Cofactor F_{420}: an expanded view of its distribution, biosynthesis and roles in bacteria and archaea

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One sentence summary: This review provides a comprehensive description of the distribution and biosynthesis of the redox cofactor F_{420}, as well as its enzymology, physiological roles and biotechnological applications.

This review is written in honor of Prof Ralph S. Wolfe (1921-2019), a pioneer of archaeal biology and biochemistry who co-discovered F_{420} in 1971.

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ABSTRACT

Many bacteria and archaea produce the redox cofactor F_{420}. F_{420} is structurally similar to the cofactors FAD and FMN but is catalytically more similar to NAD and NADP. These properties allow F_{420} to catalyze challenging redox reactions, including key steps in methanogenesis, antibiotic biosynthesis and xenobiotic biodegradation. In the last 5 years, there has been much progress in understanding its distribution, biosynthesis, role and applications. Whereas F_{420} was previously thought to be confined to Actinobacteria and Euryarchaeota, new evidence indicates it is synthesized across the bacterial and archaeal domains, as a result of extensive horizontal and vertical biosynthetic gene transfer. F_{420} was thought to be synthesized through one biosynthetic pathway; however, recent advances have revealed variants of this pathway and have resolved their key biosynthetic steps. In parallel, new F_{420}-dependent biosynthetic and metabolic processes have been discovered. These advances have enabled the heterologous production of F_{420} and identified enantioselective F_{420}H_{2}-dependent reductases for biocatalysis. New research has also helped resolve how microorganisms use F_{420} to influence human and environmental health, providing opportunities for tuberculosis treatment and methane mitigation. A total of 50 years since its discovery, multiple paradigms associated with F_{420} have shifted, and new F_{420}-dependent organisms and processes continue to be discovered.

Keywords: cofactor 420; redox chemistry; enzymology; cofactor biosynthesis; redox cofactor; cofactor distribution

ABBREVIATIONS

2PL: 2-phospho-L-lactate
3PG: 3-phospho-D-glycerate
Adf: F_{420}-dependent secondary alcohol dehydrogenase
ANME: anaerobic methanotrophic archaea
AOA: ammonium-oxidizing archaea
APDs: 4-alkyl-L-proline derivatives
BGC: biosynthetic gene cluster
CoM: coenzyme M
CoB: coenzyme B
CoB-S-S-CoM: coenzyme B, coenzyme M heterodisulfide
ddn: deazaflavin-dependent nitroreductase
DFTR: F_{420}H_{2}-dependent flavin-containing thioredoxin reductase

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INTRODUCTION

Cofactors play a fundamental role in biological chemistry. When bound to enzymes, they provide chemical reactivity and specificity that is otherwise unattainable via protein sidechain and backbone chemistry (Begley 2010). Cofactors that mediate redox reactions often contain heterocyclic ring structures, which can accept and donate electrons at physiologically relevant redox potentials (Eicher, Hauptmann and Speicher 2013). In addition to the important heterocyclic riboflavin cofactors FAD and FMN (Fig. 1A), bacteria and archaea produce the structurally related deazaflavin cofactor, F_{420} (Factor 420; Fig. 1B; Cheeseman, Toms-Wood and Wolfe 1972; Eirich, Vogels and Wolfe 1979; Walsh 1980; Joosten and van Berkel 2007; Ney et al. 2017a). While F_{420} structurally resembles FAD and FMN, it is chemically more similar to the nicotinamide cofactors NAD and NADP (Fig. 1C; Jacobson and Walsh 1984; Walsh 1986; de Poorter, Geerts and Keltjens 2005; Huang et al. 2012; Buckel and Thauer 2013). Like NAD(P), F_{420} functions as a cellular hydride carrier (Hendrickson and Leigh 2008). It is reduced by dedicated F_{420}− reducing dehydrogenases, with low potential electrons provided by catabolic substrates or NADPH (Schauer and Ferry 1986; Purwantini and Daniels 1996; Berk and Thauer 1997; Warkentin et al. 2001; Bashiri et al. 2008; Allegretti et al. 2014). The resulting reduced cofactor, termed F_{420}H_{2}, is then utilized by diverse F_{420}H_{2}-dependent reductases to reduce substrates in both catabolic and anabolic pathways (Wang et al. 2013; Ahmed et al. 2015; Purwantini, Daniels and Mukhopadhyay 2016; Greening et al. 2017; Mascotti et al. 2018; Steiningerova et al. 2020).

F_{420} was first described in methanogenic archaea of the phylum Euryarchaeota (Cheeseman, Toms-Wood and Wolfe 1972; Tzeng, Bryant and Wolfe 1975) by the Wolfe group in 1971. Its production was subsequently shown to be universal among methanogenic Euryarchaeota and widespread among other members of this phylum (Eirich, Vogels and Wolfe 1979; van Beelen, Dijkstra and Vogels 1983; Li and White 1986; De Wit and Eker 1987; Gorris and Voet 1991; Gorris 1994; Purwantini, Gillis and Daniels 1997). F_{420} biosynthesis genes are also encoded by diverse other archaea, including members of the TACK and Asgard archaeal superphyla (Evans et al. 2015; Kerou et al. 2016; Vanwongterghem et al. 2016; Ney et al. 2017a; Jay et al. 2018; Spang et al. 2019; Wang et al. 2019). Independent from its discovery in methanogens, F_{420} was isolated from antibiotic-producing streptomycetes belonging to the phylum Actinobacteria (Miller et al. 1960; McCormick and Morton 1982), and was then shown to be widely produced by members of this phylum, including all members of the genus Mycobacterium (Naraoka et al. 1984; Daniels, Bakhiet and Harmon 1985; Purwantini, Gillis and Daniels 1997). Outside of Actinobacteria, F_{420} biosynthesis genes have been detected in a diverse range of bacteria, and its production has been biochemically confirmed in both Proteobacteria and Chloroflexi (Ney et al. 2017a; Braga et al. 2019, 2020). Until recently, it was thought that the F_{420} biosynthesis pathway was identical in all producing organisms (Ney et al. 2017a). However, recent studies have uncovered variation in the substrates and enzymes utilized for F_{420} biosynthesis between bacteria and archaea, as well as a new variant of the mature cofactor in Proteobacteria (Bashiri et al. 2019; Braga et al. 2019; Grinter et al. 2020). This variability reflects the diversity of the organisms that produce F_{420}, as well as the complex evolutionary history of the biosynthesis pathway, which is characterized by both vertical and horizontal gene transfer events (Weiss et al. 2016; Ney et al. 2017a).

In addition to its role in microbial physiology, F_{420} has garnered interest for its industrial, medical and environmental applications. The cofactor and its analogs have potential in industrial biocatalysis (Taylor, Scott and Grogan 2013; Greening et al. 2017; Bashiri et al. 2019; Drenth, Trajkovic and Fraaije 2019). The low redox potential and obligate hydride transfer chemistry of F_{420} enable reduction of otherwise recalcitrant organic molecules (Greening et al. 2017; Mathew et al. 2018; Martin et al. 2020). Numerous F_{420}-dependent enzymes are present in microbial genomes, providing an inventory for industrial biocatalysis (Selenkut and Haft 2010; Ahmed et al. 2015; Mascotti et al. 2018; Steiningerova et al. 2020). Some progress has been made towards use of F_{420}-dependent enzymes in industrial catalysis, including the first heterologous production of the cofactor (Bashiri et al. 2019; Braga et al. 2019; Ney 2019), though further advances are required. With respect to medical applications, the F_{420}-dependent enzyme deazaflavin-dependent nitroreductase (Ddn) from Mycobacterium tuberculosis activates the recently approved antitubercular drugs pretomanid and delamanid and F_{420} has been shown to play a role in antimicrobial resistance in mycobacterial pathogens (Hasan et al. 2010; Cellitti et al. 2017a).
Figure 1. Structural comparison of F420 with flavins and nicotinamides. (A) Structures of the riboflavin head group and tail groups of the flavins FMN and FAD. (B) Structures of the 5-deazaflavin head group and tail groups of F420 and 3PG-F420. Locations of chemical substitutions between riboflavin and 5-deazaflavin are highlighted. N = 2–9 depending on the microbial species. (C) Structural similarity between the nicotinamides NAD(P)H and the central redox-active portion of F420H2. For F420H2, R represents the phospholactyl and oligoglutamate tail shown in panel B. For NAD(P)H, R2 represents the ribose-5-phosphate of the nicotinamide nucleotide and the adenosine nucleobase as shown in (Bogan and Brenner 2013).

Significant progress has been made in understanding F420 in the five years since this topic was last reviewed comprehensively (Greening et al. 2016). We now have a much-improved understanding of the distribution, biosynthesis and roles of F420. These new findings have challenged several paradigms in the field, including the idea that F420 is restricted to a few microbial lineages and is synthesized through a universal pathway. This review provides a new synthesis of our understanding of F420, by integrating recent and historical literature while outlining remaining knowledge gaps. We also discuss how these fundamental advances facilitate applications, for example heterologous F420 production for biocatalysis.

CHEMISTRY, DISTRIBUTION AND ROLES OF F420

Chemical properties

Like the universal nicotinamide cofactors NAD(P) and flavin cofactors FMN/FAD, the primary role of F420 is to transfer electrons between compounds within the cell (Walsh 1986; Munro and McLean 2013). Chemically, F420 consists of three components: the redox-active isoalloxazine head group F0, a phosphoorganic acid linker and a γ-linked polyglutamate tail of variable length (Fig. 1B; Eirich, Vogels and Wolfe 1978; Braga et al. 2019).

As a 5-deazaflavin moiety, the F0 head group contains three chemical substitutions compared to flavins (Fig. 1A and B) that give F420 unique spectral and electrochemical properties (Fig. 2A and B). The key change is the substitution of the redox-active N-5 atom of the isoalloxazine ring for a carbon. In contrast to flavins, this substitution precludes F420 from forming a stable semiquinone, given unpaired electrons cannot delocalize through a C-5 isoalloxazine ring in low-energy states (O’Brien, 2012; Gurumurthy et al. 2013; Lee et al. 2020). Additionally, methanogenic archaea that reside in environments such as livestock rumen, rice paddies and waste landfill produce a significant portion of global methane emissions via a process that requires F420 (Kirschke et al. 2013; Greening et al. 2019). As such, inhibition of F420 biosynthesis or F420-dependent enzymes in livestock has been proposed as a strategy to reduce global greenhouse gas emissions (Attwood et al. 2011; Patra et al. 2017).
Weinstock and Cheng, 1967, 1970; Edmondson, Barman and Tollin, 1972; Xia, Shen and Zhu, 2015. As a result, F420 is an obligate hydride carrier similar to nicotinamides and does not readily undergo single-electron reactions such as autoxidation in air (Fisher, Spencer and Walsh, 1976; Spencer, Fisher and Walsh, 1976; Jacobson and Walsh, 1984; Walsh, 1986). In addition, when compared to flavins, C-7 and C-8 of F420 are demethylated and C-7 is hydroxylated, further altering the redox properties of the cofactor (Eirich, Vogels and Wolfe, 1978). As a result of these three substitutions, F420 has a much lower standard redox potential (−340 mV) than riboflavin (−210 mV), FAD (−220 mV) or FMN (−190 mV; Thauer, Jungermann and Decker, 1977; Walsh, 1986). This redox potential is modulated by physiological conditions, resulting in a redox potential of −380 mV in hydrogenotrophic methanogens that maintain a 10:1 ratio of reduced to oxidized F420 (Jacobson and Walsh, 1984; de Poorter, Geerts and Keltjens, 2005). This makes F420 well suited to mediate the low potential reactions of anaerobic metabolism, as well as reductions that require a low potential electron donor (Thauer, Jungermann and Decker, 1977; Hartzell et al., 1985).

F420 can exist in a range of protonation states as summarized in Fig. 2. The resonance structure of the isoalloxazine ring of oxidized F420 lowers the pKₐ of the C-7 hydroxyl group to 6.3, favoring its deprotonation under basic conditions. Deprotonation of the F420 C-7 hydroxyl leads to delocalization of the resulting unbonded electron and the formation of a conjugated paraquinoid anion, which is the species that exhibits the classic F420 spectral properties of strong absorbance at 420 nm (Fig. 2A; Walsh, 1980, 1986). In this paraquinoid state, F420⁻ exhibits reduced electrophilicity, making it resistant to reduction via hydride acquisition at its C-5 carbon (de Poorter, Geerts and Keltjens, 2005). Protonation of the F420 C-7 hydroxyl group results in a shift of its absorption maxima to ∼400 nm, as well as a decrease in the overall absorption coefficient (Fig. 2B; Mohamed et al., 2016a). During reduction in biological systems, F420 receives a hydride ion at its C-5 carbon with reductant derived from H₂, glucose-6-phosphate (G6P), NADPH, or other low-potential electron donors, via the action of dedicated F420H₂-dependent reductases (Fig. 2A; Aufhammer et al., 2004; Vit et al., 2014; Le et al., 2015; Oyugi et al., 2018). N-1 of reduced F420 possesses an unbonded electron pair and a net negative charge, facilitating its protonation, hence the F420H₂ nomenclature applied to the reduced compound (Jacobson and Walsh, 1984; Walsh, 1986). The pKₐ for the proton association with N-1 of reduced F420H₂ is 6.9,
meaning that the deprotonated reduced form, $\text{F}_{420}^\text{H}^-$, may be the physiologically relevant state of this cofactor in many $\text{F}_{420}$-dependent reductases, which has mechanistic implications as discussed below (Mohamed et al. 2016a). The changes to the bond structure of the isooxazoline ring of $\text{F}_{420}$H$_2$ lead to a corresponding change in its optical properties (Fig. 2A and C; Eirich, Vogels and Wolfe 1979; Walsh 1986; Mohamed et al. 2016a). $\text{F}_{420}$H$_2$ exhibits weak absorbance at 320 nm, with deprotonation to $\text{F}_{420}^\text{H}^-$ causing minimal further changes to its absorbption profile in the visible spectrum (Fig. 2C; Mohamed et al. 2016a). $\text{F}_{420}$H$_2$ formation interrupts conjugation across the isooxazoline ring and isolates the benzenoid portion of the molecule, preventing deprotonation of the C-7 hydroxyl at physiological pH ($pK_a$ 9.7; Walsh 1980, 1986; Jacobson and Walsh 1984).

$\text{F}_{420}$ is a fluorescent molecule, named for the absorbance of its oxidized $\text{F}_0$ head group at 420 nm, with corresponding fluorescence emission at 470 nm mediated by a $\pi \rightarrow \pi^*$ transition upon photon absorption (Cheeseman, Toms-Wood and Wolfe 1972; Mohamed et al. 2016a). $\text{F}_0$ spectral properties are blue-shifted relative to flavin and give $\text{F}_{420}$ a characteristic golden-yellow color and blue-green fluorescence (Cheeseman, Toms-Wood and Wolfe 1972; Eirich, Vogels and Wolfe 1978). The blue-shifted fluorescence of $\text{F}_0$ allows it to efficiently transfer photons to flavin via Förster resonance energy transfer (FRET). In addition to its incorporation into $\text{F}_{420}$, $\text{F}_0$ is synthesized independently and its fluorescent properties are exploited by a class of DNA photolyases, which bind $\text{F}_0$ and FMN as cofactors to mediate the reductive cleavage of DNA pyrimidine dimers (Malhotra et al. 1992; Tamada et al. 1997). $\text{F}_0$-utilizing DNA photolyases are present in cyanobacteria, unicellular algae and possibly higher eukaryotes including Drosophila (Mayerl et al. 1990; Sancar 1990; Glas et al. 2009). Like $\text{F}_0$, $\text{F}_{420}$ exhibits analogous autofluorescence and these properties can be used to identify $\text{F}_{420}$-producing organisms such as methanogens and mycobacteria by fluorescence microscopy (Doddema and Vogels 1978; Maglica, Özdemir and McKinney 2015; Lambrecht et al. 2017), or sort them by flow cytometry. However, $\text{F}_{420}$ is not used by DNA photolyases and its physiological role appears to be restricted to acting as a redox cofactor (Sancar 1990; Kiontke et al. 2014; Greining et al. 2016).

