Antonie van Leeuwenhoek (2018) 111:717–725
https://doi.org/10.1007/s10482-018-1036-6

REVIEW

The role of corynomycolic acids in *Corynebacterium*-host interaction

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Received: 9 October 2017 / Accepted: 29 January 2018 / Published online: 12 February 2018

Abstract Within the *Actinobacteria*, the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* form the so-called CMNR group, also designated as mycolic acid-containing actinomycetes. Almost all members of this group are characterized by a mycolic acid layer, the mycomembrane, which covers the cell wall and is responsible for a high resistance of these bacteria against chemical and antibiotic stress. Furthermore, components of the mycomembrane are crucial for the interaction of bacteria with host cells. This review summarizes the current knowledge of mycolic acid synthesis and interaction with components of the immune system for the genus *Corynebacterium* with an emphasis on the pathogenic species *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* as well as the biotechnology workhorse *Corynebacterium glutamicum*.

Keywords Cord factor · Corynomycolic acids · Diphtheria · Lipidomics · Mycomembrane

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Mycolic acids-containing *Actinobacteria*

The class of *Actinobacteria*, one of the largest groups in the domain of bacteria, has been subdivided based on 16S rRNA gene sequence data and supragenic relationships into 15 orders, 43 families and 203 genera of Gram-positive bacteria with a high G + C content in their DNA base composition (Ventura et al. 2007; Goodfellow 2012). Within the *Actinobacteria*, the CMNR group, formed by the genera *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus*, can be distinguished. Almost all members of this group—also designated as the mycolic acid-containing actinomycetes—are characterized by a mycolic acid layer, the mycomembrane, which covers the cell wall and is in many aspects functionally equivalent to the outer membrane of Gram-negative bacteria (Niederweis et al. 2010; Burkovski 2013).

The genus *Corynebacterium* comprises a collection of morphologically similar, irregular- or club-shaped non-sporulating (micro-)aerobic microorganisms. To date, 90 species were taxonomically classified (Tauch and Sandbote 2014; Sangal et al. 2014). More than half of these, i.e. 52 species, are occasional or rare causes of infections, while only a few evoke severe diseases in humans. The most prominent member of the latter group is *Corynebacterium diphtheriae*, which is also the type species of the genus. *C. diphtheriae* is the etiological agent of respiratory diphtheria, which is restricted to about 5000 annual cases, mainly in

developing countries today. Nevertheless, diphtheria is still a worldwide threat as exemplified by a severe outbreak with more than 157,000 cases in the states of the former Soviet Union in 1990 to 1998. Furthermore, a number of smaller outbreaks have been reported from different countries (Burkovski 2014, 2016; Sangal and Hoskisson 2016).

The robustness of corynebacterial cells in respect to physical, salt, metal, oxidative and membrane stress seems to be the basis of successful colonization of various environments including animals and humans (Tauch and Burkovski 2015). The mycomembrane is a key structure in this respect. It confers resistance to detergents and antibiotics and protects the cell against other harmful compounds such as lysozyme. The major components of the mycomembrane are mycolic acids, which are α-alkylated β-hydroxylated fatty acids with a short α-alkyl and a meromycolate side chain (Daffé 2005). On the one hand, the mycolic acids are covalently linked to the arabinogalactan-peptidoglycan meshwork of the cell wall, and on the other hand to distinct sugars forming glycolipids, which are located in the outer leaflet of the mycomembrane (Fig. 1). A prominent member of these glycolipids is trehalose dimycolate, which is known to be involved in the interaction of *Mycobacterium tuberculosis* and *Rhodococcus equi* with their specific hosts (Indrigo et al. 2003; Axelrod et al. 2008; Sydor et al. 2013) and consequently of high interest in respect to pathogenicity of these bacteria. While for *M. tuberculosis* the role of trehalose dimycolates in virulence is well studied (Takayama et al. 2005; Bhatt et al. 2007; Lee et al. 2012; Lang 2013; Nataraj et al. 2015), only very limited information is available for the interaction of corynebacterial mycolates with host immune system components, although the synthesis of corynomycolic acids is rather well investigated (Burkovski 2013). This review summarizes the current knowledge of mycolic acid synthesis and interaction with components of the immune system. Additionally, the few available data for epithelial cells are presented.

