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Molecular armory or niche factors: virulence determinants of *Corynebacterium* species

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One sentence summary: Review of data supporting the concept of niche factors in the genus *Corynebacterium*.

Running title: Virulence versus niche factors in corynebacteria

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ABSTRACT

For successful colonization of a host, pathogenic bacteria are equipped with a plethora of proteins and other components, often designated as virulence factors. However, many of these factors are involved in general adaptation to ecological niches rather than being specific for interaction of a bacterial pathogen with a distinct host. Therefore, a designation of these components as niche factors was proposed. While originally developed for different species colonizing the gastro-intestinal tract, in this communication the concept of niche factors is discussed in respect to a single genus, i.e. *Corynebacterium* species.

INTRODUCTION

The interaction of pathogenic bacteria with their respective hosts at the molecular level relies on cellular components typically designated as virulence factors. Based on the observation that they are often necessary to colonize a distinct ecological niche, rather than having a specific detrimental function, the reconsideration of many virulence factors was discussed recently (Hill 2012). It was argued that these proteins might better be designated as 'niche factors', since they have a function in pathogenicity only in combination with other proteins such as toxins and in a certain environment. Consequently, the number of true virulence factors might be limited. To support this concept, different probiotic and pathogenic microbes colonizing the gastro-intestinal tract and using similar proteins for this purpose were presented, including selected members of the genera *Bifidobacterium*, *Yersinia*, *Lactococcus*, *Enterococcus* and *Listeria*.

If valid, this concept should also hold true within a distinct taxonomical group of bacteria. The genus *Corynebacterium* might be especially interesting in this respect due to its highly diverse composition. In 2014, 90 *Corynebacterium* species were described. About 50 species were initially isolated from humans or human clinical material, 32 species appeared to be associated with animals and a few have been implicated in the transmission to humans probably causing zoonotic infections. Sixteen *Corynebacterium* species were isolated from different environments such as synthetic surfaces, food, water and soil (Tauch and Sandbote

2014). Within the group of medically relevant corynebacteria, many species rarely cause infections, while only a very limited number of species are potent human pathogens. Interestingly, broad host spectra were observed for some pathogenic species, e.g. *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*. *C. diphtheriae* have not only been isolated from humans but also, for example, from cats, cows and horses, while *C. ulcerans* colonizes a wide variety of mammals (Mattos-Guaraldi *et al*. 2014). Recent studies showed that hosts might not be restricted to mammals, since *C. diphtheriae*, *C. ulcerans* and *Corynebacterium glutamicum* can colonize *Caenorhabditis elegans* and induce morphological changes typical for nematode pathogens like *Leucobacter* isolates (Clark and Hodgkin 2015) and *Microbacterium nematophilum* (Hodgkin *et al*. 2000) (Antunes *et al.*; unpublished observation). Taken together, these observations make a highly specific host-pathogen interaction rather unlikely and hint to a more general adhesion mechanism in corynebacteria.

This review focuses on four well-studied *Corynebacterium* species, representing different ecotypes colonizing humans, animals and soil environments. The most prominent member of these is *C. diphtheriae*, the etiological agent of diphtheria. *C. ulcerans* is a closely related zoonotic pathogen, which seems to be mainly a commensal in mammals. However, *C. ulcerans* cause diseases such as mastitis in cattle, skin lesions, abscesses and ulcers in platypus, wild boars and water rats as well as diphtheria-like illness in humans (Mattos-Guaraldi *et al.* 2014). *Corynebacterium jeikeium* is a member of the human skin flora and involved in body odor formation. In intensive care units, it is of medical importance as a multidrug-resistant nosocomial pathogen (Tauch *et al*. 2005). *C. glutamicum* is a non-pathogenic member of the genus, which plays an important role in biotechnology as producer of amino acids, organic acids, alcohols and other industrially important metabolites (Burkovski 2015).

VIRULENCE VERSUS NICHE FACTORS

Lifestyle and pathogenicity

C. jeikeium is a lipid-requiring auxotrophic bacterium, due to the absence of a fatty acid synthase-encoding gene in its genome. In addition to biomass production, fatty acids are metabolized by *C. jeikeium* as carbon and energy source. While the human skin and especially the axilla provide sufficient fatty acids, supply may become limiting in host tissue. In this case, surface-anchored enzymes such as ceramidase, cholesterol oxidase and cholesterol esterase as well as acid phosphatase might be responsible to ensure fatty acid supply (Tauch *et al.* 2005). Unfortunately, activity of these enzymes will damage the tissue of the host. Nevertheless, these proteins might better be considered as niche factors rather than virulence factors, as they reflect the adaptation of the microbe to its lipophilic lifestyle in the human axilla. A similar example of a lipophilic *Corynebacterium* species encoding ceramidase and cholesterol oxidase and exhibiting multidrug resistance is *Corynebacterium resistens*, which can cause bacteremia (Schröder *et al*. 2012).