While the $\text{F}_0$ deazaflavin headgroup is solely responsible for $\text{F}_{420}$ redox function, the phospho-organic acid linker and polyglutamate tail modulate cofactor functionality by imparting negative charge and mediating interactions with $\text{F}_{420}$ dependent enzymes (Fig. 1B; Ney et al. 2017b). Bacterial $\text{F}_{420}$-dependent enzymes from at least two superfamilies form electrostatic interactions with the phosphate group of the $\text{F}_{420}$ linker via conserved motifs, enhancing their specificity for the cofactor (Ahmed et al. 2015; Purwantini, Daniels and Mukhopadhyay 2016). The polyglutamate tail of $\text{F}_{420}$ varies in maximum length among producing organisms and exists as a population of different tail lengths from one to nine residues (Gorris and Voet 1991; Gorris 1994; Ney et al. 2017a, b). In archaea, the relative abundance of $\text{F}_{420}$ with different tail lengths varies depending on culture conditions and growth phase, suggesting tail length may modulate $\text{F}_{420}$ function (Peck 1989). Recently we investigated the effect of $\text{F}_{420}$ polyglutamate tail length on the function of mycobacterial $\text{F}_{420}$-dependent enzymes (Ney et al. 2017b). $\text{F}_{420}$ containing both short (two) and long (five to eight) polyglutamate chains were compatible with these enzymes, though long-chain $\text{F}_{420}$ bound these enzymes with six to 10-fold greater affinity. Chain length also significantly modulated the kinetics of the enzymes, with long-chain $\text{F}_{420}$ increasing the substrate affinity (lower $K_m$) but reducing the turnover rate (lower $k_{cat}$).

Molecular dynamics simulations indicated that $\text{F}_{420}$-dependent enzymes make multiple dynamic electrostatic interactions with the $\text{F}_{420}$-polyglutamate tail via conserved surface residues, likely explaining the observed differences in activity between short and long chain $\text{F}_{420}$ (Ney et al. 2017b). These data suggest that variable $\text{F}_{420}$ polyglutamate tail length may have evolved to modulate the activity of $\text{F}_{420}$-dependent enzymes. Additionally, these findings have significant implications for the use of $\text{F}_{420}$ in industrial applications, where a high catalytic turnover is likely to be desirable.

**$\text{F}_{420}$-dependent enzymes**

$\text{F}_{420}$-dependent enzymes are broadly classified as $\text{F}_{420}$-reducing dehydrogenases or $\text{F}_{420}$H$_2$-dependent reductases based on the direction of the redox reaction they perform under physiological conditions (Greining et al. 2016). However, due to the relatively similar redox potentials of many $\text{F}_{420}$-substrate pairs, some $\text{F}_{420}$-dependent enzymes are bidirectional depending on the organism and physiological conditions (Eker, Hessels and Meerwaldt 1988; Berk and Thauer 1997; Afting, Hochheimer and Thauer 1998; Hendrickson and Leigh 2008). $\text{F}_{420}$-dependent enzymes can be further divided into two classes based on their mechanism of electron transfer. In the first of these classes, bound $\text{F}_{420}$ accepts or donates hydride directly to or from the enzyme substrate. In the second class, bound flavin (FAD or FMN) acts as an intermediate, either accepting a hydride from or donating a hydride to $\text{F}_{420}$ (Shima et al. 2000; Ceh et al. 2009; Allegretti et al. 2014; Ahmed et al. 2015; Joseph et al. 2016; Ouygi et al. 2018). $\text{F}_{420}$-dependent oxidoreductases of this second class often contain additional subunits with multiple iron-sulfur (FeS) clusters, which transfer electrons between the enzyme-substrate (i.e. H$_2$ or formate) and $\text{F}_{420}$, via FNM/FAD. In this role, the bound flavin acts as a modulator between the single-electron chemistry of the FeS clusters and the hydride chemistry of $\text{F}_{420}$ (Wood, Haydock and Leigh 2003; Seedorf et al. 2007; Vitt et al. 2014).

$\text{F}_{420}$-dependent enzymes are structurally diverse and can be classified into several families, which possess distinct folds and evolutionary histories (Greining et al. 2016). These families are often distributed in both $\text{F}_{420}$-producing archaea and bacteria (Ney et al. 2017a) and have evolved to utilize $\text{F}_{420}$ as a cofactor independently (Ahmed et al. 2015; Mascotti et al. 2018; Mascotti, Ayub and Fraaije 2020). $\text{F}_{420}$-dependent enzyme families often include both $\text{F}_{420}$-reducing and $\text{F}_{420}$H$_2$-oxidizing enzymes and are members of broader groups of oxidoreductases that utilize FNM, FAD, NAD(P)H, or heme as cofactors (Ahmed et al. 2015; Mascotti et al. 2018). Some of these groups contain multiple distinct lineages of enzymes that utilize $\text{F}_{420}$, indicating that specificity for the cofactor arose on multiple occasions (Ahmed et al. 2015; Mascotti et al. 2018). The currently identified enzyme families that utilize $\text{F}_{420}$ as a cofactor are summarized in Table 1, with functionally characterized $\text{F}_{420}$-dependent dehydrogenases and $\text{F}_{420}$H$_2$-dependent reductases cataloged in Tables 2 and 3 respectively. The structures of representative examples of each family are shown in Figs 3 and 4. We have previously comprehensively reviewed the structure and function of these enzymes (Greining et al. 2016), and so we will not detail these aspects here.

**Taxonomic distribution**

Until recently $\text{F}_{420}$ was thought to be a rare cofactor, taxonomically restricted to the members of archaeal phylum Euryarchaeota and the bacterial phylum Actinobacteria (Ney...
| F420-dependent protein family | Acronym | Protein fold | Mechanism of hydride transfer | Phylogenetic distribution | Characterized function(s) | Key references |
|-------------------------------|---------|--------------|-------------------------------|---------------------------|---------------------------|----------------|
| Flavin/deazaflavin oxidoreductase | FDOR-A, FDOR-B | Split β-barrel | Direct F420-substrate | Actinobacteria, Chloroflexi | F420H2-dependent reduction of diverse substrates (e.g. menaquinone, tetracycline and biliverdin) with promiscuous activity often observed | Cellitti et al. (2012); Lapaíkar et al. (2012); Ahmed et al. (2015); Greening et al. (2017) |
| Luciferase-like hydride transferase | LLHT | TIM-Barrel | Direct F420-substrate | Broadly found in F420 producing bacteria and archaea | F420-dependent oxidation or F420H2-dependent reduction of diverse substrates (e.g. G6P, mycolic acids and CH2=H4MPT) Hydride transfer between F420/F420H2 and NADP/NADPH | Aufhammer et al. (2004, 2005); Bashiri et al. (2008); Nguyen et al. (2017); Mascotti et al. (2018) |
| F420-dependent NADPH oxidoreductase | Fno | Rossmann fold | Direct F420-substrate | Broadly found in F420 producing bacteria and archaea | | |
| F420-dependent H4MPT oxidoreductase | Mtd | Rossmann fold | Direct F420-substrate | Euryarchaeota: methanogens, ANME, Archaeoglobales | Hydride transfer between F420/F420H2 and CH=H4MPT/CH2=H4MPT Reduction of dioxigen (O2) to water (2 H2O) to detoxify O2 Couples the reduction/oxidation of F420/F420H2 to that of formate, H2, methanophenazine, quinone or sulfite, via association with structurally diverse protein subunits. | Shim et al. (2000); Hagemeyer et al. (2003a); Warkentin et al. (2005); Ceh et al. (2009); Seedorf et al. (2004, 2007) |
| F420-dependent bifurcating reductase | FdcC | β-lactamase/flavodoxin fold | Indirect F420-flavin-2Fe-O2 | Euryarchaeota: methanogens, ANME, Archaeoglobales | | Baron and Ferry (1989); Bäumer et al. (2000); Brüggenmann, Falinski and Deppenmeier (2000); Johnson and Mukhopadhayay (2005, 2008b); Welte and Deppenmeyer (2011a); Allegretti et al. (2014); Vitt et al. (2014) |
| Deazaflavin-dependent thioredoxin reductase | DFTR | Thioredoxin reductase fold | Indirect F420-flavin-disulfide | Euryarchaeota: Methanococcales | Couples F420H2 oxidation to the reduction of thioredoxin | Susanti, Loganathan and Mukhopadhayay (2016) |
| F420-dependent bifurcating reductase | HdrA2 | HdrA-like fold | Indirect F420-flavin-FeS-substrate | Euryarchaeota: Methanosarcinales | Couples F420H2 oxidation to the reduction of CoM-S-S-CoB and ferredoxin via bifurcation | Yan, Wang and Ferry (2017) |
| Oxidoreductase and domain | Physiological role | Taxonomic distribution | Family | EC no. | PDB ID | References |
|--------------------------|-------------------|------------------------|--------|--------|--------|------------|
| **Archaea**              |                   |                        |        |        |        |            |
| Fdh: F$_{420}$-reducing hydrogenase | Methanogenic growth on H$_2$. Couples oxidation of H$_2$ to the reduction of F$_{420}$. May be physiologically reversible. | All orders of methanogens | FDRC | 1.12.98.1 | 4OMF, 4C10, 3ZFS, 6QGT | Tzeng, Wolfe and Bryant (1975); Jacobson et al. (1982); Muth, Morschel and Klein (1987); Kulkami et al. (2009); Mills et al. (2013); Allegrètii et al. (2014); Vitt et al. (2014); Ilina et al. (2019) |
| Ffd: F$_{420}$-reducing formate dehydrogenase | Methanogenic growth on formate. Couples oxidation of formate to the reduction of F$_{420}$. May be part of electron bifurcating complex. | Many Eurarchaeota (e.g. Methanobacterales, Methanococcales, Methanopyrales, Methanomicrobiales and Methanocellales) | FDRC | 1.2.99.9 |        |            |
| Adf: F$_{420}$-reducing secondary alcohol dehydrogenase | Growth on secondary alcohols. Couples oxidation of secondary alcohols (e.g. isopropanol) to the reduction of F$_{420}$. | Some Eurarchaeota (Methanomicrobiales and Methanocellales) | LLHT | 1.1.98.5 | 1RHC | Widdel and Wolfe (1989); Bleicher and Winter (1991); Aufhammer et al. (2004); Martin et al. (2020) |
| **Bacteria**             |                   |                        |        |        |        |            |
| Fno: F$_{420}$-reducing NADPH dehydrogenase | Exchanges electrons between NADP and F$_{420}$. F$_{420}$ reduction direction dominant in bacteria, as F$_{420}$ is the secondary cofactor. | Many Actinomycetales (e.g. Streptomyces, Thermobifida, Rhodococcus, Noeadia and Noeadiales), Chloroflexi?, Alphaproteobacteria?, Betaproteobacteria? | Fno | 1.5.1.40 | 5N2I | Eker, Hessels and Meerwaldt (1989); Heiss et al. (2002); Kumar et al. (2017) |
| Fgd: F$_{420}$-reducing glucose-6-phosphate dehydrogenase | Heterotrophic growth. Couples oxidation of glucose-6-phosphate to the reduction of F$_{420}$ via the pentose phosphate pathway. | Many Actinomycetales (e.g. Mycobacterium, Actinoplanes, Microbacterium and Amycolatopsis), Chloroflexi, Alphaproteobacteria?, Betaproteobacteria? | LLHT | 1.1.98.2 | 3B4Y | Bashiri et al. (2008); Oyugi et al. (2018) |
| Fsd: F$_{420}$-reducing sugar-6-phosphate dehydrogenase | Heterotrophic growth. Couples oxidation of glucose-, fructose- or mannose-6-phosphate to the reduction of F$_{420}$. Similar to Fgd, with a catalytic preference for glucose-6-phosphate, but an expanded substrate specificity. | Some Actinomycetales (e.g. Noeadiales and Cryptosporangium) | LLHT | 1.1.98.2 |        | Mascotti et al. (2018) |
| fimAD: F$_{420}$-reducing hydroxymycolic acid dehydrogenase | Cell wall biosynthesis. Catalyzes F$_{420}$-dependent oxidation of hydroxymycolic acids to ketomycolic acids. | Few Mycobacterium (primarily pathogenic species) | LLHT |        |        | Bashiri et al. (2012); Purwantini and Mukhopadhyay (2013) |
| Amm4: F$_{420}$-dependent ammosamide dehydrogenase | Putative dehydrogenase involved in primary amide formation in the pyrroloquinoline alkaloid ammosamide. Details of reaction mediated and the product formed are unresolved. | Few Actinomycetales (e.g. Streptomyces and Amycolatopsis) | FDOR-B |        |        | Jordan and Moore (2016) |
Table 3. Functionally characterized F$_420$H$_2$-dependent reductases. This table updates and expands upon the enzymes previously summarized and reviewed by Greening et al. (2016).