Synthesis of mycolic acids and formation of the mycomembrane in corynebacteria

Within the CMNR group, corynebacterial mycolic acids have the shortest chain length with 16 to 38 carbon atoms, while the most complex mycolic acids with 60 to 90 carbon atoms and specific modifications such as cyclopropanes and oxygenated groups are found in mycobacteria (Lanéelle et al. 2013; Marrakchi et al. 2014) (Fig. 2).

Two types of fatty acid synthases can be distinguished based on their general composition: the FAS-II type, typically found in bacteria, contains the seven functional domains necessary for fatty acid synthesis organized in one polypeptide, whereas the FAS-I type, typically found in eukaryotes, comprises of a large multifunctional protein complex. As an exception from this rule, FAS-I proteins are found in members of the *Corynebacterianae*. *Corynebacterium glutamicum* and *Corynebacterium efficiens* contain even two FAS-I-type complexes, FAS-IA and FAS-IB, which were functionally characterized in detail for *C. glutamicum* (Radmacher et al. 2005). Interestingly, *C. glutamicum* not only has genes coding for two FAS-I, but also three genes coding for FAS-II, which are involved in elongation of mycolic acid chains in mycobacteria. Since *C. glutamicum* does not contain elongated mycolic acids and FAS-II is absent in other corynebacteria such as *C. diphtheriae*, it was concluded that these might not be functional in *C. glutamicum* (Lanéelle et al. 2013).

Mycolic acids are α-branched β-hydroxy fatty acids, requiring carboxylation and condensation of two fatty acids for their synthesis. In *C. glutamicum* two carboxylases, AccD2 and AccD3, were identified, which are essential for fatty acid and mycolic acid synthesis and conserved among the *Corynebacterinae*. Further proteins involved in mycolic acid synthesis in corynebacteria are AccD1, involved in malonyl-CoA synthesis, Pks, a ketoacyl synthase involved in fatty acid elongation, and FadD, a fatty acid acyl-AMP ligase (Eggeling et al. 2008; Burkovski 2013; Lanéelle et al. 2013).

To form the mycomembrane, mycolic acids have to be linked to the arabinogalactan layer of the cell wall and to trehalose, the major mycolic acid acceptor in corynebacteria, by mycoloyl transferases (Burkovski 2013; Lanéelle et al. 2013). Six corresponding genes were identified in *C. glutamicum*, five in *C. efficiens* and four in *C. diphtheriae* (Brand et al. 2003; De Sousa-D'Auria et al. 2003; Daffé 2005). The main transfer of mycolic acids onto trehalose was shown to take place outside the cytoplasm within the cell wall (Tropis et al. 2005). Recently, it was shown that acetylation of trehalose mycolates is crucial for