Multidrug resistance

For some members of the genus *Corynebacterium*, a multidrug resistance phenotype was observed. As described above, *C. jeikeium* is part of the normal human skin flora. Unfortunately, the bacterium carries a number of antibiotic-resistance genes, and while being harmless when colonizing the skin of healthy individuals, in hospital settings, especially in intensive care units, *C. jeikeium* may become a serious threat as nosocomial pathogen. In this setting, *C. jeikeium* is notoriously difficult to combat since it is resistant against macrolides, lincosamides, tetracyclines, chloramphenicol and many other medically relevant drugs (Tauch *et al.* 2005).

For other pathogens, such as *C. diphtheriae*, wide variations in respect to antibiotic resistances has been observed, which are in general rare and seem to be coupled to prescription practices in different countries. Multidrug-resistant *C. diphtheriae* strains were observed only recently in global literature (for review, see Zasada 2014).

As exemplified by *C. jeikeium*, antibiotic resistance *per se* might be considered as a niche factor for the adaptation to soil and other environments, where competitors might excrete bacteriostatic or bactericidal compounds (Tauch *et al*. 2005).

Influence of cell envelope components

Surface structures are important players in host-pathogen interactions, working for example as receptors, effector or signal molecules. Corynebacteria are characterized by a complex cell wall architecture: In these bacteria, the plasma membrane is surrounded by a peptidoglycan layer, which is covalently linked to arabinogalactan. Bound to this, an outer layer of mycolic acid-sugar conjugates is present which is functionally equivalent to the outer membrane of Gram-negative bacteria (Burkovski 2013a).

Although the corynomycolic acid layer might play an important role in stress resistance and pathogenicity (Burkovski 2013a), it could be considered a niche factor rather than a true virulence factor since almost all *Corynebacterium* species possess trehalosyldimycolates.

Between 20 and 70 surface-located proteins were detected for *C. diphtheriae*, *C. jeikeium*, *C. glutamicum* and *Corynebacterium efficiens* (Hansmeier *et al*. 2006a, 2006b, 2007) and a few of these were investigated in respect to their contribution to virulence, for example *C. diphtheriae* DIP1281, which raised interest due to its annotation as invasion-associated protein. In fact, DIP1281 mutants were unable to invade epithelial cells. However, subsequent experiments hint to a general function of this protein, since besides cell elongation and separation, surface organization was strongly influenced by lack of DIP1281 (Ott *et al*. 2010a). Similar effects were observed for the corresponding protein in *C. glutamicum* (Tsuge *et al*. 2008). In summary, these studies show that DIP1281 and its homologs in other corynebacteria are involved in cell division rather than being virulence factors.

A similar conclusion can be drawn for DIP0733. Based on *in vitro* studies, DIP0733 was described as multifunctional virulence factor of *C. diphtheriae* involved in

hemagglutination, adhesion, invasion and induction of apoptosis (Sabbadini *et al.* 2012). In fact, a DIP0733 mutant strain lacking the corresponding protein was attenuated for colonization of *C. elegans*, survival in macrophages, adhesion and invasion of epithelial cells as well as binding to extracellular matrix proteins (Antunes *et al*. 2015). However, DIP0733 homologs are widely distributed in corynebacteria and related species including non-pathogenic members of the genus such as *C. glutamicum* and *Corynebacterium ammoniagenes*, while in several pathogens, DIP0733 homologs are absent, i.e. *Corynebacterium afermentans*, *Corynebacterium appendicis*, *Corynebacterium bovis*, *Corynebacterium mycetoides* and *Corynebacterium xerosis* (Antunes *et al*. 2015). Taken together, the results might support a function of DIP0733 proteins in adhesion to biotic surfaces and thus working as niche factor, rather than having a distinct role in pathogenicity. A role of DIP0733 as virulence factor based on a specific induction of apoptosis remains unclear, since a detailed molecular analysis is hampered by a completely lacking annotation of functional domains within this protein (Sabbadini *et al.* 2012).

A third example is the thiol-disulfide oxidoreductase MdbA of *C. diphtheriae*, which is crucial for viability, pilus assembly, toxin production and virulence, based on its proposed function as part of the general oxidative folding machinery (Reardon-Robinson *et al*. 2015).