| Oxidoreductase and domain | Physiological role | Taxonomic distribution | Family | EC no. | PDB ID | References |
|---------------------------|-------------------|------------------------|--------|--------|---------|------------|
| **Archaea**               |                   |                        |        |        |         |            |
| Mtd: F$_420$-reducing methylene-H$_4$MPT dehydrogenase | Reduces CH≡H$_4$MPT to CH$_2$=H$_4$MPT with F$_420$H$_2$ during CO$_2$-reducing methanogenesis. Performs the opposite reaction during methylotrophic methanogenesis and anaerobic methane/alkane oxidation. | Various Euryarchaeota including: all orders of methanogens, Archaeoglobales, ANME and Halobacterales; various TACK and Asgard archaea | Mtd | 1.5.98.1 | 1QV9, 1U61, 3IQF, 3IQE | Hartzell et al. (1985); Brümmler et al. (1991a,b); Hagemieier et al. (2003a,b); Geh et al. (2009) |
| Mer: F$_{230}$H$_2$-dependent methylene-H$_4$MPT reductase | Reduces CH$_2$=H$_4$MPT to CH$_3$-H$_4$MPT with F$_420$H$_2$ during CO$_2$-reducing methanogenesis. Performs the opposite reaction during methylotrophic methanogenesis and anaerobic methane/alkane oxidation. | Various Euryarchaeota including: all orders of methanogens, Archaeoglobales, ANME and Halobacterales; various TACK and Asgard archaea | LLHT | 1.5.98.2 | 1F07, 1EZW, 1Z69 | Te Brümmler et al. (1991b); Shima et al. (2000); Aufhammer et al. (2005); Geh et al. (2009) |
| Fpo: F$_420$H$_2$-dependent methanophenazine reductase | Proton-translocating primary dehydrogenase in respiratory chain transferring electrons from F$_{230}$H$_2$ to heterodisulfide. | Methanosarcinales | FDRC | 1.1.98.4 | | Bäumer et al. (1998); Deppenmeier, Lienard and Gottschalk (1999); Ide, Bäumer and Deppenmeier (1999); Bäumer et al. (2000); Welte and Deppenmeier (2011a); Kunow et al. (1994); Brügmann, Falinski and Deppenmeier (2000); Hallam et al. (2004); Hocking et al. (2014); Seedof et al. (2004,2007) |
| Fpo: F$_{230}$H$_2$-dependent quinone reductase | Proton-translocating primary dehydrogenase in respiratory chain transferring electrons from F$_{230}$H$_2$ to sulfate. | Archaeoglobales and ANME | FDRC | 1.1.98.4 | | |
| Fpr: F$_{230}$H$_2$-dependent oxidase | Detoxifies O$_2$ by mediating the four-electron reduction of O$_2$ to H$_2$O with F$_{230}$H$_2$. | Methanobacteria, Methanococcales, Methanomicrobiales and Methanocellales | FprA | 1.5.3.22 | 2OHH, 2OHI, 2OHI | Seedof et al. (2004,2007) |
| Fsr: F$_{230}$H$_2$-dependent sulfite reductase | Detoxifies sulfite by mediating the six electron reduction of sulfite to sulfide with F$_{230}$H$_2$. Also enables the use of sulfite as an S source. | Methanobacteria and Methanococcales | Mds | 1.8.98.3 | | Johnson and Mukhopadhyay (2005,2008a) |
| Fno: F$_{230}$H$_2$-dependent NADP$^+$ reductase | Exchanges electrons between NADP$^+$ and F$_{230}$-NADP$^+$ reduction direction dominant in archaea, as NADP$^+$ is the secondary cofactor. | Various Euryarchaeota including: all orders of methanogens, Archaeoglobales and ANME; various TACK and Asgard archaea | Fno | 1.5.1.40 | 1JAY, 1JAX | Tzeng, Wolfe and Bryant (1975); Kunow et al. (1993); Berk and Thauer (1997); Warkentin et al. (2001) |
| HdrA2B2C2: F$_{230}$H$_2$-dependent, electron-bifurcating, heterodisulfide reductase | The HdrA2 subunit of this complex oxidizes F$_{230}$H$_2$, with subunits HdrB2 and HdrC2 bifurcating the resulting electrons to ferredoxin and CoM-S-S-CoB (heterodisulfide). Thought to mediate energy conservation during acetoclastic methanogenesis. | Methanosarcinales | HdrA2 | | | Yan, Wang and Ferry (2017) |
| DFTR: F$_{230}$H$_2$-dependent thioredoxin reductase | Recycling of the thioredoxin disulfide through reduction by electrons transferred from F$_{230}$H$_2$ via a low potential FMN and disulfide redox center. | Methanococcales | DFTR | 1.8.1.9 | | Susanti, Loganathan and Mukhopadhyay (2016) |
| Oxidoreductase and domain | Physiological role | Taxonomic distribution | Family | EC no. | PDB ID | References |
|---------------------------|-------------------|-----------------------|--------|--------|---------|-------------|
| **Bacteria** | | | | | | |
| **Ddn**: F420H2-dependent menaquinone reductase | Reduction of the respiratory cofactor menaquinone for energy conservation and possibly to mitigate redox stress. Also catalyzes the promiscuous activation nitroimidazole prodrugs. | Most Actinomycetales (e.g., Mycobacterium, Streptomyces, Rhodococcus), Chloroflexi?, Methanosarcinales? | FDOR-A1 family. | 1.1.98.- | 3H96, 4Y9I, 3R5R, 3R57 | Taylor et al. (2010); Cellitti et al. (2013); Ahmed et al. (2015); Lee et al. (2020) |
| **Fbr**: F420H2-dependent biliverdin reductase | Reduction of the heme degradation product biliverdin to bilirubin. May also reduce mycobillins. | Most Actinomycetales (e.g., Mycobacterium, Streptomyces, Rhodococcus), Chloroflexi?, Methanosarcinales? | FDOR-B3 and FDOR-B4 family. | 1.1.98.- | 2FY1, 4QVB, 1W9A | Canaan et al. (2005); Biswal et al. (2006); Ahmed et al. (2015, 2016); Mashalidis et al. (2015) |
| **Fts**: F420H2-dependent tetracycline synthase | Reduction of dehydrotetracyclines to tetracyclines during streptomycete antibiotic synthesis. Role in mycobacteria unknown. | Most Actinomycetales (e.g., Mycobacterium, Streptomyces, Rhodococcus), Chloroflexi?, Thaumarchaeota? | FDOR-B family. | 1.1.98.- | 2FY1, 4QVB, 1W9A | Taylor et al. (2010); Wang et al. (2013); Ahmed et al. (2015); Lee et al. (2020) |
| **TpnL**: F420H2–dependent dehydropiperidine reductase | Reduction of the dehydropiperidine moiety to piperidine during the synthesis of thiopeptins antibiotics. | Some Actinomycetales (Streptomyces, Amycolatopsis, Micromonospora and Actinoalloteichus) | FDOR-B family. | 1.1.98.- | | Ichikawa, Bashiri and Kelly (2018) |
| **GupA**: F420H2–dependent dihydropyrazine reductase | Reduction of the dihydropyrazine ring to piperzine during the synthesis of guanipiperazines. | Some Actinomycetales (Streptomyces) | FDOR-B family. | 1.1.98.- | | Shi et al. (2021) |
| **Fht**: F420H2-dependent picrate reductase | Reduces 2,4,6-trinitrophenol (picrate) for use as a C and N source through hydride transfer to the nitroaromatic ring. | Few Actinomycetales (Rhodococcus, Nocardia, Nocardioides) | FDOR-A/B family. | 1.1.98.- | | Ebert, Rieger and Knackmuss (1999); Heiss et al. (2002) |
| **Fps/Adp6**: F420H2-dependent 4-alkyl-L-proline derivative reductases | Reduction of 4-alkyl-L-proline derivatives (APDs) in the final step in the biosynthesis of this compound. Different enzymes of this class impart structural diversity by reducing either the endocyclic imine or the exocyclic double bond of APDs. | Some Actinomycetales (Streptomyces, Micrococcus and Streptosporangium) | FDOR-A/B family. | 1.1.98.- | | Li et al. (2009a,b); Steiningerova et al. (2020) |
| **fPKR**: F420H2-dependent phthiodiolone ketoreductase | Reduction of phthiodiolone keto intermediates during the synthesis of phthiocerol dimycocerosates (PDIM), a class of mycobacterial cell surface apolar lipids. | Few Mycobacterium (primarily pathogenic species) | FDOR-A/B family. | 1.1.98.- | | Purwantini, Daniels and Mudumbai (2019) |
| **LxmJ**: F420H2-dependent 2,3-didehydroalanine reductase | Stereospecific reduction of the 2,3-didehydroalanine reductase to D-alanine during class V lanthipeptide biosynthesis. | Few Streptomyces | FDOR-A/B family. | 1.1.98.- | | Tao et al. (2020) |
et al. 2017a). However, recent studies applying genomic, spectroscopic and biochemical analysis have demonstrated that F420 is much more widely distributed among bacteria and archaea than previously thought (Kerou et al. 2016; Lackner et al. 2017; Ney et al. 2017a; Braga et al. 2019, 2020). Prior to these studies, it was assumed that F0 production was more widespread than F420. Yet genomic analysis shows that, in the majority of organisms, the genes required for F0 biosynthesis co-occur with those required for its conversion to F420, indicating that F0 is generally produced as a precursor for F420 biosynthesis, with a possible secondary role as a chromophore in some F420 producers (Kiontke et al. 2014; Ney et al. 2017a). A phylogenetic tree and accompanying table outlining microbial lineages biochemically demonstrated to produce F0 and F420, as well as those predicted to produce these cofactors based on genomic data, is presented in Fig. 5 and Table 4. There is currently no evidence that F420 is synthesized as a redox cofactor by eukaryotes. The distribution of F420 biosynthesis genes among bacteria and archaea appears to be widespread in some lineages (i.e. Euryarchaeota and Actinobacteria; Cheeseman, Toms-Wood and Wolfe 1972; Eirich, Vogels and Wolfe 1979; Lin and White 1986; Bair, Isabelle and Daniels 2001), but variable among others (i.e. TACK lineages of Archaea and Proteobacteria; Kerou et al. 2016; Ney et al. 2017a). F420 biosynthesis genes are highly abundant in metagenomes.
The secondary structural elements are highlighted (blue = α-helix, yellow = β-sheet or coil), FMN or FAD colored red and FeS clusters and metal ions are shown as spheres. (A) FDFO family $F_420$-dependent flavodiiron oxidase (FprA) from *Methanothermobacter thermautotrophicus* responsible for the reduction of $O_2$ to $H_2O$ (PDB ID = 2OHJ; Seedorf et al. 2007). (B) FDRC domain-containing $F_420$-reducing NiFe hydrogenase (Frh) from *Methanothermobacter marburgensis* (PDB ID = 4CI0; Allegretti et al. 2014). (C) $F_420$-H$_2$-dependent thioredoxin reductase (DFTR) from *M. jannaschii* (homology model; Susanti, Loganathan and Mukhopadhyay 2016).

from diverse soil, marine and some host-associated ecosystems, further indicating that $F_420$ biosynthesis is a widespread trait (Ney et al. 2017a).

**$F_420$ production and roles within archaea**

Within archaea, $F_420$ production has only been biochemically confirmed in Euryarchaeota and much of our understanding of the physiological roles of the cofactor is derived from these organisms (Jacobson and Walsh 1984; Schmitz et al. 1991; Vaupel and Thauer 1995; Berk and Thauer 1997; Thauer 1998; Brüggemann, Falinski and Deppenmeier 2000). Currently, available genomic and metagenomic datasets show that a complete complement of genes necessary for $F_420$ biosynthesis is also present in members of two other major archaeal groups, the TACK and Asgard archaea (Kerou et al. 2016; Ney et al. 2017a;
Figure 5. Phylogenetic distribution of F0 and F420 producing organisms. A simplified two-domain tree of life depicted the organisms shown or predicted to produce the 5-deazaflavins F0 or F420. This is based on currently available data from published work (Greening et al. 2016; Ney et al. 2017a), and genomic and metagenomic data in the NCBI database (as of October 2020). Tree topography is based on Hug et. al. (Hug et al. 2016) and Castelle and Banfield (2018), with additional reference to Zhou et al. (2020), Wang et al. (2019) and Momper, Aronson and Amend (2018). ∗ = F420 biosynthesis genes detected only in multiple metagenome-assembled genomes (MAGs) or single-amplified genomes (SAGs) from these archaea and bacteria, rather than genomes derived from pure culture.
Table 4. Confirmed and predicted F_{420}-producing organisms. Experimentally confirmed F_{420} producers are highlighted in yellow, while predicted F_{420} producers with a full complement of F_{420} biosynthesis genes based on analysis of assembled pure culture genomes or multiple MAGs/SAGs, are highlighted in green.

| Taxonomy             | FbiC | CofH | CofG | CofC/ FbiD | CofD/ FbiA | CofE | FbiE | FbiB |
|----------------------|------|------|------|------------|------------|------|------|------|
| **Bacteria**         |      |      |      |            |            |      |      |      |
| Actinobacteria       |      |      |      |            |            |      |      |      |
| Actinobacteria       |      |      |      |            |            |      |      | X    |
| Rubrobacteria        |      |      |      |            |            |      |      | -    |
| Thermoleophilia      |      |      |      |            |            |      |      | -    |
| Acidimicrobia        |      |      |      |            |            |      |      | -    |
| Nitriliruptoria      |      |      |      |            |            |      |      | -    |
| **Chloroflexi**      |      |      |      |            |            |      |      |      |
| Thermomicrobia       |      |      |      |            |            |      |      | -    |
| Ardenticaenae        |      |      |      |            |            |      |      | -    |
| Ktedonobacteriales   |      |      |      |            |            |      |      | -    |
| Caldilineae          |      |      |      |            |            |      |      | -    |
| Anaerolineae         |      |      |      |            |            |      |      | -    |
| Dehalococoidia       |      |      |      |            |            |      |      | -    |
| Chloroflexia         |      |      |      |            |            |      |      | -    |
| SAR202 cluster       |      |      |      |            |            |      |      | X    |
| **Proteobacteria**   |      |      |      |            |            |      |      |      |
| Alphaproteobacteria  | X    | X    | X    | X           | X           | X    | X    | X    |
| Betaproteobacteria   | X    | X    | X    | X           | X           | X    | X    | -    |
| Gammaproteobacteria  | X    | X    | X    | X           | X           | X    | X    | -    |
| Deltaproteobacteria  | X    | X    | X    | X           | X           | X    | X    | -    |
| **Firmicutes**       |      |      |      |            |            |      |      |      |
| Clostridia           |      |      |      |            |            |      |      | -    |
| Bacilli              |      |      |      |            |            |      |      | -    |
| Acidobacteria        |      |      |      |            |            |      |      | -    |
| Candidatus Rokubacteria | -  | X    | X    | X           | X           | X    | X    | -    |
| Nitrospinae          |      |      |      |            |            |      |      | X    |
| Candidatus Tectomicrobia | -  | X    | X    | X           | X           | X    | X    | -    |
| Candidatus Dormibacteraeota | -  | X    | X    | X           | X           | X    | X    | -    |
| Candidatus Hydrogenedentes | -  | X    | X    | X           | X           | X    | X    | -    |
| Spirochaetales       |      |      |      |            |            |      |      | X    |
| Verrucomicrobia      |      |      |      |            |            |      |      | X    |
| Gemmatimonadetes     |      |      |      |            |            |      |      | X    |
| Candidatus Methylomirabilis (NC10) | -  | X    | X    | -           | X           | X    | X    | -    |
| Candidatus Dadabacteria | -  | X    | X    | X           | X           | X    | X    | -    |
| Candidatus Lindowbacteria | -  | X    | X    | X           | X           | X    | X    | -    |
| Candidatus Schekmanbacteria | -  | X    | X    |            |            |      |      |      |

Roles in Euryarchaeota

Methanogenic Euryarchaeota Methanogens are a diverse group of obligately anaerobic archaea that produce methane as the end product of their energy generation pathways (Liu and Whitman 2008). Methanogens encompass at least seven orders within the Euryarchaeota (Brochier, Forterre and Gribaldo 2004; Bapteste, Brochier and Boucher 2005; Brochier-Armanet, Forterre and Gribaldo 2011; Borrel et al. 2013; Evans et al. 2019), though Adam, Borrel and Gribaldo 2019; Evans et al. 2019; Orsi et al. 2020). The central role of F_{420} in this pathway likely goes some way to explain its widespread production by the archaeal domain. However, the role of F_{420} goes well beyond one-carbon metabolism and the diversity of F_{420}-producing archaea indicates that many additional functions likely remain to be discovered (Kozubal et al. 2013; Kerou et al. 2016; Susanti, Loganathan and Mukhopadhyay 2016; Ney et al. 2017a; Jay et al. 2018).
Table 4. Continued

| Archaea                  | Euryarchaeota group |  |  |  |  |  |  |  |
|--------------------------|---------------------|---|---|---|---|---|---|
| Euryarchaeota            |                     |  |  |  |  |  |  |
| Methanococci             | X                   | X | X | X | X | - | - |
| Methanobacteria          | X                   | X | X | X | X | - | - |
| Methanomicrobia          | X                   | X | X | X | X | - | - |
| Halobacteria             | X                   | X | X | X | X | - | - |
| Archaeoglobi             | X                   | X | X | X | X | - | - |
| Methanopyri              | X                   | X | X | X | X | - | - |
| Methanoratronarchaeia    | X                   | X | X | X | X | - | - |
| Candidatus Methanoliparia| X                   | X | X | X | X | - | - |
| Candidate Hadesarchaeia  | X                   | X | X | X | X | - | - |
| Candidate Theionarchaeia | X                   | X | X | X | X | - | - |
| Candidate division MSBL1 | X                   | X | X | X | X | - | - |
| Candidate Hydrothermarchaeia | X               | X | X | X | X | - | - |
| TACK group               |                     |  |  |  |  |  |  |
| Crenarchaeota            |                     |  |  |  |  |  |  |
| Thermoprotei             | X                   | X | X | X | X | X | - |
| Thaumarchaeota           |                     |  |  |  |  |  |  |
| Nitrososphaeria          | X                   | X | X | X | X | X | - |
| Nitrospumilales          | X                   | X | X | X | X | X | - |
| Candidatus Bathyarchaeota| X                   | X | X | X | X | X | - |
| Candidatus Marsarchaeota | X                   | X | X | X | X | X | - |
| Candidatus Geothermarchaeota | X               | X | X | X | X | X | - |
| Candidatus Verstraetearchaeota | X           | X | X | X | X | X | - |
| Candidatus Nezhaarchaeota| -                   | - | - | - | X | X | - |
| Candidatus Korarchaeota  | -                   | - | - | X | X | X | X |
| Asgard group             |                     |  |  |  |  |  |  |
| Candidatus Lokiarchaeota | X                   | X | X | X | X | X | X |
| Candidatus Heimdallarchaeota | X            | X | X | X | X | X | - |
| Candidatus Helarchaeota  | X                   | X | X | X | X | X | X |
| Candidatus Odinarchaeota | -                   | - | - | X | X | X | X |
| Candidatus Thorarchaeota | -                   | - | - | X | X | X | X |

Genome-resolved metagenomics indicates there are potentially methanogenic archaea outside this phylum (vanwonterghem et al. 2016; sorokin et al. 2017; spang and ettema 2017; berghuis et al. 2019). All cultured methanogens synthesize F420, which serves as a central redox cofactor for two of the three major routes of methanogenesis: the CO2-reducing and methylotrophic pathways (Cheeseman, toms-wood and Wolfe 1972; edwards and mcbride 1975; dodderma and vogels 1978; eirich, vogels and Wolfe 1979; van beelen, dijkstra and vogels 1983; dolfing and mulder 1985). As such, it is often present at high concentrations (up to 2.0 \mu mol per g dry weight) in these methanogens (eirich, vogels and Wolfe 1979; isabelle, simpson and daniels 2002).

