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MINI-REVIEW

Occurrence, function, and biosynthesis of mycofactocin

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Abstract

Mycofactocin is a member of the rapidly growing class of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products. Although the mycofactocin biosynthetic pathway is widely distributed among *Mycobacterial* species, the structure, function, and biosynthesis of the pathway product remain unknown. This mini-review will discuss the current state of knowledge regarding the mycofactocin biosynthetic pathway. In particular, we focus on the architecture and distribution of the mycofactocin biosynthetic cluster, *mftABCDEF,* among the Actinobacteria phylum. We discuss the potential molecular and physiological role of mycofactocin. We review known biosynthetic steps involving MftA, MftB, MftC, and MftE and relate them to pyrroloquinoline quinone biosynthesis. Lastly, we propose the function of the remaining putative biosynthetic enzymes, MftD and MftF.

Keywords Mycofactocin · Peptide modification · RiPP · Redox cofactor · Biosynthesis

Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPP) are a large class of natural products that play significant biological roles. The growing interest in RiPPs is particularly focused on the immense bioavailability of untapped and diverse classes of bacteriocins such as sactipeptides, lasso peptides, thiopeptides, and lanthipeptides as well as the ability to engineer such systems (for a review see (Arnison et al. 2013; Hegemann et al. 2015; Ortega and van der Donk 2016; De Veer et al. 2017; Craik et al. 2018; Hudson and Mitchell 2018). In addition to antibiotics (Brötz et al. 1998; Stein et al. 2002; Phelan et al. 2013), RiPPs also participate in physiological roles such as quorum sensing and as redox cofactors (Anthony 2001; Datta et al. 2001; Ibrahim et al. 2007; Fleuchot et al. 2011). The latter category is exemplified by the sole bona fide member, pyrroloquinoline quinone (PQQ). PQQ is a redox cofactor used by alcohol and sugar dehydrogenases in some prokaryotes (Goodwin and Anthony 1998; Anthony 2001 and is synthesized by the gene cluster *pqqABCDE*(*FG*) (Klinman and Bonnot 2014). Recently, it was proposed that another uncharacterized biosynthetic pathway could also encode for a RiPP-derived redox cofactor. As will be discussed later, this gene cluster is distributed among the *Mycobacterium* genera and shares a striking resemblance to the PQQ cofactor and bacteriocin biosynthetic pathways, and thus the putative product of the pathway has been named mycofactocin.

Currently, the structure, function, and biosynthesis of mycofactocin are all unknown. However, it is likely that mycofactocin is synthesized through extensive modification of the precursor peptide MftA by proteins encoded in the gene cluster *mftABCDEF* (Haft 2011). MftA is a small ribosomally synthesized peptide that contains a strictly conserved C-terminal sequence-IDGXCGVY. In addition, the cluster encodes for MftB, a small protein with predicted structural homology to the RiPP recognition element (RRE) PqqD of the PQQ biosynthetic pathway (Latham et al. 2017). The protein MftC is a putative radical S-adenosylmethionine (RS) enzyme with low sequence homology to the PQQ biosynthetic enzyme PqqE (Haft and Basu 2011). The remainder of the proteins consists of MftD, a putative FMN dependent protein, and MftF, a putative glycosyltransferase. This review will discuss the association and distribution of the *mft* genes, it will discuss the potential physiological role of mycofactocin, and it will discuss known and predicted biosynthetic steps of mycofactocin.

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Architecture and occurrence of the mycofactocin biosynthetic pathway

The mycofactocin biosynthetic pathway consists of the six strictly conserved genes, *mftABCDEF*. The association of these genes was first identified by a bioinformatic study which described the sporadically distributed pathway (Haft 2011). It was observed that MftC belongs to an RS enzyme subfamily that contains an elongated C-terminal domain annotated as a SPASM domain (subtilosin A, pyrroloquinoline quinone, anaerobic sulfatase-maturating enzyme, and mycofactocin) (Haft and Basu 2011). The proteins associated with the RS-SPASM subfamily are known peptide modifiers that have been shown to catalyze intramolecular C–S bonds and C–C bonds and oxidative decarboxylation reactions on the precursor peptide (Flühe et al. 2012, 2013; Wieckowski et al. 2015; Barr et al. 2016; Bruender et al. 2016; Bruender and Bandarian 2016; Khaliullin et al. 2016, 2017; Latham et al. 2017; Schramma and Seyedsayamdost 2017). Using the MftC clade as a starting point, Haft identified the remaining five *mft* genes in the immediate neighborhood localized around *mftC* (Fig. 1). Indeed, the association of *mftABCDEF* is such that they are found all together or none at all, implying that they are part of the same biosynthetic pathway (Haft 2011). Significantly, Haft also found other genes, mainly a variety of dehydrogenases, that are dependent upon the presences the *mft* cluster (Haft 2011). However, unlike the *mft* genes, these ancillary genes do not share the same strict association with the pathway. Instead, they are found in various combinations only when the *mft* cluster is present, suggesting that they are dependent upon the pathway product, mycofactocin. This finding provided insight to the possible physiological function of mycofactocin, which will be discussed later.