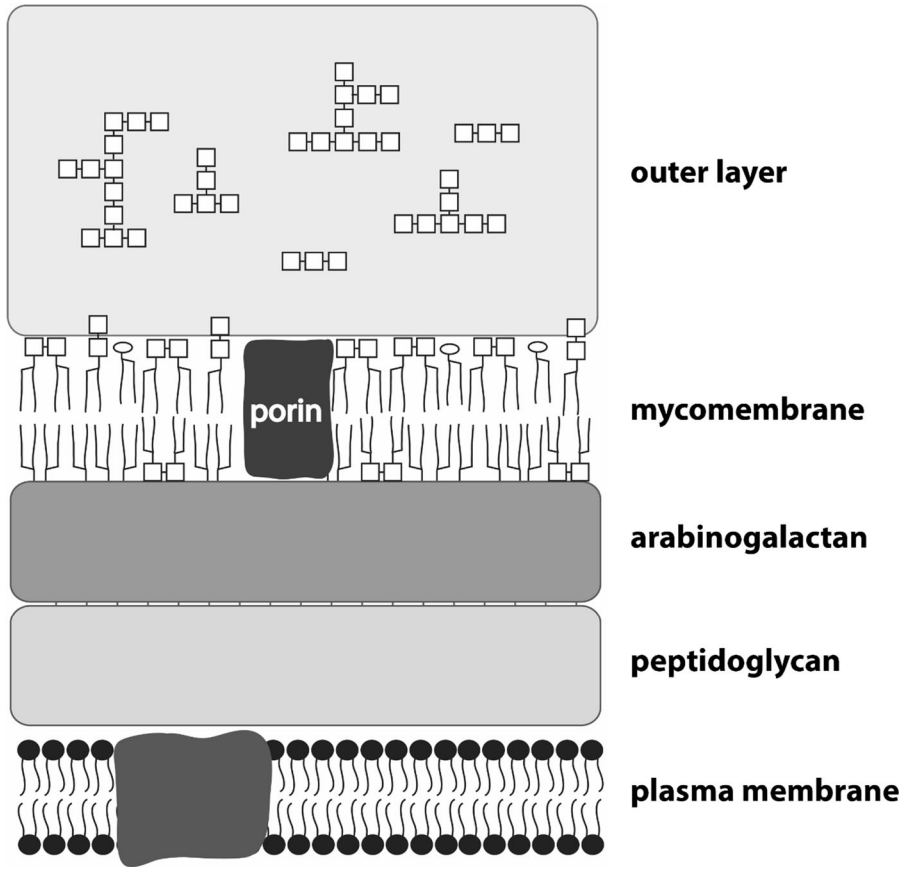

Fig. 1 Schematic of the envelope structure of members of the CMNR group. The different layers are found in almost all species of the group, while their specific composition might differ. The outer layer consists predominantly of glucans and proteins. Based on infection experiments, in *C. diphtheriae* LAM seems to be localized in this layer and also porins may also be present in this layer. While the outer leaflet of the mycomembrane is composed of free lipids including TDM (see also Fig. 2), TMM and various glycolipids, in the inner leaflet of the mycomembrane mycolic acids are covalently linked to the arabinogalactan layer (adapted from Burkovski 2013)

MmpL-mediated membrane transport in *C. glutamicum* and that the corresponding gene is conserved not only in *M. tuberculosis* but in all sequenced *Corynebacterinae* (Yamaryo-Botte et al. 2014).

Interestingly, mycolic acids are also involved in targeting and localization of proteins to the mycomembrane. As well as the porins PorA and PorH, O-mycoloylation was described in *C. glutamicum* for a small protein of unknown function (Huc et al. 2010; Rath et al. 2011, 2013; Carel et al. 2017). Very recently, two other porins of *C. glutamicum*, PorB and PorC, were found to be mycoloylated by a metabolic labelling approach (Issa et al. 2017). A specific mycoloyl transferase of *C. glutamicum* MytC, was identified, which seems to be responsible for O-mycoloylation of these proteins. Moreover, a defect in *C. glutamicum* MytC could be complemented by expression of mycoloyl transferases from *C. diphtheriae* and *Rhodococcus erythropolis* (Huc et al. 2013), implicating a wider distribution of this posttranslational modification among members of the CMNR group.

Analysis of corynomycolic acids

The isolation and partial characterization of trehalose dicorynomycolate from *C. diphtheriae* was first described more than 50 years ago (Ioneda et al. 1963) and in the last decades, a number of methods have been successfully applied to analyze trehalosyl mycolates and other glycolipids from different corynebacteria. These include one- and two-dimensional thin layer chromatography (TLC) (e.g. Gande et al. 2004; Tropis et al. 2005; Gebhardt et al. 2007; Bansal-Mutalik and Nikaido 2011; Yang et al. 2012; Ott et al. 2017; Schick et al. 2017), ^{1}H NMR (Datta and Takayama, 1993; Chami et al. 2002) and different mass spectrometric methods (e.g. Senn et al. 1967; Azuma et al. 1976; Tropis et al. 2005; Bansal-Mutalik and Nikaido 2011; Shui et al. 2012; Yang et al. 2012;