In the CO2-reducing pathway, CO2 is progressively reduced to methane using electrons derived from exogenous substrates such as H2, formate and less commonly secondary alcohols (tzeng, bryant and Wolfe 1975; widdel and Wolfe 1989; fig. 6). F420H2 donates a hydride for two of these reaction steps, after first being reduced by F420-reducing dehydrogenases utilizing H2 (Frh; jacobsen et al. 1982; muth, morschel and klein 1987; fiebig and friedrich 1989; de poorter, geerts and keljens 2005; allegré et al. 2014), formate (Ffd; schauer and ferry 1986; shuber et al. 1986; baron and ferry 1989), or secondary alcohols (Adf; widdel and Wolfe 1989; bleicher and winter 1991). In this pathway, CO2 is first condensed with the cofactor methanofuran, before being transferred to tetrahydromethanopterin (H4MPT) to form 5-formyltetrahydrodethanopterin (CHO-H4MPT). CHO-H4MPT then undergoes enzymatically mediated intramolecular condensation to form 5,10-methylenetetrahydrodethanopterin (CH≡H4MPT). The CO2-derived methyl group resulting from these reactions is then transferred to coenzyme M (CoM),
Figure 6. F420-dependent reactions of one-carbon metabolism in archaea. F420 is a cofactor involved in key steps in hydrogenotrophic methanogenesis, methylotrophic methanogenesis, anaerobic methanotrophy and anaerobic alkane oxidation in archaea. Hydride transfer reactions involving F420-dependent enzymes are indicated as is the enzyme responsible. F420H2 reduced through the oxidation of formate (Ffd), H2 (Frh), or secondary alcohols (Adf) can be utilized for reactions mediated by Mtd, Mer, or for other cellular processes. Only reactions mediated by F420-dependent enzymes are shown in detail. For a full outline of methanogenesis pathways, refer to the following reviews on the subject (Deppenmeier 2002; Thauer et al. 2008; Timmers et al. 2017; Evans et al. 2019).

before it is substituted by coenzyme B (CoB), forming the heterodisulfide CoB-S-S-CoM and releasing methane (Fig. 6; Thauer 1998). The methyl transfer from CH3-H4MPT to CoM is mediated by the MtrA-H membrane protein complex, which conserves energy through the pumping of sodium ions out of the cell (Welander and Metcalf 2005; Thauer et al. 2008). In addition to the MtrA-H complex, energy is also conserved through respiratory reduction of CoB-S-S-CoM in methanogens with cytochromes (i.e. Methanosarcinales) or by electron bifurcation in methanogens without cytochromes (Thauer et al. 2008; Kaster et al. 2011; Welte and Deppenmeier 2014). In the case of methanogens with cytochromes, F420H2 can serve as a direct electron donor to the respiratory chain; this depends on the activity of F420H2-dependent methanophenazine reductase (Fpo), a 14-subunit complex similar to bacterial complex I that directly pumps protons using the energy released from electron transfer from F420H2 to the membrane-diffusible cofactor methanophenazine (Deppenmeier et al. 1990; Abken and Deppenmeier 1997; Bäumer et al. 1998, 2000; Welte and Deppenmeier 2011b).
F420 has distinct roles in the methylo trophic and acetoclastic methanogenesis pathways. In the methylo trophic metha nogenesis pathway, methyl groups (from methanol, methylated amines and methylated sulfides) are alternatively converted into CH₄ (reductive route) and CO₂ (oxidative route; Fig. 6; Krzycki et al. 1987; Ferry and Kastead 2007). The oxidative route likely occurs through the reverse direction of the CO₂-reducing pathway, with the methyl group first transferred to CoM, then H₂MPT, before being oxidized sequentially by Mer and Mtd, yielding F₂O₂H₂. The reductive route proceeds from CH₃-S-CoM in the same fashion as the CO₂-reducing pathway (Fig. 6; Deppenmeier 2002; Thauer et al. 2008). Methanogens with cytochromes use F₂O₂H₂ generated through the oxidative arm of the methylo trophic pathway as an input to the respiratory chain via Fpo (Welte and Deppenmeier 2011b). Aceto castic methanogenesis is F₂O₂H₂-dependent, producing CO₂ and CH₄ from acetate utilizing a largely distinct set of enzymes to the hydrogenotrophic or methylotrophic pathways (Smith and Mah 1978; Barber et al. 2011). However, despite not being required for this process, F₂O₂H₂ is present in facultatively acetoclastic Methanosarcina when grown solely on acetate and in the obligately acetoclastic genus Methanothrix, indicating that the cofactor has roles in methanogenesis physiology beyond those of methanogenesis (Baresi and Wolfe 1981; Barber et al. 2011; Zhu et al. 2012). In support of this, a potential electron-bifurcating heterodisulfide reductase that uses ferredoxin and F₂O₂H₂ as electron donors has been identified in Methanosarcina acetivorans (Yan, Wang and Ferry 2017).

Dedicated F₂O₂H₂-dependent enzymes have been shown to mediate other diverse reactions in methanogens, as detailed in Tables 2 and 3. These include reduction of the redox cofactors NADP⁺ (F₂O₂H₂-dependent NADP reductase; Fno) and thioredoxin (F₂O₂H₂-dependent flavin-containing thioredoxin reductase; DFTR; Spaans et al. 2015; Susanti, Loganathan and Mukhopadhyay 2016), mobilization of sulfate as a sulfur source (F₂O₂H₂-dependent sulfate reductase; Fsr; Johnson and Mukhopadhyay 2008a) and detoxification of atmospheric O₂ (F₂O₂H₂-dependent oxidase; FprA; Seedorf et al. 2007).

Methane-, ethane- and butane-oxidizing Euryarchaeota Anaerobic methanotrophy is a biogeochemically significant process in which methane of biological or abiotic origin is oxidized to CO₂, with nitrate, sulfate, or transition metal ions as terminal electron acceptors (Bhattarai, Cassarini and Lens 2019). Up to 90% of the methane produced by marine sediment is estimated to be internally recycled by this process, thereby moderating methane release into the atmosphere (Reeburgh 2002; Timmers et al. 2017). Outside of central metabolism, little is known about the role of F₂O₂H₂ in Archaeoglobi. However, A. fulgidus possesses Fno, which is thought to be the sole route for NADP reduction (Kunow et al. 1993; Warkentin et al. 2001). F₂O₂H₂ production has also been experimentally determined in the halophiles Halobacterium and Halococcus, though its physiological role remains undefined (Lin and White 1986; De Wit and Eker 1987).

Roles in other Archaea
TACK lineages of Archaea The TACK lineage represents a major grouping of archaea originally containing the phyla Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota, but now expanded to contain several other recently identified phyla (Guy and Ettema 2011; Spang, Caceres and Ettema 2017; Wang et al. 2019). Diverse members of the TACK group contain a full complement of genes for F₂O₂ (Spang et al. 2012; Zhalnina et al. 2014; Evans et al. 2015; Kerou et al. 2016; Vanwonterghem et al. 2016; Nys et al. 2017a; Jay et al. 2018; Berghuis et al. 2019; Table 4), though no definitive experimental evidence confirming the production and roles of F₂O₂ has been presented. Putative F₂O₂ producing species adopt diverse aerobic and anaerobic lifestyles (Jay et al. 2018; Yu et al. 2018; Berghuis et al. 2019). F₂O₂ production appears to be a common trait in Thaumarchaeota (Tourn et al. 2011; Kozlowski et al. 2016; Ren et al. 2019;
Based on the analysis of metagenome-derived genomes (MAGs) that mediate nitrification in soil and marine ecosystems (Kuyers, Marchant and Kartal 2018). Genomic analysis and fluorescence microscopy indicate both Nitrosospira gargensis and Nitrospira viennensis synthesize F420 in significant quantities (Spang et al. 2012; Kerou et al. 2016), though the presence and role of F420 in this phylum has not been biochemically confirmed. Given Nitrospira are aerobes that cannot perform methano genesis, the cofactor is unlikely to play a role in one-carbon transformations (Kerou et al. 2016; Ren et al. 2019). Proteinomic analysis indicates that F420-dependent oxidoreductases of the luciferase-like hydride transferase (LLHT) and flavin/deazaflavin oxidoreductase (FDOR) families are produced at high levels, suggesting a role for the cofactor in biosynthetic or biodegradative processes (Kerou et al. 2016).

Several other TACK phyla also encode F420 biosynthesis genes. Marsarchaeota and Geaarchaeota, two closely related aerobic chemoheterotrophic phyla recently discovered in thermophilic iron-rich microbial mats, also encode F420 biosynthesis genes and F420-dependent oxidoreductases. Metatranscriptomic analysis indicates that F420-dependent oxidoreductases are highly expressed by Marsarchaeota living in microbial mats. These enzymes were hypothesized to play a role in the metabolism of extracellular sulfonates, although there is limited phylogenetic or biochemical evidence to support this (Jay et al. 2018). The candidate phyla Bathyarchaeota and Ver straearchaeota are also predicted to produce F420 (Table 4; Evans et al. 2015; Vanwonerghem et al. 2016; Zhou et al. 2018). Based on the analysis of metagenome-derived genomes (MAGs) from these species, Ver straearchaeota are predicted to be capable of F420-dependent hydrogenotrophic methanogenesis (Fig. 6), the first example of an archaeon capable of this process to be discovered outside of the Euryarchaeota (Berghuis et al. 2019; Evans et al. 2019). Based on the presence of genes homologous to the methyl-CoM reducing complex Mcr, it was originally suggested that Bathyarchaeota are also capable of methy lotropic methanogenesis (Evans et al. 2015). However, the phylogenetic grouping of the Mcr genes present in Bathyarchaeota indicates that they utilize this complex for F420-dependent anaerobic alkane oxidation (Fig. 6), rather than methanogenesis, similarly to the recently identified candidate genus Syn trophoarchaem and potentially the candidate phylum Hela rchaeota (Laso-Pérez et al. 2016; Chen et al. 2019; Evans et al. 2019; Seitz et al. 2019).

**F420 production and roles within bacteria**

In bacteria, F420 has been primarily studied in Actinobacteria. It has been biochemically identified in members of the genera *Mycobacterium*, *Streptomyces*, *Rhodococcus*, *Nocardia* and *Nocardioidea* (Daniels, Bakhiet and Harmon 1985; Eker, Hessels and Meerwaldt 1989; Purwantini, Gillis and Daniels 1997; Ebert, Rieger and Knackmuss 1999; Selengut and Haft 2010), the majority of which are soil saprophytes. F420 is not essential for central metabolism in Actinobacteria, though the cofactor is used for a wide range of purposes that provide a growth or survival advantage (Ebert, Rieger and Knackmuss 1999; Hasan et al. 2010; Taylor et al. 2010; Wang et al. 2012; Gurumurthy et al. 2013; Greening et al. 2017; Lee et al. 2020). In addition to Actinobacteria, recent biochemical evidence indicates that F420 is produced by members of the phylum Chloroflexi and the classes Alphaproteobacteria and Betaproteobacteria (Ney et al. 2017a; Braga et al. 2019). Spectroscopic analysis suggests members of the candidate phylum Tectomicrobia also produce the cofactor (Lackner et al. 2017). The genes required for F420 biosynthesis are also encoded in multiple genomes from the cultivated phyla Acidobacteria, Firmicutes and Nitrospinae and the candidate phyla Rokubacteria, Tectomicrobia and Dadabacteria (Wilson et al. 2014; Hug et al. 2016; Becraft et al. 2017; Lackner et al. 2017; Ney et al. 2017a).

Presently, no experimental studies have been performed investigating its biochemical and physiological role in bacterial species outside of Actinobacteria.

**Roles in Actinobacteria**

*Mycobacteria* The genetic complement for F420 biosynthesis is present in all cultured environmental and pathogenic mycobacteria. F420 production has been experimentally confirmed in many *Mycobacterium* species including *M. tuberculosis*, *M. smegmatis*, *M. phlei*, *M. bovis* and *M. avium* (Bair, Isabelle and Daniels 2001). Two fast-growing saprophytic members of the genus, *M. smegmatis* and *M. phlei*, produce F420 in large quantities (0.3–0.6 μmol per g dry weight; Isabelle, Simpson and Daniels 2002), indicating it plays a significant role in mycobacterial physiology. In addition, F420 is produced by the obligate pathogens *M. tuberculosis* and *M. leprae* (Purwantini, Gillis and Daniels 1997; Bair, Isabelle and Daniels 2001), which suggests a conserved physiological function for the cofactor among mycobacteria, as well as a role in survival in the host. A further indication of its significance is that all mycobacterial species contain numerous enzymes known or predicted to utilize F420 as a cofactor (Selengut and Haft 2010; Ahmed et al. 2015). *M. smegmatis* is predicted to encode 75 F420-dependent enzymes (30 of FDOR family, 45 of LLHT family), while *M. tuberculosis* is predicted to encode 33 F420-dependent enzymes (15 of FDOR family, 17 of LLHT family; Selengut and Haft 2010; Ahmed et al. 2015). In addition to these known classes of F420-dependent enzymes, further F420-dependent enzymes may be present in mycobacteria, which belong to novel enzyme families and thus cannot be readily identified based on amino acid sequence homology (Kumar 2018). While the function of the majority of F420-dependent enzymes in mycobacteria remains poorly understood, recent phenotypic and biochemical studies have shed light on some...
of their physiological roles (Hasan et al. 2010; Bashiri et al. 2012; Gurumurthy et al. 2013; Ahmed et al. 2015; Jirapanjawat et al. 2016; Purwantini, Daniels and Mukhopadhyay 2016; Lee et al. 2020).