A completely unclassified gene involved in the adhesion to epithelial cells is DIP1621 (Kolodkina *et al.* 2011). Initially identified from a transposon mutant pool screened for reduced adherence, the corresponding protein is part of the secreted proteome of *C. diphtheriae* (Hansmeier *et al.* 2006a), but is also widely distributed among other pathogenic and non-pathogenic corynebacteria, e.g. in *C. ulcerans*, *C. jeikeium*, *C. glutamicum*, *Corynebacterium variabile* and *Corynebacterium halotolerans*.

Adhesion to abiotic and biotic surfaces

In the laboratory, microbes are predominantly grown in liquid media. However, many bacteria switch between free-living and sessile states or form biofilms. As described above, many *Corynebacterium* species were originally isolated either from abiotic and biotic surfaces and

consequently, adhesion mechanisms, either specific or non-specific, can be expected in this group of bacteria.

In fact, binding of *C. diphtheriae* strains to polystyrene, polyurethane and glass was reported (Gomes *et al*. 2009). Based on general hydrophobic surface properties and slime formation also medically relevant devices such as catheters can be colonized non-specifically (Gomes *et al*. 2009; Mattos-Guaraldi *et al*. 2000).

Binding to fibrinogen, fibronectin and collagen was observed in many studies for toxigenic and non-toxigenic *C. diphtheriae* strains as well as *C. ulcerans* isolates (Sabbadini *et al*. 2010; Simpson-Louredo *et al*. 2014). Interestingly, mutation of DIP0733 impairs binding to these proteins to different extent, but without blocking it completely (Antunes *et al.* 2015) indicating alternative binding mechanisms. In summary, surface binding seem to be a multifactorial process, depending at least partially on general corynebacterial properties such as hydrophobicity or sugar content of the outer cell envelope layer.

In contrast, pili seem to be specific adhesion factors. The genome of the first sequenced *C. diphtheriae* strain, NCTC 13129, encodes three distinct adhesive pili (SpaABC, SpaDEF, SpaGHI). Mutant strains lacking proper pilus structures, showed decreased adhesion rates to different epithelial cell lines; however, adhesion was not lost completely (Mandlik *et al.* 2007). Atomic force microscopy revealed that pilus formation is highly variable in *C. diphtheriae* and even strains lacking visible pili structures show adhesion to epithelial cells (Ott *et al*. 2010b). Moreover, a recent pangenomic study provided evidence for a high degree of genetic variability in pilus gene clusters of different *C. diphtheriae* isolates (Trost *et al*. 2012). A fourth type of pili was observed in this analysis, and despite the fact that all sequenced strains contained at least two pilus gene clusters, the single clusters were differently distributed among the isolates. The high variability and the location of pilus genes on genomic islands indicated frequent horizontal gene transfer. The genes are not part of the *C. diphtheriae* core genome and the main function of pili seems to ensure specific adhesion to distinct cell types. The same conclusion can be drawn for the different pilus types of the animal pathogen *Corynebacterium pseudotuberculosis* (Soares *et al*. 2013).

However, adhesive pili are not only encoded in pathogenic corynebacteria such as *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans*, but also found in non-pathogenic species such as *Corynebacterium casei*, *C. efficiens*, *C. glutamicum* and *Corynebacterium vitaeruminis*, and consequently are not virulence factors in *sensu stricto* according to the definition by Wassenaar and Gaastra (2010) but virulence life-style proteins.

Toxins

Diphtheria toxin is the main virulence factor of *C. diphtheriae* and responsible for the life-threatening symptoms of respiratory diphtheria (Burkovski 2013b). The toxin is encoded on a temperate bacteriophage, which during lysogeny is capable of toxin production (Sangal and Hoskisson 2014). Interestingly, non-toxigenic *C. diphtheriae* strains are increasingly recognized as a cause of systemic infections (Mattos-Guaraldi *et al*. 2014) and a recent study of German *C. diphtheriae* isolates showed that only about 12 % of isolates carry *tox* genes (Meinel *et al*. 2014). Obviously, this toxin dramatically increases tissue damage due to *C. diphtheriae* infection; however, it is not essential for pathogen-host interaction.

Besides *C. diphtheriae* two closely related species, *C. pseudotuberculosis* and *C. ulcerans*, can be lysogenized by *tox*-positive corynephages and can produce diphtheria toxin (Riegel *et al*. 1995; Sangal and Hoskisson 2014). Recently, a new transmission pathway for the *tox* gene was described for *C. ulcerans* (Meinel *et al*. 2014). Based on next generation sequencing of nine *C. ulcerans* isolates a novel putative diphtheria toxin-encoding pathogenicity island was identified. This corynephage-independent transmission pathway might explain the high number of diphtheria toxin-encoding *C. ulcerans* strains (Meinel *et al*. 2014).