Expression of the mycofactocin biosynthetic pathway is controlled by the regulator MftR, a member of the TetR family of regulators (TFRs) (Minch et al. 2015). TFRs are a large family of single component regulators responsible for the regulation of a wide variety of biosynthetic and response pathways (for a review see Cuthbertson and Nodwell 2013). Indirect evidence that MftR is the mycofactocin regulator is based on a bioinformatic analysis that suggests that the orientation and proximity of *mftR* to the mycofactocin biosynthetic cluster is highly indicative of its regulatory role (Ahn et al. 2012). More conclusively, a recent chromatin immunoprecipitation sequencing (ChiP-Seq) study found MftR bound to a position located 5' of *mftB* (Minch et al. 2015). Although it is likely that MftR is the mycofactocin regulator, the condition under which it governs the pathway remains enigmatic.

While the mycofactocin biosynthetic pathway is one of the most widely distributed RiPP pathway, one fascinating attribute about the *mft* gene cluster is that it is highly concentrated among *Mycobacterial* genomes. A search of the UniProt database for MftC, followed by a manual inspection of the gene context of putative homologs, led us to find the pathway in 625 unique species and subspecies with representatives in the Proteobacteria, Chloroflexi, Euryarchaeota, and Firmicutes phyla (Fig. 2, Ayikpoe et al. 2019). However, the vast majority of mycofactocin-producing bacteria belong to the Actinobacteria phylum. The Actinobacteria phylum contains significant populations within the *Streptomyces* (20), *Geodermatophilus* (17), *Nocardiodes* (14), *Frankia* (17), *Pseudonocardia* (15), *Gordonia* (36), *Nocardia* (15), and

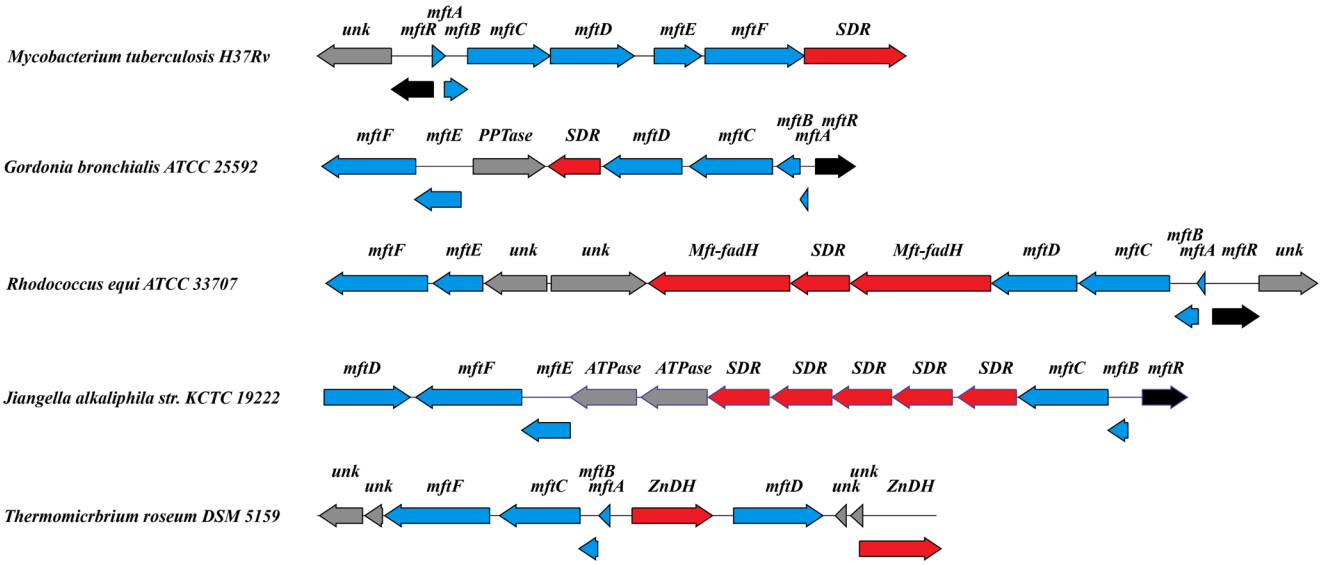


Fig. 1 The gene organization of the mycofactocin biosynthetic pathway (blue) from various species indicates that the genes *mftABCDEF* are generally found together and that MftR (black) is the regulator of the pathway. Ancillary genes (red), such as short-chain dehydrogenases (*SDR*) and zinc-dependent dehydrogenases (*ZnDH*) can be found in various combinations but are dependent upon the presence of the mycofactocin biosynthetic cluster. Additional genes, such as the mycofactocin *fadH*, are also associated with the pathway but for unclear reasons

Rhodococcus (66) genera that were found to have the requisite *mft* genes. Strikingly, about half of all discovered mycofactocin-producing species were found to be concentrated in the *Mycobacterium* genus, with 300 represented species and subspecies. The *mft* cluster is evenly distributed in the genomes of both slow and rapid growing *Mycobacteria* (Stahl and Urbance 1990) including notable species such as *M. tuberculosis*, *M. avium*, *M. bovis*, *M. abscessus*, *M. kansasii*, *M. smegmatis*, *M. ulcerans*, *M. marinum*, and *M. vanbaalenii*.