F420 is not essential for mycobacterial growth, with mutants deficient in its synthesis or reduction successfully generated in M. smegmatis (Purwantini and Mukhopadhyay 2009; Taylor et al. 2010; Grinster et al. 2020), M. tuberculosis (Darwin et al. 2003; Manjunatha et al. 2006; Gurumurthy et al. 2013) and M. bovis (Choi, Kendrick and Daniels 2002). However, several studies indicate that F420 contributes to the ability of mycobacteria to persist in response to oxygen deprivation, oxidative stress, nitrosative stress, or treatment with antimicrobial compounds (Purwantini and Mukhopadhyay 2009; Gurumurthy et al. 2013; Jirapanjawat et al. 2016; Lee et al. 2020; Rifat et al. 2020). F420 reduction in the cytoplasm of Mycobacterium appears to be solely mediated by the F420-reducing glucose 6-phosphate dehydrogenase (Fgd), rather than by Fno, which is employed by most other Actinobacteria (Purwantini, Gillis and Daniels 1997; Bashiri et al. 2008; Jirapanjawat et al. 2016). In mycobacteria, Fgd is one of two entry points into the reductive pentose phosphate pathway, in addition to the canonical NADP+ -reducing enzyme. The metabolic coupling of F420 reduction of G6P oxidation represents a significant portion of the flux through the pentose phosphate pathway in mycobacteria, with Fgd activity in cell lysates roughly equivalent to NADP-dependent G6P dehydrogenase (Purwantini, Gillis and Daniels 1997, 1998). G6P levels are 100-fold higher in M. smegmatis than E. coli grown under comparable conditions and may serve as a store of reductant that is mobilized through F420 to combat oxidative stress (Hasan et al. 2010). Consistent with this hypothesis, mycobacteria use G6P when challenged with redox cycling agents (e.g. menadione), rapidly reduce such compounds using F420H2-dependent reductases and are hypersusceptible to challenge in strains unable to make or reduce F420 (Hasan et al. 2010; Gurumurthy et al. 2013; Jirapanjawat et al. 2016). Mycobacteria unable to produce or reduce F420 are also hypersusceptible to nitrosative stress, including from NaN3O2 and NO (Darwin et al. 2003; Purwantini and Mukhopadhyay 2009). In a chemical assay, isolated F420H2 readily reduces NO2-, leading to the suggestion that the cofactor may directly quench reactive nitrogen species (Purwantini and Mukhopadhyay 2009). However, the biochemical mechanism of F420 dependent oxidative and nitrosative stress resistance in Mycobacterium remains to be fully elucidated.

Emerging evidence suggests that F420H2 may also serve as a respiratory electron donor for mycobacteria. The FDOR-A family enzyme deazaflavin nitroreductase (Ddn) from M. tuberculosis, as well as its homologs from M. smegmatis, can reduce menaquinone at physiologically relevant rates (Fig. 7A; Lee et al. 2020). Furthermore, heterologous expression of Ddn stimulated the O2 consumption of isolated M. smegmatis membranes in an F420H2-dependent fashion, indicating it supplies F420 derived reductant to the respiratory chain. An M. tuberculosis mutant lacking this enzyme is impaired in its ability to recover from hypoxia-induced dormancy (Lee et al. 2020). However, more systematic studies are required to unravel the contribution of F420H2 compared to other electron donors in maintaining energy and redox homeostasis in mycobacterial cells.

F420 also plays a role in the biosynthesis of the complex lipids that compose the outer envelope of Mycobacterium, thereby contributing to the virulence and intrinsic antibiotic resistance of the genus (Cox et al. 1999; Dubnau et al. 2000; Jain et al. 2007; Purwantini and Mukhopadhyay 2013; Purwantini, Daniels and Mukhopadhyay 2016). The outer envelope of pathogenic mycobacteria contains ketomycolic acids, which are important virulence factors (Yuan et al. 1998; Dubnau et al. 2000; Sambandan et al. 2013). Ketomycolic acids are produced by the oxidation of hydroxymycolic acids, after their transport to the extracellular side of the cellular membrane by fHMAD, an F420-reducing dehydrogenase of the LLHT family (Fig. 7B; Purwantini and Mukhopadhyay 2013). fHMAD is secreted from the cell via the TAT secretion system in complex with F420. As a dehydrogenase, fHMAD does not require a pool of reduced F420 to mediate ketomycolic acid formation, allowing it to function extracytoplasmically (Bashiri et al. 2012). Phthiocerol dimycolocerosates (PDM) are another family of lipids prevalent in the cell envelope of pathogenic mycobacteria. While likely absent from saprophytic species like M. smegmatis (Bansal-Mutalik and Nikaido 2014), in M. tuberculosis PDM constitutes 46% of the total lipids (Wang et al. 2020), contributing to cell envelope impermeability and phagosomal escape from host cells (Quigley et al. 2017). In M. bovis, conversion of phthiodiolone dimycolocerosates to PDM is dependent on reduced F420H2 provided either enzymatically by Fgd or added exogenously to cell lysates. Based on sequence analysis, it is predicted that f420H2-dependent LLHT (phthiodiolone ketoreductase, fPKR) is responsible for the reduction of phthiodiolone dimycolocerosates to phthiotriol dimycolocerosates, the penultimate step in PDM synthesis (Fig. 7C; Siméone et al. 2007; Purwantini, Daniels and Mukhopadhyay 2016). Suggestive of further roles for F420-dependent enzymes in lipid biosynthesis, proteomic analysis of the FDOR-AA family in mycobacteria indicates these enzymes are membrane-associated and their genetic context suggests they play a role in lipid synthesis (Ahmed et al. 2015). Synthesis of the complex lipid-rich mycobacterial outer envelope requires a high level of biosynthetic complexity (Kolattukudy et al. 1997; Bansal-Mutalik and Nikaido 2014; Marrakchi, Lanéelle and Daffé 2014), which may be provided by F420-dependent enzymes, thereby helping to explain their abundance and diversity in mycobacterial species.

F420H2-dependent reductases also provide a reductive detoxification system in mycobacteria, providing the ability to inactivate a range of exogenous compounds with antimicrobial activity (Jirapanjawat et al. 2016). M. smegmatis ΔfbiC and Δfsgd strains are hypersensitive to a range of antimicrobial compounds, including quinone analogs (e.g. menadione), coumarin derivatives (e.g. methoxsalen), arolymethane dyes (e.g. malachite green) and quinolones (e.g. oxolinic acid; Guerra-Lopez, Daniels and Rawat 2007; Hasan et al. 2010; Jirapanjawat et al. 2016). The intrinsic resistance of wild-type M. smegmatis to these compounds is attributed to the large number of FDORs it uses. Numerous purified FDORs from M. smegmatis have been shown to promiscuously reduce members of the above chemical classes to varying degrees (Jirapanjawat et al. 2016; Greening et al. 2017). In support of the role of FDORs in reductive detoxification, wildtype M. smegmatis can reduce methoxsalen, malachite green and methyl violet added to cultures, but ΔfbiC and Δfsgd strains cannot (Guerra-Lopez, Daniels and Rawat 2007; Jirapanjawat et al. 2016). Importantly, M. smegmatis ΔfbiC and Δfsgd strains only display a modest increase in sensitivity to the clinically utilized antimycobacterials, including rifampicin, isoniazid and clofazimine, suggesting it lacks F420-dependent enzymes capable of reducing them (Jirapanjawat et al. 2016). While some FDORs from M. tuberculosis also promiscuously reduce exogenous compounds (Taylor et al. 2010; Cellitti et al. 2012), it remains to be determined whether F420H2-dependent reductases provide an analogous detoxification system in obligately pathogenic mycobacteria.

Despite our growing understanding of the general role of F420-dependent processes in Mycobacterium, few F420-dependent
enzymes have a defined physiological function (Selengut and Haft 2010; Ahmed et al. 2015). In addition to those discussed above, an FDOR-B enzyme purified from M. tuberculosis is proposed to be an F420H2-dependent biliverdin reductase; the enzyme reductively converts biliverdin to bilirubin, a potent antioxidant that may play a role in resisting host-induced oxidative stress, though it remains to be established if this activity occurs physiologically (Fig. 7D; Biswal et al. 2006; Ahmed et al. 2015). To fully understand the role of F420 in Mycobacterium, further work is required to systematically characterize the phenotypes associated with this cofactor, including its role in resistance to antimicrobials, redox stress and hypoxia. Additionally, while F420 plays a role in mycobacterial physiology, the extent to which the cofactor is required for the long-term persistence of M. tuberculosis in the host is unclear. To reconcile the physiology with biochemical mechanisms, the role of specific F420-dependent enzymes in mediating the reactions behind these phenotypes needs to be determined through genetic and biochemical analysis.

Streptomyces In Streptomyces species, F420 plays an important role as a cofactor for enzymes involved in the synthesis of structurally diverse antibiotics and secondary metabolites (Wang et al. 2013; Ichikawa, Bashiri and Kelly 2018; Steiningerova et al. 2020). F420 plays a role in the biosynthesis of structurally diverse antibiotics and secondary metabolites (Wang et al. 2013; Ichikawa, Bashiri and Kelly 2018; Steiningerova et al. 2020). APDs are biosynthetic precursors for lincosamide and griselimycin antibiotics (Peschke et al. 1995; Lukat et al. 2017), several pyrrolobenzodiazepines (PBDs) with antitumorigenic and antibiotic properties (e.g. tomaymycin, sibiromycin, anthranmycin; Li et al. 2009a, b; Steiningerova et al. 2020), and the quorum-sensing peptide hormaomycin (Höfer et al. 2011). These F420H2-dependent LLHT reductases, named Apd6s, are present in the biosynthetic gene clusters (BGCs) for these secondary metabolites and perform the final reduction step in APD biosynthesis (Fig. 8B; Steiningerova et al. 2020). While it was not formally identified at the time, one of the earliest instances of F420 isolation was from Streptomyces aureofaciens, where it was shown to mediate the final hydrogenation step in chlorotetracycline biosynthesis (McCormick et al. 1958; Miller et al. 1960). More recently, it was shown that an F420H2-dependent FDOR-B family enzyme catalyzes the final reduction of the C5a-C11a double bond of the dehydroxytetracycline precursor of several tetracycline variants (Fig. 8A; Wang et al. 2013). These enzymes are designated OxyR, CtcR and DacO4 in the oxytetracycline/tetracycline, chlorotetracycline and dactylocycline biosynthesis pathways respectively (Wang et al. 2012, 2013).

Figure 7. Physiological reactions proposed to be mediated by F420-dependent enzymes in mycobacteria. The bond oxidized or reduced is highlighted in orange for each substrate, with the enzyme responsible for the reaction indicated. For the reactions shown in A, B and D, F420H2 is generated by Fgd through oxidation of G6P. For the reaction shown in C, F420 oxidizes hydroxymycolic acid to ketomycolic acid at the extracellular face of the cytoplasmic membrane, yielding F420H2.
Figure 8. Reactions proposed to be mediated by F₄₂₀-dependent reductases in streptomycetes. The bond reduced is highlighted in orange for each substrate, with the enzyme responsible for the reaction indicated. F₄₂₀H₂ for the reactions shown is generated by the enzyme Fno via the oxidation of NADPH.
4-substituted Δ1-pyrroline-2-carboxylic acid substrate (Fig. 8B; Steiningerova et al. 2020). These differences in Adp6 specificity lead to variably saturated APD moieties that help mediate the biological function of the final compound that contains them (Steiningerova et al. 2020). Bioinformatic analysis indicates that Adp6 homologs are widely distributed within several bacterial phyla and often associated with BGCs of unknown function, suggesting they mediate the formation of novel APD-containing molecules (Steiningerova et al. 2020).

An F420H2-dependent reductase of the FDOR-B superfamily from Streptomyces chrestomyceticus, designated GupA, forms part of the BGC for guanipiperazines A and B. These compounds are formed through the condensation of two L-tyrosine molecules forming a dihydropyrazine ring that is reduced by GupA to form the piperazine ring found in the final product (Fig. 8C). While the function of these compounds is unknown, homologues of GupA and other components of the guanipiperazine BGC are widespread in Streptomyces species (Shi et al. 2021).

Other F420-dependent enzymes form part of the biosynthetic pathways of diverse posttranslationally modified peptide antibiotics. The BGC of the recently discovered lexapeptide, a class V lanthipeptide produced by Streptomyces rochei, contains the F420H2-dependent LLHT LxmJ that catalyzes the reduction of a lexapeptide dehydroalanine moiety to D-Ala (Fig. 8D). This reduction increases the potency of lexapeptide towards a panel of Gram-positive bacteria (Tao et al. 2020). Additionally, an F420H2-dependent FDOR-B family reductase designated TpnL is present in the BGC of the thiopeptide thiopeptin produced by Streptomyces tetayamensis. TpnL mediates the reduction of an imine within the dehydropyrazine moiety of thiopepin, yielding a modified piperidine-containing product (Fig. 8E; Ichikawa, Bashiri and Kelly 2018). TpnL homologs are present in many known or predicted peptide BGCs and form a distinct clade from other FDOR-B sequences (Ahmed et al. 2015; Ichikawa, Bashiri and Kelly 2018). Also of note is the BGC of the pyrroloquinoline alkaloid ammosamide from Streptomyces sp. CNR-698, which contains the putative F420-dependent FDOR-B protein Amm4. The authors predict Amm4 is an oxidase involved in primary amide biosynthesis, based on the accumulation of an ammosamic acid shunt product in an Δamm4 producing strain (Jordan and Moore 2016).

The chemically diverse nature of the secondary metabolites with biosynthetic pathways containing F420-dependent enzymes, as well as the involvement of the structurally unrelated FDOR and LLHT enzyme families, demonstrates that F420-dependent enzymes are versatile biosynthetic tools for streptomyces. An abundance of BGCs containing predicted F420-dependent enzymes indicates that F420 is likely to be utilized in the biosynthesis of many more secondary metabolites than those currently identified experimentally. For example, putative F420-dependent LLHTs are also encoded in the BGCs for a coronafacoyl phytotoxin produced by the plant pathogen Streptomyces scabiei (Bown et al. 2016), the aminoglycoside kasugamycin produced by Streptomyces kasugaensis (Ikeno et al. 2006), and mitomycin C produced by Streptomyces laeudulaceae (Mao, Varoglu and Sherman 1999). However, further experimentation is required to support the F420-dependence of these enzymes and their specific role in the biosynthesis of these compounds. At odds with our increasing knowledge of the role of F420 in secondary metabolism in streptomyces, virtually nothing is known about its role in primary metabolism, or whether it shares some physiological roles to those described for its fellow actinobacteria genus Mycobacterium.

Other Actinobacteria F420 is widely produced by other Actinobacteria including Rhodococcus, Nocardia and Nocardioides (Daniels, Bakheit and Harmon 1985; Purwantini, Gillis and Daniels 1997; Ebert, Rieger and Knackmuss 1999). Bacteria from these genera utilize F420H2-dependent reductases from the LLHT superfamily to mobilize the explosive picrate (2,4,6-trinitrophenol) and related compounds (e.g., 2,4-dinitrophenol, 2,4-dinitroanisole) for degradation (Ebert, Fischer and Knackmuss 2001; Fida et al. 2014). Due to this capability, several actinobacterial strains such as Rhodococcus opacus and Nocardioidea simplex can grow on picrate as their sole carbon and nitrogen source (Lenke et al. 1992; Ebert, Fischer and Knackmuss 2001). Other than its role in the remediation of nitroaromatic xenobiotics (Ebert, Rieger and Knackmuss 1999; Ebert, Fischer and Knackmuss 2001; Fida et al. 2014), little is known about the physiological roles of F420 in these Actinobacteria. Consistent with its role in picrate degradation, F420 likely contributes to the well-documented ability of soil actinomycetes to biodegrade a wide variety of complex organic compounds, including polycyclic aromatic hydrocarbons (McCarthy and Williams 1992; Schrijver and Mot 1999).

**Roles in other bacteria**

The production of F420 by bacteria outside of the phylum actinobacteria was only recently experimentally verified (Ney et al. 2017a; Braga et al. 2019, 2020). As such, no F420H2-dependent reductases or F420-reducing dehydrogenases have been investigated experimentally in these bacteria. However, based on the levels of F420 production and analysis of the genomes of F420-producing bacteria, some inferences can be made regarding the role of F420 in these species. Of the species shown experimentally to produce F420, Thermomicrobium roseum (Chloroflexi) and Paraburkholderia rhizoxinica (Betaproteobacteria) are abundant producers, suggesting F420 plays a significant role in the physiology of these organisms (Braga et al. 2019). In contrast, F420 was only detected in Oligotropha carboxidovorans and Paracoccus denitrificans in trace quantities, indicating a minor or niche-specific role for the cofactor in these Alphaproteobacteria (Ney et al. 2017a). F420 producers within Betaproteobacteria and Tectomicrobia only encode Fno among known F420-reducing dehydrogenases, suggesting that NADPH is the major or only compound utilized for F420 reduction in these bacteria (Ney et al. 2017a). Predicted alphaproteobacterial F420-producers encode Fgd in addition to Fno, suggesting that G6P is also utilized for F420 reduction. In Chloroflexi, Fgd is the most prevalent F420-reducing dehydrogenase, but Adf and Fno homologs are also present, suggesting diverse substrates enable F420 reduction (Ney et al. 2017a).