Instead of diphtheria toxin, the human isolate *C. ulcerans* 809 carries a gene designated *rbp*, which encodes a putative ribosome-binding protein with striking structural similarity to Shiga-like toxins. Moreover, the *cpp* gene of 809 encodes a protein with similarities to the α-domain of extracellular endoglycosidase of the EndoE family. The human isolate *C. ulcerans* KL387 harbors a prophage with a putative novel virulence factor, which

shares homology with the RhuM virulence factor from *Salmonella enterica* at the molecular level (Meinel *et al*. 2014). A *rhuM* mutant of *S. enterica* exhibited a significant decrease in epithelial cell invasion (Tenor *et al*. 2004). The functions of these putative toxins have not been characterized at the molecular level (Trost *et al*. 2011; Meinel *et al*. 2014).

C. ulcerans and *C. pseudotuberculosis* are the only members of the genus carrying a *pld* gene for phospholipase D (PLD). Functioning as sphingomyelinase (phosphatidylcholine phosphohydrolase), detrimental effects of PLD on erythrocytes and neutrophils infected with *C. pseudotuberculosis* due to sphingomyelin depletion were reported (Brogden *et al*. 1990). Furthermore, *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 (Muckle and Gyles 1983; McNamara *et al*. 1994), and plays a role in macrophage death (McKean *et al*., 2007). Studies with *pld* mutants of *C. pseudotuberculosis* demonstrated the necessity of PLD for establishment of caseous lymphadenitis and revealed that mutant strains are unable to cause abscesses of the lymph nodes (McNamara *et al*. 1994). However, the general role of phospholipases in disease and pathogenesis must be interpreted with caution, since they might have primarily a metabolic function. This idea is in accordance with the observation that phospholipase D in *C. ulcerans* seems to have no crucial effect on the interaction of the bacterium with epithelial cell lines (Hacker *et al.* 2015).

CONCLUSIONS

Bacterial pathogens are subject to selective pressure to grow and multiply and, consequently, nutrient uptake mechanisms and mechanisms to evade host defense are crucial for their evolutionary success. As a result, metabolism is closely linked to pathogenicity and metabolic enzymes with so-called moonlighting function might also be directly involved in virulence regulation (Henderson and Martin 2011; Commichau and Stülke 2015). A mechanism designated pathoadaptive mutation might be responsible for the development of proteins with second functions and other genetic adaptations, which can result in enhanced pathogenicity. In this case, mutations in pre-existing genes increase of the

246 fitness of a bacterium and drive the evolution of the species torwards a more pathogenic
247 lifestyle (Sokurenko *et al*. 1999). In consequence, many proteins may be involved in
248 metabolism and pathogenicity, making a clear functional distinction difficult (Wassenaar and
249 Gaastra 2001). The definition of microbial components acting on host cells as virulence
250 factors might not only need differentiation but might also be dependent on the context of
251 bacterium-host interaction and host damage (Wassenaar and Gaastra 2001; Hill 2012;
252 Casadevall and Pirofski 2014). As one attempt for differentiation, the concept of 'niche
253 factors' was introduced by Hill (2012) and was originally deduced from a data set of different
254 microbial genera and species colonizing the human gastro-intestinal tract. In this study, we
255 transferred this concept to a single genus (Figure 1). Many compounds shown to be
256 important for interaction of *Corynebacterium* species with host organisms so far are also
257 present in non-pathogenic corynebacteria and consequently seem to be niche factors rather
258 than virulence factors in *sensu stricto*. The robustness of corynebacterial cells in respect to
259 physical, salt, metal, oxidative and membrane stress etc. and their special surface properties
260 seem to be the basis of successful colonization of various environments. Cell envelope
261 compounds such as surface proteins and glycolipids seem to favor adhesion to abiotic and
262 biotic surfaces. Together with a high capacity for gene uptake by horizontal gene transfer,
263 many members of the genus *Corynebacterium* developed into rare pathogens, or 'pathogens
264 by chance', while only a few species became a real threat for human health due to the
265 uptake of different toxin genes.
266 In summary, based on data obtained for members of the genus *Corynebacterium*
267 presented here, a reconsideration of many virulence factors as niche factors (Hill 2012)
268 seems to be a valid concept. It might help to understand the development of pathogens as
269 well as virulence factors and might be important in the light of growing number of studies to
270 characterize the composition and function of human microbiota. To expand this concept
271 further, an attractive group of bacteria is the genus *Enterococcus* inhabiting humans and
272 animals and being isolated from several environmental sources (Fisher and Phillips 2009),
273 like the aforementioned corynebacteria. In addition, group A streptococci produce numerous