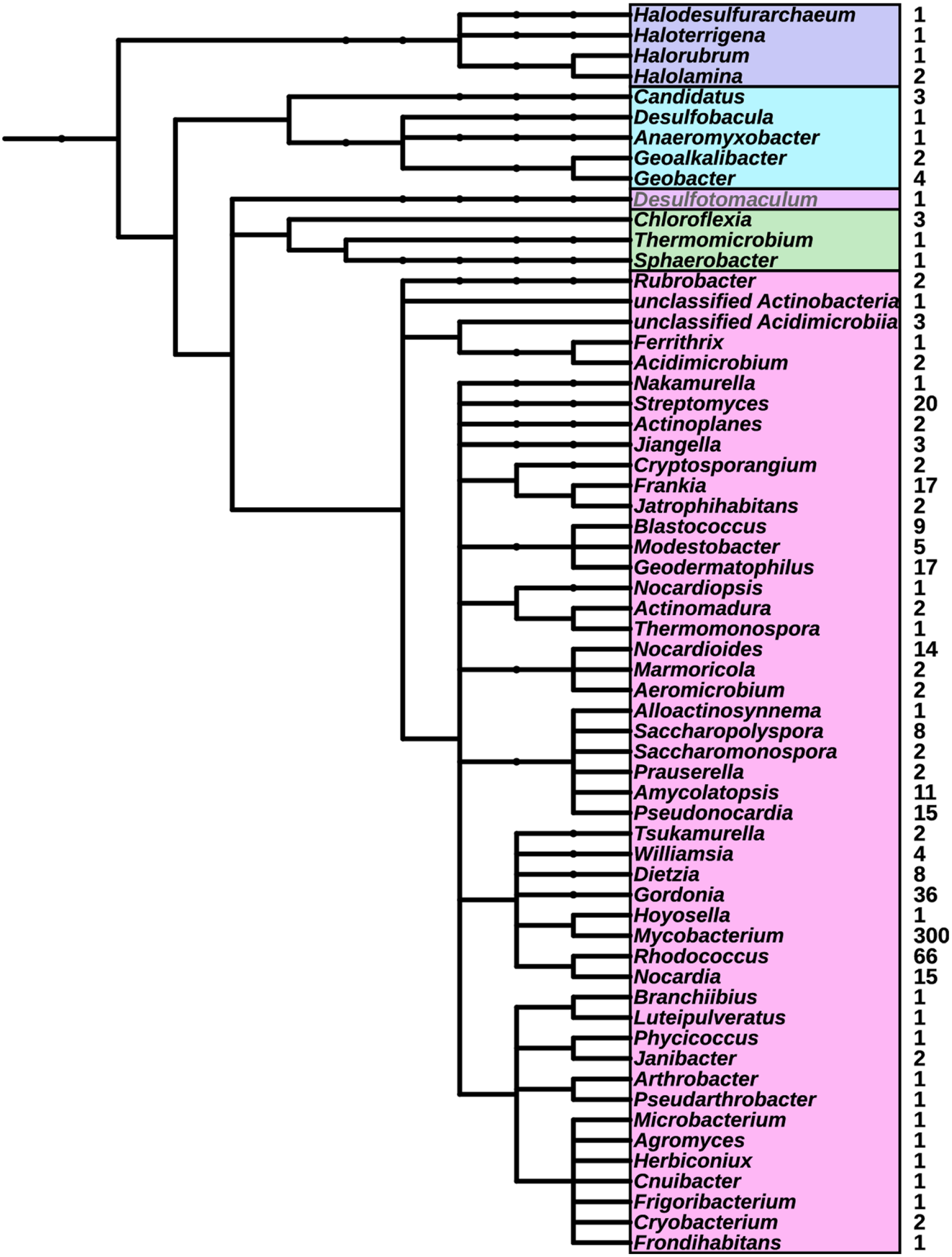
The concentrated nature of the *mft* cluster within the *Mycobacterium* genus is highly provocative and could suggest that the pathway originated in the genus. It is certainly tempting to think that the mycofactocin biosynthetic pathway proliferated to ecologically and genetically similar organisms through horizontal gene transfer, a mechanism that has been associated for a variety of secondary metabolic pathways transfers (for a review see ref. (Soucy et al. 2015)). Indeed, the average sequence identity of all MftC proteins within the *Mycobacterium* genus to the *M. tuberculosis H37Rv* (*Mtb*) MftC is an astonishing 89%. Within the Actinobacteria phylum, the average sequence identity of MftC proteins to that of *Mtb* MftC remains quite high in genera such as *Rhodococcus* (79%), *Nocardia* (77%), *Gordonia* (79%), and *Streptomyces* (74%). However, the sequence identity of MftC outside of the Actinobacteria undergoes a significant drop-off with averages in a range of 42 to 59%. While the *mft* pathway is concentrated in *Mycobacterium* and the sequence identity of MftC is substantial in Actinobacteria, a more in-depth analysis of the entire pathway will be required to identify the origins of the mycofactocin biosynthetic pathway.