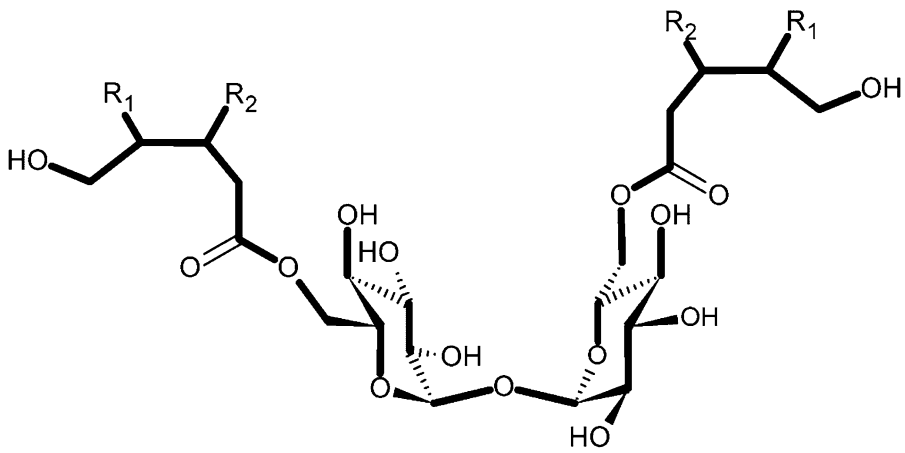

Fig. 2 Basic structure of trehalose dimycolates. Within the CMNR group, corynebacteria comprise the shortest mycolic acids with chain lengths of 22 to 38 carbon atoms (R1 and R2), followed by rhodococci with 34 to 52 and *Nocardia* species with 46–60 total carbon atoms, while the most complex mycolic acids with 60–90 carbon atoms and specific modifications such as cyclopropanes and oxygenated groups are found in mycobacteria (Marrakchi et al. 2014)

Ott et al. 2017). Although corynomycolic acids are shorter and less modified compared to mycolic acids from mycobacteria, differences in length and saturation were for example detected in *C. glutamicum* strains (Yang et al. 2012). Data on other corynebacteria are scarce and more analyses are needed especially to detect putative species and strain-dependent differences as shown for *M. tuberculosis* (Shui et al. 2012).

Other components of the mycomembrane

Besides mycolic acids, other glycolipids—lipoarabinomannan and its precursors—were observed in the cell wall of corynebacteria (Puech et al. 2001). Biochemical and molecular biological studies implicated a linear pathway from phosphatidyl-*myo*-inositol via mono- and diacetylated PIMs and lipomannan to lipoarabinomannan (Mishra et al. 2011). Distribution of LM- and LAM-like compounds seems to be species-specific: in *C. glutamicum* LM-like molecules dominate, in *Corynebacterium xerosis* and *Corynebacterium amycolatum* LAM-like substances were preferentially found, while a *C. diphtheriae* strain showed an almost equal distribution of LM and LAM derivatives (Puech et al. 2001). For this organism, a contribution of CdLAM to the adhesion of bacteria to the human respiratory epithelial cell line Hep-2 was observed (Moreira et al. 2008). Later, Mishra and coworkers demonstrated interaction LAM of *C. glutamicum* with TLR2-transfected HEK293 epithelial cells (Mishra et al. 2011).

Putative role in pathogenicity

Compounds and structures, which support the general robustness of a bacterium, can support pathogenicity unspecifically as so-called niche factors (Tauch and Burkovski 2015). In the case of pathogenic mycobacteria, TDM has long been known to induce inflammatory responses and granuloma formations and functions as adjuvant for T cell responses (Geisel et al. 2005; Hunter et al. 2006; Shenderov et al. 2013). Accordingly, besides playing a passive, protective role, the mycomembrane of different corynebacteria plays also an active role in pathogenicity and the interaction with components of the host immune system.

