Cell-surface hemin receptor
–	CULC809_02125	CULC22_02281	cpfrc_01477	*htaF*	Cell-surface hemin receptor
–	CULC809_02065	CULC22_02218	cpfrc_01987	*htaE*	Membrane protein
–	–	–	cpfrc_01476	*htaG*	Membrane protein
DIP1669	CULC809_01565	CULC22_01581	cpfrc_01487	*hmuO*	Heme oxygenase
DIP2327	CULC809_02136	CULC22_02292	cpfrc_02058	*chrA*	Two-component system transcriptional regulatory protein
[a]	CULC809_02085	CULC22_02242	cpfrc_02010	*hrr*	Two-component system transcriptional regulatory protein
[a]	CULC809_01040	CULC22_01055	cpfrc_00987	*ciuA*	Iron ABC transport system substrate-binding protein
[a]	CULC809_01041	CULC22_01056	cpfrc_00988	*ciuB*	Iron ABC transport system permease protein
[a]	CULC809_01042	CULC22_01057	cpfrc_00989	*ciuC*	Iron ABC transport system permease protein
[a]	CULC809_01545	CULC22_01058	cpfrc_00990	*ciuD*	Iron ABC transport system ATP-binding protein
[a]	CULC809_01044	CULC22_01059	cpfrc_00991	*ciuE*	Siderophore biosynthesis-related protein
[a]	CULC809_01045	CULC22_01060	cpfrc_00992	*ciuF*	Putative efflux protein
[a]	–	–	–	*ciuG*	Conserved membrane protein
–	CULC809_00665	CULC22_00672	cpfrc_00624	*ogs*	Oxygenase

Table 8.4 (continued)

ID *C. diphtheriae* NCTC 13129	ID *C. ulcerans* 809	ID *C. ulcerans* BR-AD22	ID *C. pseudotu-berculosis* FRC41	Gene name	Annotated function of deduced protein
–	CULC809_00664	CULC22_00671	cpfrc_00623	*ocd*	Ornithine cyclodeaminase
–	CULC809_00663	CULC22_00670	cpfrc_00622	*odc*	Ornithine decarboxylase
–	–	CULC22_00669	cpfrc_00621	*tsb*	Cysteine synthase
–	–	–	cpfrc_00620	*mdtk*	Putative membrane protein
–	CULC809_00666	CULC22_00673	cpfrc_00625	*stsA*	ABC transporter solute-binding protein
–	CULC809_00667	CULC22_00674	cpfrc_00626	*stsB*	ABC transporter permease protein
–	CULC809_00668	CULC22_00675	cpfrc_00627	*stsC*	ABC transporter permease protein
–	CULC809_00669	CULC22_00676	cpfrc_00628	*stsD*	ABC transporter ATP-binding protein
DIP2266	CULC809_02084	CULC22_02241	cpfrc_02009	*lsr2*	Protein Lsr2
DIP1543	CULC809_02127	CULC22_02283	cpfrc_02047	*fpg1*	Formamidopyrimidine DNA glycosylase
–	CULC809_01439	CULC22_01453	cpfrc_01339	*fpg2*	Formamidopyrimidine DNA glycosylase
[a]	CULC809_01318	CULC22_01332	cpfrc_01226	*ahpC*	Alkyl hydroperoxide reductase AhpD
DIP1419	CULC809_01317	CULC22_01331	cpfrc_01225	*ahpD*	Alkyl hydroperoxide reductase AhpD
DIP2261	CULC809_02075	CULC22_02231	cpfrc_01998	*sodA*	Superoxide dismutase
[a]	CULC809_00652	CULC22_00659	cpfrc_00610	*sodC*	Superoxide dismutase [Cu–Zn]
–	–	–	cpfrc_00128	*nor*	Nitric oxide reductase

[a] not detected in strain NCTC 13129, but in several other *C. diphtheriae* strains

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