Mycofactocin function

Currently, the molecular function of mycofactocin remains unknown. However, recent bioinformatic and experimental data has provided a glimpse into the function of the molecule. To begin with, a partial phylogenetic profile on mycofactocin containing species suggests that redox active ancillary genes are dependent on the pathway (Haft 2011). These second-tier genes encode for redox active short chain dehydrogenases (SDR, TIGR03971), Fe-dependent dehydrogenases (Fe-DH, PF00465), and Zn-dependent dehydrogenases (Zn-DH, TIGR03989). Moreover, the second-tier genes often occur in close proximity to the mycofactocin gene cluster and are found only in species-containing mycofactocin genomic markers. Because of this association, it was proposed that mycofactocin could serve as an electron carrier for these ancillary redox active enzymes (Haft 2011). The co-dependence of SDRs, Zn-DHs, and Fe-DHs is heterogeneous and does not appear to be phylum dependent. However, some peculiarities are present on the genera level. For instance, Haft found that the majority of *Mycobateria* genomes that encode for the mycofactocin biosynthetic pathway predominantly encode for SDRs and Zn-DHs. Conversely, the genomes of *Geobacter* species only encode for Fe-DHs when the mycofactocin biosynthetic pathway is present (Haft 2011). Presently, there is no clear understanding for the variability in the distribution of the mycofactocin-dependent dehydrogenases.

The speculation that mycofactocin is an electron carrier is further corroborated by a recent report on mycofactocin-associated SDRs. Crystallographic evidence suggests that the associated SDRs have a reduced solvent accessible surface area due to the presence of an insertion loop at the NAD^+ binding site, thus preventing NADH from diffusing from away from the binding site and allowing a new NAD^+ to bind (Haft et al. 2017). Furthermore, enzyme activity assays demonstrate that the NAD^+/NADH is non-exchangeable, suggesting that large protein conformational changes do not occur to release the spent cofactor (Haft et al. 2017). In addition, the non-physiologically relevant redox mediator, 2,6-dichlorophenolindophenol (DCPIP) was shown to enhance the oxidation of NADH to NAD^+ of homologous SDRs (Haft et al. 2017). This finding is an important observation since homologous SDRs have been shown to be single turnover enzymes or rely on external redox mediators, such as DCPIP, to catalyze multiple turnovers (Van Der Werf et al. 1999). Taken together with the bioinformatic analysis and the strong resemblance of mycofactocin biosynthesis with PQQ biosynthesis (discussed below), this suggests that mycofactocin could be a redox cofactor.

Although the molecular function for mycofactocin remains unclear, in vivo information is available regarding the essentiality of the pathway in *M. tuberculosis*, providing some insight into its physiological function. An initial study using transposon site hybridization, where genes in *M. tuberculosis* were mutated and the resulting mutant bacterial strains were recovered on mice spleen, demonstrated that the *mft* genes were nonessential for *M. tuberculosis* survival during infection (Sassetti and Rubin 2003). The non-essentiality of the *mft* genes in *M. tuberculosis* was further substantiated by a transposon, and sequencing (TnSeq) study where the *mft* genes were found to be nonessential when *M. tuberculosis* is grown on enriched media (DeJesus et al. 2017). However, when *M. tuberculosis* was grown on cholesterol, an important energy source for the persistence of the bacterium when enveloped by the macrophage (Pandey and Sassetti 2008; Brzostek et al. 2009), Tn-seq data indicated that the genes *Rv0693*, *Rv0694*, *Rv0695*, and *Rv0696* (*mftC*, *mftD*, *mftE*, and *mftF*, respectively) are essential (Griffin et al. 2011). Tn-seq data targeting *mftA* was not available and data for *mftB* showed a tenfold decrease in the transposon population as compared with the control (Griffin et al. 2011). In addition, it was shown that the mycofactocin-associated Zn/NAD-dependent dehydrogenase encoding gene, *Rv0761c*, is also essential for *M. tuberculosis*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Geobacter 4
Desulfotomaculum 1
Chloroflexia 3
Thermomicrobium 1
Sphaerobacter 1
Rubrobacter 2
unclassified Actinobacteria 1
unclassified Acidimicrobiia 3
Ferrithrix 1
Acidimicrobium 2
Nakamurella 1
Streptomyces 20
Actinoplanes 2
Jiangella 3
Cryptosporangium 2
Frankia 17
Jatrophihabitans 2
Blastococcus 9
Modestobacter 5
Geodermatophilus 17
Nocardiopsis 1
Actinomadura 2
Thermomonospora 1
Nocardioides 14
Marmoricola 2
Aeromicrobium 2
Alloactinosynnema 1
Saccharopolyspora 8
Saccharomonospora 2
Prauserella 2
Amycolatopsis 11
Pseudonocardia 15
Tsukamurella 2
Williamsia 4
Dietzia 8
Gordonia 36
Hoyosella 1
Mycobacterium 300
Rhodococcus 66
Nocardia 15
Branchiibius 1
Luteipulveratus 1
Phycicoccus 1
Janibacter 2
Arthrobacter 1
Pseudarthrobacter 1
Microbacterium 1
Agromyces 1
Herbiconiux 1
Cnuibacter 1
Frigoribacterium 1
Cryobacterium 2
Frondihabitans 1