Investigations of *Corynebacterium pseudotuberculosis* indicated a lethal effect of mycomembrane lipids on caprine and murine macrophages. Lipid extracts of *C. pseudotuberculosis* had negative effects on glycolytic activity, membrane integrity and viability of macrophages (Hard 1975) and lipid content was correlated to abscess formation in mice (Muckle and Gyles 1983). For synthetic trehalose dimycolates it was shown that lethality, in this case in mice, is

correlated with chain length and short-chain TDCM analogues had no toxic effect (Nishizawa et al. 2007).

By analogy to results obtained for *M. tuberculosis* (Indrigo et al. 2003) and *R. equi* (Sydor et al. 2013), which indicated a role of mycolic acids in vesicle trafficking during phagolysosome maturation, a putative role of corynomycolic acids in pathogenicity was also assumed for pathogenic corynebacteria such as *C. ulcerans* and *C. diphtheriae*. In fact, in these two species a delay of phagolysosome maturation was observed (Hacker et al. 2016; Ott et al. 2017). Unexpectedly, however, a mycolic acid-free mutant of *C. diphtheriae* was still able to delay phagolysosome formation in murine and human macrophage cell lines and showed no differences to mycolic acid-containing strains (Ott et al. 2017), indicating that at least in this species, mycolic acids do not interfere with macrophage maturation. The reason might again be based on the short chain length of 16 to 36 carbon units for corynomycolates, which are much shorter than mycolic acids from mycobacteria and rhodococci with up to 89 and 60 carbon units in length (Lanéelle et al. 2013; Sydor et al. 2013; Marrakchi et al. 2014). Moreover, *C. amycolatum* (Collins et al. 1988) and *Corynebacterium kroppenstedtii* (Collins et al. 1998; Riegel et al. 2004; Tauch et al. 2008) are pathogenic corynebacteria without a mycomembrane and lacking corresponding genes for mycolic acid synthesis. Consequently, the role of corynomycolic acids might be more complex than expected and has to be studied in more detail for a wider range of strains and species.

Recognition of mycolic acids and other glycolipids by the immune system

Glycolipids are well-known as highly effective immune-stimulatory compounds. Therefore, it is not surprising that corynebacterial glycolipids are also recognized by host immune cells through specific receptors.

Priming and activation of murine macrophages by trehalose 6,6′-dicorynomycolate (TDCM) from *C. glutamicum* was reported 15 years ago. The effect of TDCM was comparable to TDM from mycobacteria. Moreover, like lipopolysaccharide (LPS) of Gram-negative bacteria, TDCM-containing vesicles induced nitric oxide and TNFα production. Furthermore, TDCM was priming the macrophages for an enhanced secondary nitric oxide response to LPS and induction of endotoxin tolerance (Chami et al. 2002).

Using synthetically produced trehalosyl dicorynomycolate analogs Nishizawa and co-workers showed that toxicity and immunological activities of TDCM in mice was dependent on their fatty acid chain length (Nishizawa et al. 2007). Recently, the mechanism of TDCM recognition was unraveled in more detail. Using enantioselective synthesis of coryomycolic acids and different reporter cell lines, van der Peet and co-workers showed that trehalosyl dicorynomycolate and trehalosyl monocorynomycolate are as effective as the mycobacterial cord factor trehalosyl dimycolate to activate human and murine macrophages. Furthermore, also glycerol monocorynomycolate is recognized by human macrophages. Sensing of these corynomycolic acid-containing glycolipids occurs through the pattern recognition receptor Mincle (van der Peet et al. 2015).