The ecological niche and physiology of recently identified F420 producing bacteria suggest the cofactor is employed in diverse roles, which are at least partially analogous to those identified in Actinobacteria. The phylum Tectomicrobia (Candidatus Entotheonella spp.) includes uncultured bacteria that produce diverse bioactive secondary metabolites associated with marine sponges (Lackner et al. 2017). These bacteria possess large genomes (>9 Mbp) and are predicted to be F420 producers, given they are autofluorescent, possess a full set of F420 biosynthetic genes (Table 4) and encode multiple putative F420H2-dependent reductases (Wilson et al. 2014; Lackner et al. 2017; Ney et al. 2017a; Mori et al. 2018). F420 likely plays a role in secondary metabolite biosynthesis in Tectomicrobia similar to that of streptomyces discussed above. Chloroflexi are one of the dominant bacterial phyla found in soils and are reputed for their biodegradative capacities (Björnsson et al. 2002; Speirs et al. 2019). They generally encode numerous F420H2-dependent
reductases (Ney et al. 2017a), suggesting members of the phylum may use $F_{420}$ as a cofactor for biodegradative reductases in a similar manner to $M. \text{sme}$gmat $is$ or $N. \text{ocardia}$ spp. The betaproteobacterial fugal endosymbiont $P. \text{rhi}$zoxina produces a chemically distinct $F_{420}$ variant, $3PG-$ $F_{420}$ (see section 3.1.2). In $P. \text{rhi}$zoxina, $3PG-$ $F_{420}$ production is greatly enhanced when growing inside its fugal syndrome, suggesting the cofactor facilitates symbiosis. Based on the presence of genes encoding a putative ABC transporter adjacent to the $3PG-$ $F_{420}$ BCG, $P. \text{rhi}$zoxina possibly exports the cofactor to be used by its fungal partner (Braga et al. 2019). Interestingly, the genome of $P. \text{rhi}$zoxina lacks genes with homology to known $F_{420}$-dependent enzymes, suggesting that distinct mechanisms of cofactor utilization and cycling occur in this bacterium (Braga et al. 2019).

Experimental work is required to establish the role of $F_{420}$ in these bacteria. Metagenomic analysis indicates that $F_{420}$ producers are prevalent among aerobic soil bacteria (Ney et al. 2017a), suggesting it is widely used for its versatile biosynthetic and biodegradative properties. As a result, $F_{420}$-dependent enzymes may directly affect the microbial and chemical composition of soils, by providing bacteria with the ability to both synthesize and degrade antimicrobial compounds, in an ongoing arms race.

**THE MECHANISM AND EVOLUTION OF $F_{420}$ BIOSYNTHESIS**

**Diversity within the $F_{420}$ biosynthetic pathway**

Figure 9 presents the steps in the $F_{420}$ biosynthesis pathway and the enzymes that mediate them. Consistent with the initial discovery of $F_{420}$ in methanogenic Euryarchaeota (Cheeseman, Toms-Wood and Wolfe 1972), initial investigation of the structure and biosynthesis of the cofactor was performed in these organisms (Eirich, Vogels and Wolfe 1978; Jacobson and Walsh 1984; Li et al. 2003a). The $F_{420}$ biosynthesis pathway in methanogenic Euryarchaeota was established and was assumed to be universal to all $F_{420}$ producing organisms (Greening et al. 2016; Bashiri et al. 2019). However, recent investigation of $F_{420}$ biosynthesis in bacteria has revealed that divergent pathways for $F_{420}$ biosynthesis are employed in different organisms. These differences originate from variation in the substrate compound utilized to link the $F_{0}$ head group of $F_{420}$ to its polyglutamate tail (Bashiri et al. 2019; Braga et al. 2019; Grinter et al. 2020). Based on current experimental evidence, the $F_{420}$ biosynthesis pathway occurs via three variant pathways, found in Euryarchaeota, Actinobacteria and Proteobacteria, respectively (Fig. 9). However, future investigation may reveal further diversity in the $F_{420}$ biosynthesis pathway.

**A note on nomenclature**

Different nomenclature has been applied to $F_{420}$ biosynthetic enzymes from archaea and bacteria. This reflects the incremental nature of the advance in our understanding of $F_{420}$ biosynthesis, as well as the distant relationships between these domains. However, the continued use of different nomenclature is now justified with the recent discovery that $F_{420}$ is synthesized through distinct routes in these domains. Nevertheless, the nomenclature has become increasingly convoluted in light of these discoveries, together with recent evidence demonstrating multiple horizontal gene transfer of $F_{420}$ biosynthetic genes and gene fusion events. For simplicity, in this review, we will generally refer to $F_{420}$ biosynthesis proteins mediating the archaenal pathway with the prefix ‘CoF’ and those mediating the bacterial pathway with the prefix ‘Fbi’.

In methanogens, the enzymes CoF G and CoFH mediate $F_{0}$ biosynthesis, CoFC and CoFD mediate $F_{420}$-0 biosynthesis, and CoFE is responsible for the formation of mature $F_{420}$ via the addition of a $\gamma$-linked polyglutamate tail. In mycobacteria, the prefix ‘Fbi’ is utilized, with a different lettering system, where the following enzymes are analogous: FbiC is derived from a fusion of CoFC and CoFH, FbiD and FbiA are similar to CoFC and CoFD respectively, and FbiE is derived from a fusion of CoFE and the nitroreductase (NTR) superfamily protein FbiE. It should be noted that some bacteria possess individual enzymes homologous to CoFC, CoFH, or CoFE of archaeal F420 producers; we refer to these by the ‘CoF’ designation, as they are distinct from the corresponding ‘Fbi’ fusion enzymes. Likewise, at least some archaea possess some homologs of ‘Fbi’ fusion enzymes. Finally, the FbiA and FbiD variants from some Betaproteobacteria produce the chemically distinct variant $3PG-$ $F_{420}$ (Braga et al. 2019). The subscript ‘3PC’ is applied to these enzymes.

**Comparison of pathways**

In the $F_{420}$ biosynthetic pathway of Eurarchaeota, 2-phospho-$\alpha$-L-lactate (2PL) links the $F_{0}$ head group of $F_{420}$ to its polyglutamate tail (Fig. 9; Grochowski, Xu and White 2008). It has been proposed that 2PL is synthesized by the unidentifed lactate kinase CoB, using lactate produced from $L$-lactaldehyde (Graupner and White 2001; Graupner, Xu and White 2002; Grochowski, Xu and White 2006). 2PL is conjugated to $F_{0}$ via the action of the enzymes CoFC and CoFD to create $F_{420}$-0 (i.e. $F_{420}$ with no glutamate tail). CoFC activates 2PL through condensation with GTP to form the intermediate compound lactyl-diphospho-$\alpha$-guanosine (LPPG; Grochowski, Xu and White 2008). CoFD subsequently transfers 2PL from LPPG to $F_{0}$ to form $F_{420}$-0 (Graupner, Xu and White 2002). The activity of CoFC is contingent on the presence of CoFD, suggesting that these enzymes form a catalytic complex to regulate the production to the LPPG intermediate, which is unstable (Bashiri et al. 2019; Braga et al. 2019). $F_{420}$-0 is then converted to mature $F_{420}$ via the addition of a variable-length $\gamma$-linked glutamate tail by the enzyme CoFE (Li et al. 2003a; Nocek et al. 2007). In archaea, the length of the glutamate tail varies from 4 to 5 in methanogens with cytochromes or 2-3 in those without (Gorris 1994). In some Eurarchaeota, an additional terminal $\alpha$-linked glutamate is added by the $\alpha$-L-glutamate ligase CoF (Li et al. 2003b).

In mycobacteria, the central metabolic intermediate phosphoenolpyruvate (PEP), rather than 2PL, is utilized as the precursor for $F_{420}$ biosynthesis in this genus (Bashiri et al. 2019; Grinter et al. 2020). The incorporation of PEP into $F_{420}$ follows an analogous route to that of 2PL in archaea, with the enzymes FbiD and FbiA (homologs of CoFC and CoFD respectively) first converting PEP into the intermediate enolpyruyl-diphospho-$\alpha$-guanosine (EPGG) and then condensing it with $F_{0}$ to form dehydro-$F_{420}$-0 (DH-$F_{420}$-0), in which the enol group of PEP remains oxidized (Fig. 9; Grinter et al. 2020). DH-$F_{420}$-0 is then modified to form mature $F_{420}$ by the dual-functional enzyme FbiE. FbiE possesses an N-terminal domain homologous to CoF, which adds a variable-length $\gamma$-linked polyglutamate tail of 2–8 residues (Bashiri et al. 2019). The C-terminal domain of FbiB (FbiB$_{\text{term}}$) reduces the enol group of DH-$F_{420}$ Converting it into mature $F_{420}$ (Bashiri et al. 2016, 2019). The reduction of DH-$F_{420}$ improves the stability of the molecule by removing the high-energy phosphoenol bond (Braga et al. 2020). The Chloroflexi strain $T. \text{roseum}$ utilizes an independent homolog of FbiB$_{\text{term}}$ to reduce DH-$F_{420}$, herein referred to as FbiE (Braga et al. 2020). Genomic analysis indicates that independent FbiE homologs are present in the
Figure 9. Diverse routes to $F_{420}$ biosynthesis employed by bacteria and archaea. 1 = classical archaean pathway (Euryarchaeota), 2 = bacterial pathway a (Actinobacteria, Chloroflexi), 3 = bacterial pathway b (Betaproteobacteria). The substrates and mechanisms for $F_{420}$ biosynthesis are shared between all identified pathways. Abbreviated compounds are as follows: PEP, phosphoenolpyruvate; 2PL, 2-phospho-L-lactate; 3PG, 3-phosphoglycerate; EPPG, enolpyruvyl-diphospho-5'-guanosine; LPPG, lactyl-diphospho-5'-guanosine; GPPG, 3-guanosine-5'-disphospho-D-glycerate. The enzymes involved in each biosynthesis step are indicated.
genomes of several predicted bacterial and archaeal F420 producers, and putative F420-producing members of the archaeal phylum Lokiarchaeota possess a dual functional FbiB homolog (Braga et al. 2020), suggesting that diverse bacteria and archaea also employ a PEP-dependent pathway for F420 biosynthesis (Fig. 10B).

A third route for F420 biosynthesis is employed by the betaproteobacterium P. rhizoxinica, in which 3-phospho-D-glycerate (3PG) is utilized in place of 2PL or PEP for F420 biosynthesis. This leads to the formation of the chemically distinct species 3PG-F420 and depends on the action of FbiD3PG and FbiA3PG, homologs of CofC/FbiD and CofD/FbiA respectively (Fig. 9; Braga et al. 2019). The specificity for 3PG over PEP or 2PL appears to originate from FbiD3PG, which preferentially mediates the incorporation of 3PG into 3-guanosine-5′-disphospho-D-glycerate (GPPG; Braga et al. 2019). A homolog of CofE then adds a variable-length γ-linked polyglutamate tail of 1–6 residues to form mature 3PG-F420 (Braga et al. 2019).

The selective pressures underlying the rerouting of the F420 biosynthesis pathways remain unclear. Considering that CofC/FbiD/FbiD3PG evolved from a common F420-producing ancestral enzyme (Ney et al. 2017a; Bashiri et al. 2019; Braga et al. 2019), a substrate switch must have occurred in at least two lineages to yield the three observed biosynthesis pathways. This switch likely occurred to reconcile the precursor utilized for F420 production with its presence or level of availability in the metabolite pool of the F420-producing organism.

### Structural and biochemical basis

Recently, considerable progress has been made in understanding F420 biosynthesis in both bacteria and archaea. Except for CofG/CofH and FbiC, crystal structures have been determined for all enzymes in the F420 biosynthetic pathway, with biochemical analysis revealing considerable detail on the catalytic mechanisms employed during F420 biosynthesis.

#### Synthesis of F0 by CofG/CofH and FbiC

In all studied F420 producing organisms, F0 is synthesized through a universal mechanism involving two SAM-radical domain enzymes (Decamps et al. 2012). In archaea and some bacteria, these domains exist as two separate proteins CofG and CofH, while in Actinobacteria and Proteobacteria they are present in the single fusion protein FbiC (Ney et al. 2017a; Bashiri et al. 2019; Braga et al. 2019). No structures for these enzymes have been determined to date, likely due to the difficulty of working with these oxygen-sensitive proteins (Imlay 2006; Philmus et al. 2015). However, mass spectrometric analysis of CofG and CofH reaction products combined with substrate deuteration has provided considerable insight into the catalytic mechanism behind F0 synthesis (Decamps et al. 2012; Philmus et al. 2015). F0 is formed by the condensation of L-tyrosine with pyrimidine ribityldiaminouracil (5-amino-6-ribitylamino-2,4-[1H,3H]-pyrimidinedione), which is also a substrate for riboflavin biosynthesis (Bacher et al. 2000; Decamps et al. 2012). In the first step of this two-step reaction, the 5′-deoxyadenosyl radical generated by CofH abstracts a hydrogen atom from the tyrosine amine, which causes the tyrosine to fragment to form a ψ-hydroxybenzyl radical. This radical then undergoes addition to the double bond of pyrimidine ribityldiaminouracil, with this compound subsequently oxidized by the [4Fe4S] of CofH to yield an intermediate product (Fig. 11B). This product is then accepted by CofG, where the 5′-deoxyadenosyl radical formed by this enzyme extracts a further hydrogen, creating a radical intermediate that undergoes cyclization followed by oxidation by the [4Fe4S] cluster of CofG to yield F0 (Fig. 11C; Philmus et al. 2015). Despite existing as a fusion protein, the two domains of FbiC appear to function independently, with diffusion rather than direct substrate transfer responsible for the transfer of the product of the FbiC C-terminal domain to the N-terminal domain to complete F0 biosynthesis (Philmus et al. 2015).

#### Synthesis of LPPG, EPPG and GPPG by CofC, FbiD and FbiD3PG

The F0 is linked to the polyglutamate tail of mature F420 via either a 2PL for F420 or 3PG for 3PG-F420 (Graupner, Xu and White 2002; Braga et al. 2019; Grinter et al. 2020). In order to activate them for condensation with F0, 2PL, PEP or 3PG are condensed with GTP to form LPPG, EPPG and GPPG, respectively (Fig. 9). Despite the differences in their preferred substrate CofC, FbiD and FbiD3PG are homologs thought to share a common catalytic mechanism (Bashiri et al. 2019; Braga et al. 2019). The crystal structure of Apo-CofC from Methanosarcina mazei (PDB ID: 2I5E) was first determined by a structural genomics consortium (2006), demonstrating that the enzyme possesses a nucleotide-binding Rossmann fold, though the lack of substrate in this crystal structure limited the insight of the catalytic mechanism provided by this structure. Recently, the structure of FbiD from M. tuberculosis was determined in complex with PEP and two catalytic Mg2+ ions (Fig. 12A; Bashiri et al. 2019). Based on this structure, key substrate-binding residues were identified (Fig. 12C; Bashiri et al. 2019). Further, structural comparison between FbiD and the distantly related bifunctional acetyltransferase/uridylyltransferase GlmU reveals a putative GTP-binding pocket (Fig. 12B). This pocket is occluded in the crystal structure of FbiD, which is consistent with the observation that neither purified CofC nor FbiD are active in the absence of their partner enzyme CofD and FbiA (Bashiri et al. 2019; Braga et al. 2019). This suggests that conformational activation of CofC/FbiD occurs in the presence of CofD/FbiA, likely to prevent the futile production of an unstable product (Bashiri et al. 2019).