◀ **Fig. 2** An iTOL-generated taxonomy based phylogenetic tree showing the number of mycofactocin encoding species identified in each genus. The indicated genera are color coordinated by phylum where pink represents Actinobacteria, green represents Chloroflexi, lavender represents Firmicutes, cyan represents Proteobacteria, and purple represents Euryarchaeota

growth on cholesterol (Griffin et al. 2011). While it is not apparent that mycofactocin plays a direct role in cholesterol degradation (for a recent review see Wilburn et al. 2018), these findings suggest that *mft* and mycofactocin-associated genes might play a supporting role.

Mycofactocin biosynthesis

If mycofactocin is indeed a redox cofactor, then we suspect that its biosynthesis would parallel that of the redox cofactor, PQQ. PQQ belongs to a family of five peptide-based redox cofactors that include cysteinyl tryptophylquinone (CTQ), lysyl tyrosine quinone (LTQ), tryptophan tryptophylquinone (TTQ), and trihydroxyphenalanine quinone (TPQ). A commonality among this family of quinones is the extensive modifications that occur on the backbone aromatic ring. In all cases, the tyrosine or tryptophan ring is oxidized to form a quinone, and in the cases of LTQ, TTQ, CTQ, and PQQ, the ring is further modified through a substitution reaction resulting in the formation of a C–N, C–S, or C–C bond (Klinman and Bonnot 2014). However, whereas CTQ, LTQ, TTQ, and TPQ are synthesized in situ of the dependent enzyme, PQQ formation requires a dedicated biosynthetic cluster consisting of the conserved genes *pqqABCDE*(*FG*) (Goosen et al. 1992; Veltrop et al. 1995; Toyama et al. 1997; Shen et al. 2012).

The results from aforementioned bioinformatic analysis of the *mft* cluster led Haft to suggest that mycofactocin biosynthesis resembles that of PQQ (Haft 2011). PQQ is synthesized through extensive modification of conserved glutamate and tyrosine residues on the precursor peptide PqqA (Fig. 3). In the first step, the RRE protein PqqD binds PqqA and forms a ternary complex with PqqE (Wecksler et al. 2010; Latham et al. 2015). PqqE catalyzes the carbon–carbon bond formation between the sp^3 hybridized γ-carbon of glutamate and an sp^2 hybridized ortho carbon of the tyrosine aromatic ring on PqqA (Houck et al. 1988; Barr et al. 2016). The resulting glutamate-tyrosine crosslink is then proposed to be excised from the PqqA by PqqF/PqqG to yield the glutamate-tyrosine crosslinked diamino acid (Puehringer et al. 2008; Wei et al. 2016). However, it should be noted that other peptidases have been implicated in PQQ maturation such as PqqH from *P. aeruginosa* (e.g., Gliese et al. 2010). Next, it has been proposed that PqqB oxidizes the crosslinked diamino acid to form the trihydroxy-phenylalanine (Shen et al. 2012; Tu et al. 2017). The spontaneous oxidation of the trihydroxyphenyalanine could lead to a nucleophilic substitution and cyclization reaction involving the free amino group of glutamate to the paraposition of trihydroxyphenylalanine leading to the intermediate 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ). Lastly, PqqC oxidizes and cyclizes AHQQ by removing eight electrons and eight protons, resulting in the formation of PQQ (Magnusson et al. 2004; Magnusson et al. 2007; Bonnot et al. 2013).