These results were supported by a recent study using bone marrow-derived mouse macrophages. Cell wall extracts of *C. glutamicum*, *C. diphtheriae* and *C. ulcerans* stimulated the inflammatory response of macrophages in a dose-dependent manner. Macrophages deficient in Mincle or its adapter protein FC receptor gamma chain (FcRγ) showed severely reduced amounts of granulocyte colony-stimulating factor (G-CSF) and nitric oxide (NO). When a mycolic acid-deficient mutant strain was used, the immune response was also decreased, but not fully absent. Therefore, it was concluded that also lipomannan and lipoarabinomannan might be involved in recognition of cell wall extracts by murine macrophages (Schick et al. 2017).

Toll-like receptors (TLR) 2 and 4 are known to be crucial for the recognition of bacterial cell wall components, recognizing different components of Gram-negative and Gram-positive bacteria (Takeuchi et al. 1999). TLR2 but not TLR4 seems to be critical for sensing cell wall extracts from *C. diphtheriae*, *C. glutamicum* and *C. ulcerans* by primary macrophages (Schick et al. 2017). Similar results were obtained for *C. glutamicum* glycolipids (Mishra et al. 2012a) and for different *C. diphtheriae* strains using macrophage reporter cell lines (Lisa Ott, unpublished obervations). The role of lipoarabinomannan with respect to initiation of immune responses was investigated for *C. glutamicum* (Mishra et al. 2012a,b). Characterization of a *C. glutamicum* strain devoid of

α(1 → 2)arabinofuranosyltransferase AftE leads to a hypermannosylated variant of LAM, designated hLM. Both LAM and hLM were able to modulate the initiation of immune response by interacting with TLR2. As shown by different in vitro assays, arabinose branching of lipoarabinomannan impacts on T helper cell differentiation and LAM as well as hLM activate dendritic cells via TLR2. Modification of lipoarabinomannan seems to be discriminated by TLR2 and signal pathway induction by hLM was shown to be broader compared to LAM. In accordance with this observation, hLM was shown to be a stronger inducer of immune responses in mice (Mishra et al. 2012b).

While information about recognition of mycolic acids from *C. glutamicum*, *C. diphtheriae* and *C. ulcerans* by the host immune system and their effects on host defense systems have become more available in recent years, data on *C. pseudotuberculosis* are still scarce. Recently, a significant increase of white blood cells, i.e. neutrophils, lymphocytes and basophils, was observed for goats, when infected with *C. pseudotuberculosis* or when isolated cell wall extract was injected, compared to animals injected with phosphate-buffered saline for control (Odhah et al. 2017).

Conclusions

The mycomembrane of corynebacteria is a very unusual structure found in a distinct group of *Actinobacteria*. The key components of the mycomembrane, trehalosyl mycolates, are important stimuli of the innate immune system in animals and humans.

For *M. tuberculosis*, it was shown by in vitro and in vivo studies that even subtle chemical modifications of mycolic acids can substantially affect inflammatory responses and virulence (Reed et al. 2004; Rao et al. 2005; Vander Beken et al. 2011; Barkan et al. 2012). Although corynomycolic acids are shorter and carry less modifications compared to mycolic acids from mycobacteria, alterations in length and saturation were for example detected in different *C. glutamicum* strains. For function of the mycolic acid layer as a diffusion barrier, the outer membrane fatty acid composition altered to adapt to different temperatures (Meniche et al. 2009; Yang et al. 2012).

In pathogenic corynebacteria similar differences might influence the outcome of infections. For example, studies of mycolic acid structure would be interesting in respect to the differential inflammatory responses evoked by *C. diphtheriae* strains or their distinct arthritogenic potential (Puliti et al. 2006; Peixoto et al. 2014; Schick et al. 2017; Peixoto et al. 2017) and different *Corynebacterium* species (Schick et al. 2017). Furthermore, mycomembrane modifications might influence the contagious potential of strains, as shown for *M. tuberculosis* (Reed et al. 2004), and consequently be important for distribution and outbreak of diphtheria and other diseases.

Acknowledgements The help of S. Morbach and G. Seidel (Friedrich-Alexander-Universität Erlangen-Nürnberg) with the preparation of figures is gratefully acknowledged.

Conflict of Interest The author declares that he has no conflict of interest.

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