#### Synthesis of F420-O, DH-F420-O and 3PG-F420-O by CofD, FbiA and FbiA3PG

CofD, FbiA and FbiA3PG are homologous enzymes that mediate the transfer of 2PL, PEP or 3PG from the diphospho-5′-guanosine intermediate produced by CofC/FbiD to F0. This results in the formation of the intermediates F420-O, DH-F420-O and 3PG-F420-O, with no glutamate moieties (Graupner, Xu and White 2002). The crystal structures of CofD from Methanosarcina mazei and FbiA from M. smegmatis have been determined in the presence of their substrates (Forouhar et al. 2008; Grinter et al. 2020). These enzymes require a divalent cation for activation, which was absent from the crystal structure of CofD, meaning the catalytically important portions of the F0 and GDP substrates were disregarded in this structure (Forouhar et al. 2008). However, we recently determined the structure of FbiA in complex with F0, GDP and Ca2+, providing a clear picture of the catalytic complex of this enzyme (Fig. 13A; Grinter et al. 2020). The catalytic metal ion represented by Ca2+ in this structure is coordinated by aspartates 45 and 57. Aspartate 46 in CofD, which is equivalent to aspartate 57 in FbiA, is important for catalytic activity, suggesting it is also involved in catalytic metal ion coordination in this enzyme (Forouhar et al. 2008). In addition to the two aspartate residues, the catalytic Ca2+ ion is further coordinated by a H2O molecule, the terminal hydroxyl of F0 and the β-phosphate of EPPG (Fig. 13B). This coordination positions the EPPG β-phosphate for nucleophilic attack by the F0 terminal hydroxyl, leading to the transfer of PEP and the creation of the DH-F420-O product (Grinter et al. 2020).
To mediate this nucleophilic attack, FO requires activation, with likely candidate bases being the carboxyl or β-phosphate group of EPPG. A proposed reaction mechanism based on proton subtraction by the former is presented in Fig. 13C.

Addition of the poly-glutamate tail to F420 by CofE and FbiBterm

CofE and the N-terminal domain of FbiB (FbiBterm) are non-ribosomal peptide synthases that perform the final step in F420 biosynthesis, adding a variable number of γ-linked glutamate residues to form the F420 tail. The crystal structure of CofE from *A. fulgidus* revealed that the protein possesses a novel fold, consisting of an intertwined butterfly-shaped dimer (Fig. 14A; Nocek et al. 2007). The GDP and catalytic Mn$^{2+}$ ion bound version of this structure revealed the location of a Y-shaped active site with grooves hypothesized to be responsible for binding F420-0 and L-glutamate in addition to GTP (Fig. 14B; Nocek et al. 2007). A proposed catalytic mechanism for CofE and FbiBterm, based on that of the nucleotide-dependent tetrahydrofolate:L-glutamate γ-ligase (FPGS; Sheng et al. 2000) and UDP-N-acetylmuramoyl-L-alanine:glutamate ligase (MurD; Bertrand et al. 1997), involves
the activation of the terminal carboxyl of F420-0 by the addition of a phosphate group from GTP. Subsequently, the carbonyl carbon of the resulting acyl phosphate undergoes a nucleophilic attack by the glutamate amine, leading to the formation of a tetrahedral intermediate, which breaks down to the final F420 product and inorganic phosphate (Fig. 14C; Forouhar et al. 2008). Biochemical and genetic evidence indicates that CofE and FbiB_term are responsible for the addition of both the initial glutamate to F0 and extension of the poly-glutamate chain (Bashiri et al. 2016, 2019). Further research is required to resolve how the active site of these enzymes can perform both the initial and subsequent glutamate additions.

**Reduction of DH-F420 by FbiB_term and FbiE**

The reduction of DH-F420 is performed by FbiB_term in mycobacteria or FbiE in Chloroflexi, homologs that belong to the FMN-dependent NTR superfamily. The crystal structure of the isolated FbiB_term from mycobacteria has been determined, revealing an intertwined dimer, in complex with either FMN or F420, in distinct binding sites (Fig 15A; Bashiri et al. 2016). The relative positions of FMN and DH-F420-0 were modeled based on these structures, providing a plausible active site and catalytic mechanism for this enzyme (Bashiri et al. 2019), where N-5 of FMNH2 is ideally positioned for hydride transfer to the DH-F420-0 enol group (Fig. 15B).

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**Figure 11.** Production of the deazaflavin F0 is catalyzed by dual SAM-radical domains. (A) Structural model of the SAM radical domains that mediate F0 synthesis, consisting of the two separate proteins CofG and CofH (in archaea and some bacteria) or a single fusion protein FbiC (in bacteria and eukaryotes). Structural models constructed based on homology modeling using Phyre2 based on the structure of MqnE from Pedobacter heparinus (PDB ID = 6XI9; Kelley et al. 2015). (B) A summary of the proposed reaction performed by CofH. (C) A summary of the proposed reaction performed by CofG. For the full reaction scheme summarized in panels B and C refer to Philmus et al. (2015). R = The F0 ribose tail as shown in Fig. 9.
Evolution of the F420 biosynthesis pathway

While it is now clear that F420 is widely distributed in bacteria and archaea, it is not universally distributed like redox cofactors FMN/FAD and NAD/NADP (Daniels, Bakhiet and Harmon 1985; Ney et al. 2017a; Braga et al. 2019, 2020). This distribution poses the question of how F420 biosynthesis originated and how the genes responsible were disseminated across bacteria and archaea. It has been proposed that the capacity to synthesize F420 was present in the last universal common ancestor (LUCA) and was selectively retained by a subset of bacterial and archaeal lineages (Weiss et al. 2016). However, current evidence suggests that the F420 biosynthesis pathway evolved in a stepwise fashion in archaea and bacteria, with horizontal gene transfer mediating assembly of the complete biosynthesis pathway (Ney et al. 2017a). Such inferences are supported by the variable distribution (Fig. 5), genetic organization (Fig. 10) and phylogenetic analysis of the F420 biosynthesis genes (Ney et al. 2017a). In Fig. 16, we present a schematic of the gene transfer events that potentially occurred during the evolution of F420 biosynthesis. By necessity, this model focuses on well-studied F420 producers, and the direction of several gene transfer events remains unresolved.

The synthesis of F₀, as the catalytically active headgroup of F420, almost certainly evolved first. F₀ has near-identical redox properties to F420 and can function as a cofactor for F420-dependent enzymes in vitro (Jacobson and Walsh 1984; Drenth, Trajkovic and Fraaije 2019). However, the uncharged aromatic structure of F₀ allows it to readily diffuse across lipid membranes (Bashiri et al. 2010), limiting its usefulness as a redox cofactor due to its metabolically costly loss from the cell (Shah et al. 2019). The problem of diffusive loss is less acute when F₀ gets phosphorylated to produce the bifunctional FbiE. The next stage in the evolution of F420 biosynthesis was the addition of a phospho-carboxylic acid group to F₀ to form F420-0, a catalytic intermediate of the current biosynthesis pathway (Bashiri et al. 2019; Grinter et al. 2020). This modification imparts a negative charge, preventing its diffusion across cellular membranes (Bashiri et al. 2010), while not affecting the redox properties of the molecule. Phylogenetic evidence suggests that the ancestors of CofC/FbiD/FbiD₃PG and CofD/FbiA/FbiA₃PG potentially originated in an actinobacterial ancestor before being laterally acquired by other bacteria and archaea (Nelson-Sathi et al. 2015; Ney et al. 2017a). This suggests that F420 was first employed as a redox cofactor in Actinobacteria, before being horizontally acquired by other bacteria and archaea, including Eurarchaeota. The final stage in the evolution of F420 biosynthesis was the addition of the variable-length γ-linked polyglutamate tail to F420-0 by the enzyme CofE (Li et al. 2003a; Bashiri et al. 2019). The polyglutamate tail greatly enhances the affinity and specificity of interactions between F420 and F420-dependent oxidoreductases, possibly explaining why it arose (Ney et al. 2017b; Drenth, Trajkovic and Fraaije 2019). The evolutionary origin of CofE is unclear (Nocek et al. 2007; Ney et al. 2017a), though the polyglutamate tail synthesized by CofE is present in F420 from all currently investigated producing species (Gorris 1994; Li et al. 2003a; Bashiri et al. 2016; Greening et al. 2016; Ney et al. 2017a; Braga et al. 2019), indicating it is universally important for F420 function and has thus been co-inherited with other F420 biosynthesis genes. In Actinobacteria, cofE underwent a fusion with the DH-F₄₂₀-0 reductase gene fbiE to produce the bifunctional fbiE.

APPLICATIONS OF F₄₂₀ BIOSYNTHESIS

Progress and challenges for the use of F₄₂₀ in industrial catalysis

The hydrogenation reactions performed by F₄₂₀-H₂-dependent reductases are of interest for biocatalysis due to their regio- and enantioselectivity, which can generate up to two chiral centers (Greening et al. 2017; Mathew et al. 2018). Further, the low redox potential of F₄₂₀ allows it to mediate the reduction of otherwise recalcitrant bonds, including alkenes, enamines, enones, enoates and cyclic imines (Taylor et al. 2010; Jirapanjawat et al. 2016; Greening et al. 2017; Ichikawa, 2018).
Bashiri and Kelly 2018; Mathew et al. 2018; Steinningerova et al. 2020). \(F_{420}\) -dependent reductases provide an alternative to the nicotinamide-dependent Old Yellow Enzymes (OYEs) for performing these reactions (Stuermer et al. 2007; Winkler, Faber and Hall 2018), with some evidence suggesting that \(F_{420}\)-dependent reductases can generate reaction products with the opposite stereochemistry than OYEs (Mathew et al. 2018). However, while recent work has addressed a number of the challenges associated with utilizing \(F_{420}\)-dependent enzymes for industrial applications, several further challenges must be addressed before their potential for chemical synthesis can be realized.

Two plausible scenarios exist for the utilization of \(F_{420}\)-dependent reductases in the production of industrially relevant compounds. Purified \(F_{420}\)-dependent reductases can be utilized, in conjunction with an \(F_{420}\)-reducing regeneration system, to perform the biocatalytic reduction of the desired substrate. Alternatively, a synthetic biology approach could be employed, utilizing microbes engineered to produce \(F_{420}\) and express \(F_{420}\)-dependent biosynthetic pathways to produce compounds via microbial cell culture. In this section, we will discuss challenges and recent developments relating to the heterologous production of \(F_{420}\) and the development of suitable \(F_{420}\)-dependent enzymes for compound synthesis. These advances apply both to systems utilizing purified \(F_{420}\)-dependent enzymes and the development of synthetic biological systems utilizing \(F_{420}\).

**Development of high yield \(F_{420}\) production**

One of the major challenges in the development and utilization of \(F_{420}\)-dependent enzymes for biotechnological applications is the low yield of \(F_{420}\) obtained when purified from native sources (Isabelle, Simpson and Daniels 2002; Mathew et al. 2018; Bashiri et al. 2019). However, recent advances in our understanding of the \(F_{420}\) biosynthesis pathway, as well as successful heterologous production, have improved the prospects of obtaining the cofactor in industrially relevant quantities (Bashiri et al. 2019; Grinter et al. 2020).

\(F_{420}\) was initially purified from methanogenic archaea, such as *Methanobacterium thermoautotrophicum* (Eirich, Vogels and...
Wolfe 1978, 1979; Isabelle, Simpson and Daniels 2002). However, the relative technical difficulty in culturing these obligate anaerobes led to the identification and optimization of the aerobic actinomycete *M. smegmatis* as an alternative source of F420 (Isabelle, Simpson and Daniels 2002). Despite producing F420 at levels 5-fold lower than methanogens, the ease of culture and high cell densities achieved by this bacterium led to *M. smegmatis* being largely adopted as the preferred organism for F420 production, except in cases where short-chain F420-2 is required (Isabelle, Simpson and Daniels 2002; Ney et al. 2017b). Heterologous plasmid-based expression of FbiA, FbiB and FbiC in *M. smegmatis* by Bashiri et al. increased production of F420 from this bacterium 10-fold to levels greater than those produced by methanogens (Bashiri et al. 2010). This augmented F420 production in *M. smegmatis* is the currently preferred method of F420 production (Lapalikar et al. 2012; Ahmed et al. 2015; Mashalidis et al. 2015; Ney et al. 2017a; Oyugi et al. 2018; Drenth, Trajkovic and Fraaije 2019; Steiningerova et al. 2020). However, yields from this method are still unlikely to be compatible with economically viable production on an industrial scale. The estimated maximum yield of F420 from this process is ~3 g/kg dry weight (~0.9 g/kg wet cell weight; Isabelle, Simpson and Daniels 2002; Bashiri et al. 2010). Therefore, considerable further optimization of this system or alternative processes for F420 production are required.

One option for large-scale production of F420 is the use of a heterologous expression system, which is amenable to optimization through metabolic engineering. Until recently, a perceived bottleneck for heterologous production was the use of 2PL as a substrate in the F420 biosynthetic pathway, as it is not produced in significant quantities by bacteria (Graupner and White 2001; Graupner, Xu and White 2002; Bashiri et al. 2019). However, the discovery that mycobacterial F420 biosynthesis utilizes the abundant metabolite PEP paved the way for its heterologous production (Bashiri et al. 2019; Grinter et al. 2020). Concurrently, Ney and Greening first successfully produced F420 in *E. coli* through the heterologous expression of FbiC, FbiA, CofD and FbiB (Ney 2019). However, the yields for F420 produced in *E. coli* were lower than that achieved for purification from *M. smegmatis* or methanogens (Isabelle, Simpson and Daniels 2002; Bashiri et al. 2010).}

**Figure 14.** Crystal structure of CofE from *A. fulgidus* in complex with GDP and Mn2+. (A) Cartoon view of the crystal structure of the functional dimer of CofE [PDB ID = 2PHN]. CofE subunits are shown in pink (Mol. A) and red (Mol. B). Bound GDP and catalytic Mn2+ ions are shown for Mol. B only, as stick and sphere representation respectively. (B) Electrostatic surface view of the CofE active site showing bound GDP, catalytic Mn2+ ions and predicted F420-0 binding pocket. (C) Proposed catalytic mechanism for the first γ-linked glutamate addition mediated by CofE, R = F4 minus terminal hydroxyl.
Figure 15. Crystal structure of FbiBC-term, from M. tuberculosis. (A) Cartoon view of the crystal structure of the functional dimer of the FbiB C-terminal domain responsible for the FMNH$_2$ mediated reduction of DH-F$_{420}$-0 [PDB IDs = 4X0O, 4X0Q] FbiBC-term is shown in green and FMN and DH-F$_{420}$-0 (modeled based on the co-crystal structure of F$_C$) are shown as sticks. (B) A zoomed view of the FbiBC-term active site in complex with FMN and DH-F$_{420}$-0 as in panel A, with a cartoon and transparent atomic surface of the FbiBC-term shown.

Figure 16. Schematic showing possible events in the evolution of F$_{420}$ and its acquisition by different bacterial and archaeal lineages. Arrows with solid lines indicate potential horizontal transfer of ancestral F$_{420}$ biosynthetic genes. Dashed lines with bidirectional arrows indicate a likely gene transfer event of unknown directionality. Phyla labels are simplified for clarity. Note the figure is a speculative model drawn based on data from sources discussed in the main text and other models are also consistent with these data.
et al. 2019; Shah et al. 2019), meaning considerable further engineering is required to make the system compatible with industrial production. Through the course of their discovery of 3PG-F420 production by P. rhocincola, Braga et. al. independently hetero logously produced this chemical F420 variant in E. coli (Braga et al. 2019). While 3PG-F420 was only produced in low quantities, it is compatible with F420-dependent enzymes from organisms producing the classical version of the cofactor (Braga et al. 2019). As such, this work provides an alternative set of enzymes and precursor substrates for heterologous F420 production, which may assist in increasing production levels.