When viewing mycofactocin biosynthesis through the lens of PQQ biosynthesis, several parallels can be drawn. In both cases, the first step requires the RS enzymes PqqE and MftC, respectively. Both enzymes catalyze carbon–carbon bond formation between an sp^2 and an sp^3 hybridized carbon on their precursor peptides, in the presence of an accessory RRE domain (PqqD and MftB, respectively), resulting in a chemically crosslinked peptide (Latham et al. 2015; Barr et al. 2016; Bruender and Bandarian 2016; Khaliullin et al. 2016, 2017; Latham et al. 2017). However, while PqqE catalyzes the carbon–carbon bond formation between glutamate and tyrosine on its precursor peptide PqqA in a single step (Fig. 3), MftC catalyzes a two-step modification of the precursor peptide MftA (Fig. 4) (Barr et al. 2016; Khaliullin et al. 2017). In the first step, MftC reductively cleaves SAM to form a 5′-deoxyadenosine radical which in turn abstracts a hydrogen from the Cβ of the C-terminal tyrosine. Following a subsequent abstraction of an electron from the substrate and the loss of the C-terminal carboxylate a new α/β unsaturated bond is formed on the intermediate MftA** (Khaliullin et al. 2017). In the next step of MftC catalysis, a second 5′-deoxyadenosine radical abstracts a hydrogen from the Cβ of the penultimate valine residue. The Cβ radical on the valine side chain forms a new bond with the sp^2 hybridized C2 of the *p*-(2-aminoethenyl)phenol. As a result, the product from MftC catalysis, MftA*, contains a C-terminal 3-amino-5-[(p-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone moiety (Khaliullin et al. 2017). Interesting, PqqE and MftC belong to a select group of RS-SPASM proteins that have been shown to form carbon–carbon bonds on their respective precursor peptide. This group of RS-SPASM proteins consists of one other known member that is involved in streptide biosynthesis. Indeed, the StrB family of proteins were shown to catalyze the radical-SAM–mediated formation of an unusual lysine-tryptophan crosslink on the peptide StrA such that the β-carbon of lysine is crosslinked to the C7 of tryptophan (Schramma et al. 2015; Schramma and Seyedsayamdost 2017). The remaining RS-SPASM proteins that have been characterized to date, with the exception of AnSME, belong to sactipeptide biosynthesis and have been shown to install thioether bond linkages between Cα or Cβ carbons of amino acid side chains and thiols of cysteine residues (Benjdia et al. 2008; Flühe et al. 2012; Nakai et al. 2015; Wieckowski et al. 2015; Bruender et al. 2016; Caruso et al. 2018).

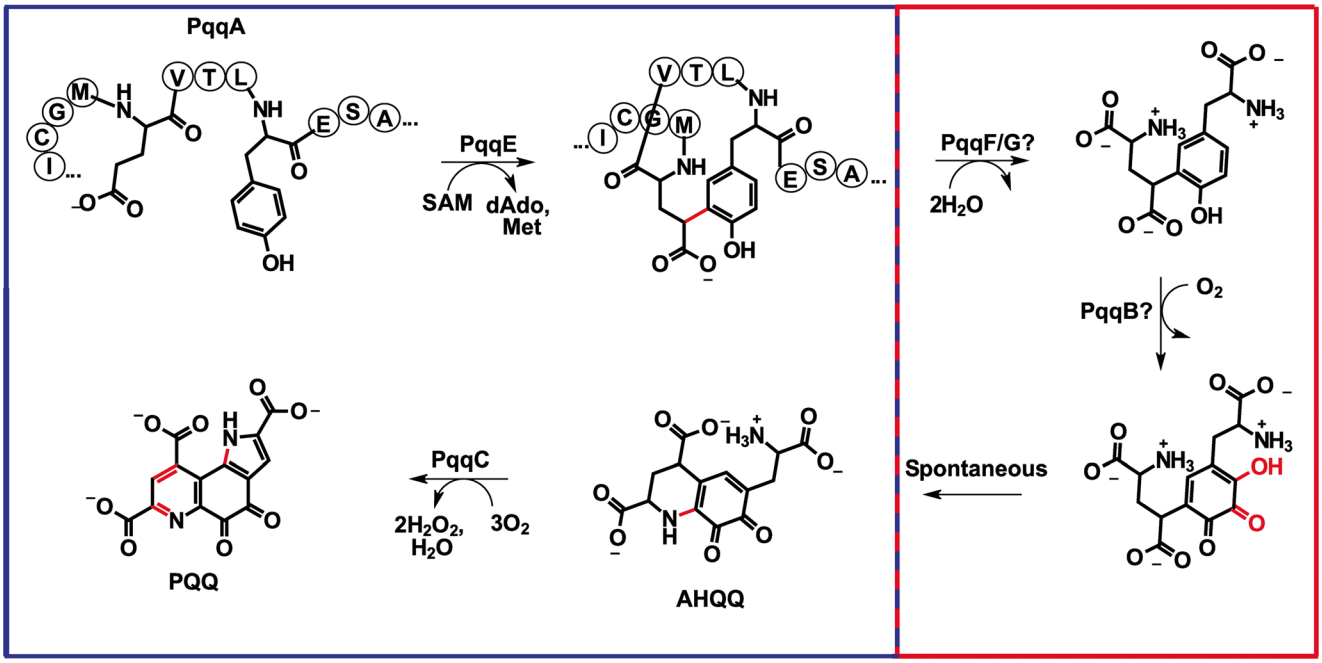

Fig. 3 The biosynthesis of pyrroloquinoline quinone begins with the crosslinking of a conserved glutamate and tyrosine by PqqE. The crosslink is then putatively excised from the peptide by PqqF and/or PqqG. PqqB has been proposed to oxidize the tyrosine ring and a subsequent spontaneous oxidation and cyclization results in the formation of 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ). The last oxidative and cyclization steps are catalyzed by PqqC which result in the formation of PQQ