extracellular factors attributed to virulence and integrated into different regulatory systems sensing the environment (Hynes 2004). A dissection of these factors and their influence on virulence can be revisited in respect to the 'niche factor' concept. Another interesting group to proof the 'niche factor' concept is the genus *Staphylococcus* with the prominent *Staphylococcus aureus* as a member and its membrane-damaging factors (Vandenesch *et al*. 2012). Together with other conceptual considerations (Casadevall and Pirofski 2015), these kind of studies will hopefully improve our understanding of the ecology of virulence.

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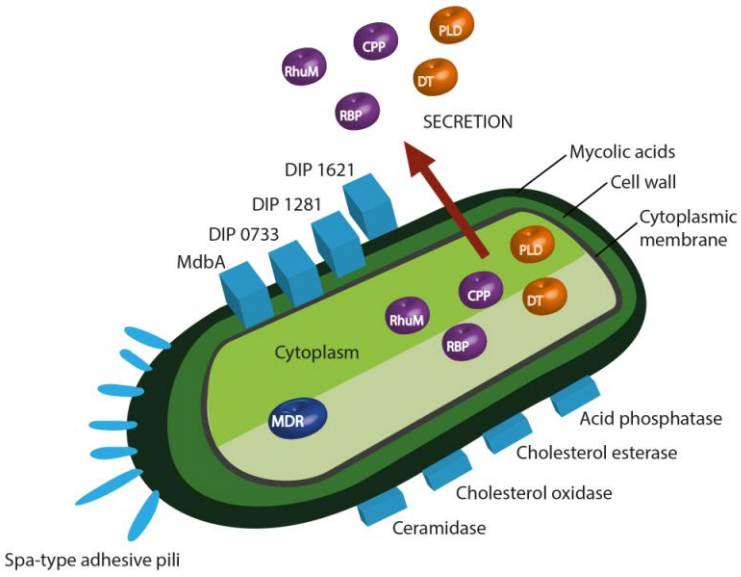
Legends to figures



Factor	Species
Acid phosphatase	**Cj**
Ceramidase	**Cj**, **Cr**
Cholesterol esterase	**Cj**
Cholesterol oxidase	**Cj**, **Cr**
CPP	**Cu**
DIP 0733	Ca, **Cd**, Cg
DIP 1281	**Cd**, Ce, Cg, **Cj**
DIP 1621	**Cd**, Cg, Ch, **Cj**, **Cu**, Cva
DT	**Cd**, **Cu**, **Cp**
MdbA	**Cd**
MDR	**Cj**, **Cr**
Mycolic acids	Ca, Cc, **Cd**, Ce, Cg, Ch, **Cj**, **Cp**, **Cr**, **Cu**, Cva, Cvi
PLD	**Cu**, **Cp**
RBP	**Cu**
RhuM	**Cu**
Spa-type adhesive pili	Cc, **Cd**, Ce, Cg, **Cp**, **Cu**, Cvi

Figure 1: Niche and virulence factors of corynebacteria. Niche factors are indicated in light blue for proteins and dark green for mycolic acids, true virulence factors in orange, candidate virulence factors in violet and ambiguous cases in dark blue. The occurrence of the components in different species (as discussed in this paper) is indicated as follows: Ca, *C. ammoniagenes*; Cc, *C. casei*; **Cd**, ***C. diphtheriae***; Ce, *C. efficiens*; Cg, *C. glutamicum*; Ch, *C. halotolerans*; **Cj**, ***C. jeikeium***; **Cp**, ***C. pseudotuberculosis***; **Cr, *C. resistens***; **Cu**, ***C. ulcerans***; Cva, C. *variabile*; Cvi, *C. vitaeruminis*; with pathogenic species shown in bold. Further abbreviations used: CPP, endoglycosidase of the EndoE family; DT, diphtheria toxin; MdbA, thiol-disulfide oxidoreductase; MDR, multi-drug resistance; PLD, phospholipase D; RBP, ribosome-binding protein/Shiga-like toxin; RhuM, RhuM-like protein. Figure 1 was kindly provided by S. Morbach (Friedrich-Alexander-Universität Erlangen-Nürnberg).