A second parallel between PQQ and mycofactocin biosynthesis is that it has been shown or proposed that a peptidase acts on the crosslinked PqqA or MftA* peptide. Common among RiPP biosynthesis is the processing of the functionalized peptide by peptidases. This step occurs as a general mechanism of cleaving the leader sequence to liberate the backbone of the RiPP molecule for further processing or transport. An example can be found in subtilosin A biosynthesis where AlbE and AlbF are proposed to be peptidases that cleave the leader sequence of the precursor peptide SboA after it has undergone thioether crosslinking catalyzed by the radical SAM enzyme AlbA (Zheng et al. 2000; Flühe et al. 2012). However, whereas conical peptidases in RiPP biosynthesis are generally multifunctional, removing the peptide leader

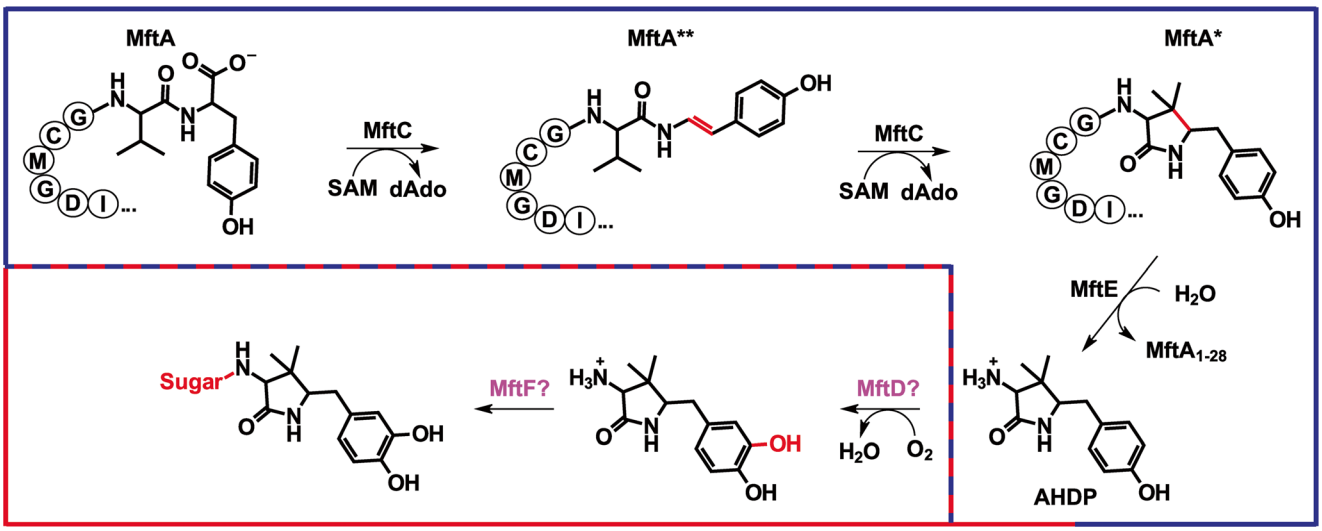

Fig. 4 Known steps (blue) in mycofactocin biosynthesis begins with the two-step modification of MftA by MftC forming the intermediate MftA*. The peptidase MftE, hydrolyzes MftA* yielding 3-amino-5-[(p-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone (AHDP). The involvement of MftD and MftF in mycofactocin biosynthesis is unknown (red) but could include the hydroxylation of the phenol of AHDP and a sugar attachment to the free amine

sequence, exporting the mature peptide product, and/or catalyzing the macrocyclization of the peptide (Havarstein et al. 1995; Duquesne et al. 2007; Ortega et al. 2014; Lagedroste et al. 2017; Ongpipattanakul and Nair 2018), the only apparent function of the mycofactocin and PQQ peptidases is to excise the carbon skeleton from the peptide backbone. In mycofactocin biosynthesis, MftE is the peptidase that cleaves the leader sequence of MftA* (Bruender and Bandarian 2017; Ayikpoe et al. 2018). Recently, MftE was shown to be an Fe-dependent peptidase that selectively hydrolyzes the amide bond between Val29 and Gly28 of MftA*, liberating the putative carbon skeleton of mycofactocin, 3-amino-5-[(p-hydroxyphenyl) methyl]-4,4-dimethyl-2-pyrrolidinone, herein referred to as AHDP (Ayikpoe et al. 2018). Similarly, in PQQ biosynthesis, PqqF and PqqG have been proposed to be the likely peptidases that free the glutamate-tyrosine crosslink from the backbone peptide PqqA (Arnison et al. 2013; Wei et al. 2016).

Following the formation of AHDP by MftE, the remainder of mycofactocin biosynthesis remains unknown. Nevertheless, if mycofactocin belongs to the same family of redox cofactors as PQQ, then a third parallel involving the oxidation of the AHDP phenol may exist. In the PQQ biosynthetic pathway, the oxidation and cyclization reactions of the putative glutamate-tyrosine crosslink has been shown to be carried out by PqqC, and presumably PqqB. Mycofactocin biosynthesis could follow precedent where the aromatic ring of AHDP is oxidized to form a di- or trihydroxy phenyl ring. Of the remaining uncharacterized *mft* genes, the putative FMN binding protein, MftD, could be the enzyme that carries out this hydroxylation/oxygenation (Fig. 4). The remaining protein, MftF, is predicted to be a member of the glycosyltransferase family (Haft 2011), a protein superfamily known to catalyze the transfer of sugar moieties from activated nucleotide diphospho-sugars, nucleotide monophospho-sugars, or sugar phosphates to a range of substrates (Campbell et al. 1997). This implies that mycofactocin, unlike PQQ, may contain a sugar or sugar-phosphate moiety. Although our current working model for mycofactocin biosynthesis does include the hydroxylation of the phenyl ring by MftD and a sugar attachment to the free amine by MftF, no evidence has been presented to suggest that these modifications are correct and other possibilities cannot be ruled out.

Conclusions and future directions

Currently, information regarding the structure and function of mycofactocin has been achieved through bioinformatic analysis and in vitro derived enzymatic data. Haft's bioinformatic analysis on the mycofactocin gene cluster provided the initial account that the molecule could exist and has consequently provided the foundation for subsequent studies. Efforts to elucidate the structure of mycofactocin have predominantly been made through the in vitro reconstitution of pathway enzymes. Similar to the elucidation of PQQ biosynthesis, this strategy has been successful due to the increased knowledge of working with RS-SPASM enzymes and through careful analysis of reaction products. However, the in vitro strategy applied to elucidate mycofactocin biosynthesis poses inherent risks that has not been apparent in the elucidation of PQQ biosynthesis. Whereas the final structure for PQQ has been known for decades, no structural information for mycofactocin exists. Without a guiding structure, accurate prediction of the chemistry for each biosynthetic enzyme becomes increasingly difficult and validating the observed in vitro chemistry is not possible. The latter concern has already been challenging. Initially, two independent reports, one by our lab, suggested that the MftA** was the product of the MftC reaction (Bruender and Bandarian 2016; Khaliullin et al. 2016). However, upon further characterization of the mechanism of MftC, it was found that MftA*, containing an AHDP moiety, was, in fact, the final product (Khaliullin et al. 2017). Although seemingly innocuous, the initial incorrect chemical annotation of MftC led to a subsequent report suggesting that MftE hydrolyzed MftA** to form Val-*p*-(2-aminoethenyl)phenol (Bruender and Bandarian 2017). After the report that MftC catalyzed the conversion of MftA to MftA* and that MftA** is an intermediate of the reaction, the activity of MftE was revisited. Indeed, it was shown that AHDP is the product of MftE hydrolysis of MftA* and that it is inactive towards MftA** (Khaliullin et al. 2017; Ayikpoe et al. 2018). Still, the substrate for MftE hydrolysis could undergo further revision should MftD or MftF act on MftA* and not AHDP or a derivative thereof. This example highlights the complexity of elucidating a biosynthetic pathway of a structurally uncharacterized molecule. While putative activities have been assigned to both MftC and MftE, there is a possibility that further revisions of the first two biochemical steps may be required once MftD and MftF are solved.

While the structure and biosynthesis of mycofactocin could be solved in the near future, efforts towards understanding the physiological function of the molecule have been minimal. TnSeq data (Griffin et al. 2011) is highly suggestive that mycofactocin is an important molecule for the ability of *M. tuberculosis* to catabolize cholesterol. However, understanding how mycofactocin participates in cholesterol catabolism will require further investigations through a combination of in vivo and in vitro techniques. Indeed, the mycofactocin associated protein Rv0761c has yet to be characterized and could be a suitable in vitro target to enhance our understanding of the physiological function for mycofactocin. Similar efforts have already been made on the mycofactocin associated SDRs (Haft et al. 2017), which has indirectly corroborated the redox role of mycofactocin.

Regardless of which direction that research on mycofactocin may go, any information gained will be important for understanding the structure, function, and biosynthesis of the molecule. In less than half a decade, work on the pathway has already discovered new chemistry for a RS-SPASM protein (MftC) and a new natural product (AHDP). With momentum building, it will be exciting to see what unique discoveries about mycofactocin will be made in the near future.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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