Synthetic or semisynthetic synthesis of F420-like cofactors is a promising alternative strategy for large quantities of cofactor compatible with industrial applications. F0 can be produced synthetically in large quantities and is catalytically compatible with some F420-dependent enzymes (Hossain et al. 2015). However, it generally exhibits much lower catalytic efficiency, making it a less than ideal cofactor for industrial applications (Drenth, Trajkovic and Fraaije 2019). Recently, Drenth et al. utilized a biosynthetic approach to enzymatically phosphorylate the terminal hydroxyl of F0, yielding the F420 analog F0-5′-phosphate (FOP). FOP was functional as a cofactor for both F420-reducing dehydrogenases and F0H2-dependent reductases with a higher catalytic efficiency than F0. However, a reduction in catalytic efficiency compared to F420 was observed depending on the enzyme employed (2- to 22-fold reduction; Drenth, Trajkovic and Fraaije 2019; Martin et al. 2020). This reduction in efficiency reinforces the importance of the F420 polyglutamate tail in protein cofactor interactions (Ney et al. 2017b) and suggests that FOP use will be limited to compatible enzymes or those which have been engineered to suit this cofactor. Currently, no definitive solution exists to cheaply and efficiently produce F420 or a suitable analog that can be utilized by enzymes with a high level of catalytic efficiency. However, the recent progress discussed above can likely be built upon to provide a solution to this bottleneck in the near future.

**Development of an efficient F420 reduction system**

To harness their potential for asymmetric hydrogenation on an industrial scale, F420H2-dependent reductases require a source of reduced F420H2. Ideally, F420H2 would be regenerated from enzymatically oxidized F420 via a mechanism integral to the reaction system, providing a high F420H2/F420 ratio and a sustained source of the reduced cofactor to maximize reaction yields. While it may be possible to directly reduce F420 by electrochemical or photochemical means (Wichmann and Vasic-Racki 2005), this has not been comprehensively investigated. As such, enzymatic regeneration of F420 using existing F420-reducing dehydrogenases is currently the most practical means of cofactor regeneration.

F420-reducing dehydrogenases that utilize a number of diverse substrates have been identified (Table 2). In their physiological context, these dehydrogenases produce a free pool of reduced cytoplasmic F420H2, which is bound by F420H2-dependent reductases and utilized to directly transfer hydride to their substrate (Ahmed et al. 2015, 2016; Greening et al. 2017; Mathew et al. 2018). This system differs mechanistically from OYEs, which tightly bind a FMN molecule that is first reduced by NAD(P)H before substrate reduction (Stuermer et al. 2007; Toogood, Gardiner and Scrutton 2010). The independent nature of F420-reducing dehydrogenases and F420H2-dependent reductases provides flexibility compared to OYEs, allowing for the use of different substrates for cofactor regeneration by F420-reducing dehydrogenases. Several F420-reducing dehydrogenases have been produced recombinantly and structurally characterized (Table 2; Warkentin et al. 2001; Aufhammer et al. 2004, 2005; Bashiri et al. 2008; Allegretti et al. 2014), facilitating enzyme production and optimization via structure-guided protein engineering. However, to provide an economically viable solution to F420 reduction for industrial catalysis, the enzyme employed must be stable and readily producible in large quantities. Additionally, it must reduce F420 with reasonable catalytic efficiency and its substrate must be cheap and readily obtainable. Considering these criteria, some F420-reducing dehydrogenases are more attractive targets than others for industrial cofactor regeneration.

Enzymes originating from methanogenic archaea, which utilize H2 (Fdh) or formate (Fdh) for F420 reduction, are superficially attractive targets due to the low cost of their substrates and the lack of contaminating solutes resulting from their oxidation (Shah et al. 2019). However, both of these enzymes are multi-subunit proteins that utilize complex transition metal cofactors for substrate oxidation and transfer the resulting electrons to F420 via multiple iron-sulfur clusters (Schauer and Ferry 1986; Vitt et al. 2014). The complexity of these enzymes, combined with the oxygen sensitivity of their metal-containing functional groups, means they are unlikely to be a practical solution for F420-reduction (Baron and Ferry 1989; Vitt et al. 2014). F420 reduction utilizing NADPH as a hydride donor, via the enzyme Fno, represents a more attractive means of cofactor regeneration (Berk and Thauer 1997; Kumar et al. 2017). Fno is a small single subunit protein, which is produced by a wide range of bacteria and archaea, providing numerous homologs from which to select an industrially compatible enzyme (Eirich and Dugger 1984; Kunow et al. 1993; Le et al. 2015; Kumar et al. 2017). Representative crystal structures of Fno from thermophilic species have been determined, providing the basis for optimization via protein engineering (Kunow et al. 1993; Kumar et al. 2017). Drawbacks for the use of Fno for cofactor regeneration include the relative expense of NADPH, which is required in stoichiometric quantities to the target for reduction (unless an additional enzyme and substrate is added for NADP+ regeneration), as well as the presence of contaminating NADP+ in the final reaction mix. The use of G6P for F420 regeneration via the enzyme Fgd is another option that has similar advantages and disadvantages to NADPH. Fgd is a single-chain protein that can be recombinantly produced and for which a crystal structure has been determined (Purwarianti and Daniels 1996; Bashiri et al. 2008). However, G6P is relatively expensive and its use in F420 reduction leads to the generation of the by-product 6-phosphoglucono-D-lactone. Recently homologs of Fgd with significant activity towards other sugar phosphates were identified (Mascotti et al. 2018). These enzymes, named Fsd, reducing sugar-6-phosphate dehydrogenases (Fsd), also exhibit low levels of F420 reductase activity with non-phosphorylated sugars (Mascotti et al. 2018). While the rates of F420 reduction with these sugars were too low to be catalytically useful, they suggest that through protein engineering Fsd could be adapted to utilize more economical non-phosphorylated sugars as substrates (Mascotti et al. 2018).

The F420-reducing secondary alcohol dehydrogenase Adf is the most promising target for F420H2 regeneration on an industrial scale. Adf is a relatively small (37 kDa) single chain enzyme produced by thermostatable organisms, which can utilize inexpensive secondary alcohols like isopropanol to reduce F420 with a reasonable catalytic efficiency (Bleicher and Winter 1991). The product of this reaction is a volatile ketone (e.g. acetone), which
can be readily separated from the reaction product. The standard potential for the reduction of acetone to isopropanol is ~290 mV, higher than that of F420/F420H2 (~340 mV; Thauer, Jungemmann and Decker 1977; Jacobson and Walsh 1984), meaning that a relatively high concentration of substrate (~100 mM) would be needed to ensure efficient F420 reduction. However, Adf has been shown experimentally to tolerate isopropanol up to 100 mM with no significant reduction in activity (Martin et al. 2020). Relatively high concentrations of secondary alcohols are often well tolerated by enzymes and this tolerance could be improved for F420H2-dependent reductases by protein engineering (Doukyu and Ogino 2010). Moreover, the addition of secondary alcohols could increase the solubility of enzyme substrates with poor solubility. In summary, Adf is a simple protein with high stability, that can be produced recombinantly, has good catalytic efficiency, an inexpensive substrate and produces a volatile product. As such, of the currently characterized F420 reducing enzymes, it is the only candidate that fulfills all the above criteria for industrial applications.

**Discovery and engineering of F420H2-dependent reductases**

The range of biological reduction reactions performed by F420-dependent enzymes discussed above indicates their potential for performing reduction reactions. The diversity of molecules identified as physiological substrates for F420H2-dependent reductases, encompassing large and small soluble molecules, as well as hydrophobic molecules and lipids, also reflects their versatility as biocatalysts (Figs 7 and 8; Wang et al. 2013; Purwan-tini, Daniels and Mukhopadhay 2016; Greening et al. 2017; Lee et al. 2020; Steiningerova et al. 2020; Tao et al. 2020). Furthermore, the abundance of predicted F420-dependent enzymes encoded in microbial genomes indicates that currently-characterized F420-mediated reactions represent a small subset of those that exist in nature (Ahmed et al. 2015; Ney et al. 2017a; Mascotti et al. 2018). This indicates that there is a wealth of F420-dependent enzymes with potential for use in industrial catalysis.

The use of F420H2-dependent reductases to perform industrially important reduction reactions currently represents the most promising application of F420 to industrial processes (Greening et al. 2017; Mathew et al. 2018; Drenth, Trajkov and Frawrie 2019). F420H2-dependent reductases can reduce a range of activated alkenes (quinones, coumarins, enones, enals, pyrones and pyrans), unsaturated nitrogen-containing compounds (imines, pyrones, pyrans and triaryl methane dyes), with activity and substrate specificity varying widely between enzymes (Fig. 17 and Table 2; Greening et al. 2017; Mathew et al. 2018). FDOR-A family enzymes investigated displayed relatively high levels of activity towards quinones, in line with their proposed physiological role as menaquinone reductases (Ahmed et al. 2015; Lee et al. 2020). Mathew et al. showed that three diverse actinobacterial FDOR-A enzymes exhibited high levels of enantioselectivity towards a panel of α/β-unsaturated ketones and aldehydes, as well as regioselectivity towards a benzyl-denial compound (Mathew et al. 2018). The observed enantioselectivity of these enzymes towards several substrates was the opposite of that observed when the substrates were reduced by OYEs, indicating that FDORs could provide stereochemical flexibility for enzymatic catalysis (Mathew et al. 2018). However, the catalytic rates of purified FDORs towards these substrates, where reported, are low, meaning that significant engineering is required to render them suitable for industrial catalysis (Jirapanjawat et al. 2016; Greening et al. 2017; Mathew et al. 2018). Interestingly, a panel of diverse FDORs showed no activity against compounds containing functional groups (nitroimidazoles and imines) known to be reduced by F420H2-dependent FDORs in a physiological context (Greening et al. 2017). This observation, combined with the vastly different activities observed for FDORs towards different substrates with the same functional group, indicates that protein-substrate interactions are important for determining enzyme activity and significant engineering will be required to adapt the substrate binding sites of these enzymes to industrially relevant substrates.

Further investigation into the mechanisms of substrate binding and reduction by FDORs will assist in engineering enzymes with higher catalytic efficiency and predicting product stereochemistry. It has been proposed that proton donation to the F420-substrate after initial hydride transfer from F420 occurs from N1 of F420H2 (Fig. 1B; Shah et al. 2019). If true, both hydrogenation events occur on the same face of the activated alkene substrate, leading to cis-hydrogenation when a substituted alkene is reduced by the enzyme. This is in contrast to OYEs where trans-hydrogenation products are formed (Hollmann, Opperman and Paul 2020). However, cis-hydrogenation by FDORs has not been demonstrated experimentally, and spectroscopic and computational analysis suggests that F44H– (in which N1 is deprotonated) is the form of the cofactor utilized by the FDOR-A Ddn (Mohamed et al. 2016a). If F420H2– is the general physiological form of the cofactor used by FDORs, then substrate protonation must instead proceed from solvent or an enzyme sidechain (Mohamed et al. 2016a,b; Greening et al. 2017). A better understanding of the structural and biochemical basis for the binding of physiological and industrially relevant substrates to FDORs is also required. No crystal structures of FDOR-substrate complexes are available, though some docking analysis has been performed, indicating residues important for substrate binding...
Classes of compounds shown to be reduced by purified F420-dependent enzymes. The reduced bond is highlighted in orange for each compound. The enzymes tested, as well as their substrate range and relative activity levels, are provided in Table 5.

Figure 17. Proposed reaction schemes for the reduction of menaquinone and malachite green by F420H2-dependent reductases of the FDOR-A superfamily. In both cases, initial hydride transfer to one carbon atom of an activated alkene is followed by tautomerization yielding the final reaction product.

(A) Quinones

Menadione 1,4-Naphthoquinone 1,2-Naphthoquinone

Pyrones/Coumarins

5,6-Dihydro-2H-pyran-2-one Coumarin 3-Cyanocoumarin Fraxetin Afatoxin B1

Enones

3-Methyl-2-cyclopentenone 2-Cyclohexen-1-one (R)-Carvone β-ionone

Secondary Alcohols

Methoxyacetone Methyl isobutyl ketone 2-Heptanone Propiophenone

Triarylmethanes

Crystal Violet Malachite Green

(B) Menaquinone

Malachite Green

Hydrophobic molecules, suggests their biosynthetic potential. Notably, the TIM-barrel fold of LLHTs is unrelated to that of FDORs, possessing a structurally divergent substrate-binding pocket (Fig. 4; Mascotti et al. 2018). As such, LLHTs are likely to provide complementary substrate specificity and stereoselectivity to FDORs. Like FDORs, sequenced microbial genomes contain a wealth of putative F420-dependent LLHTs with unknown function, many of which are contained within hypothetical secondary metabolite BCGs (Selengut and Haft 2010; Mascotti et al. 2018; Steiningerova et al. 2020).

In summary, while our current understanding of F420-dependent enzymes sets the stage for the use of F420 in industrial catalysis, considerable further work is required to develop enzymes suitable for such processes. To realize this goal these enzymes will require a high level of activity towards economically relevant substrates, with favorable kinetic parameters. Also, they will likely need to display high levels of stereoselectivity, yielding a commercially relevant enantiomer. Further, for use in synthetic chemistry, the enzymes will need to be robust, able to withstand relatively harsh extremes of temperature, pH, ionic strength and concentrations of non-polar solvents. Currently, no enzymes with these properties have been described or developed. A possibly promising strategy for the development of such enzymes would be to identify commercially relevant substrates, with chemistry amenable to reduction
Table 5. Purified F420H2-dependent reductases that have been explored for substrate specificity and range.

| Enzyme name          | Originating organism | Sequence ID | Physiological substrate | In vitro activity | Enzyme class | PDB ID | Quinones | Coumarins | Enones | Enals | Pyrones | Pyrans | Triarylmethanes | Secondary alcohol |
|----------------------|----------------------|-------------|--------------------------|-------------------|--------------|--------|----------|-----------|--------|-------|---------|--------|-----------------|------------------|
| **MSMEG-5998**       | M. smegmatis         | ABK71916    | Menaquinone              | +++               | FDOR-A1      | ND     | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-2027**       | M. smegmatis         | ARK5354     | Menaquinone              | +++               | FDOR-A1      | 4Y91   | +++      | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-2850**       | M. smegmatis         | ARW37737    | Menaquinone              | +++               | FDOR-A1      | 3186   | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-2004**       | M. smegmatis         | ABK7467     | Menaquinone              | +++               | FDOR-A1      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-3356**       | M. smegmatis         | ABK7579     | Menaquinone              | ++                | FDOR-A1      | 3H96   | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-3004**       | M. smegmatis         | ABK74167    | Menaquinone              | ++                | FDOR-A1      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-5030**       | M. smegmatis         | ABK74375    | Menaquinone              | +++               | FDOR-A2      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-3380**       | M. smegmatis         | ABK72884    | Menaquinone              | +++               | FDOR-B1      | 3F7E   | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-0048**       | M. smegmatis         | ABK73917    | Menaquinone              | ++                | FDOR-B1      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-6325**       | M. smegmatis         | ABK73368    | Menaquinone              | ++                | FDOR-B1      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-5170**       | M. smegmatis         | ARK72943    | Menaquinone              | +++               | FDOR-B1      | 4Y91   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-5717**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 5Y4V   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-4030**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 4ZKY   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-6526**       | M. smegmatis         | ARK76717    | Menaquinone              | +++               | FDOR-B2      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-5717**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 5Y4V   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-4030**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 4ZKY   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-6526**       | M. smegmatis         | ARK76717    | Menaquinone              | +++               | FDOR-B2      | ND     | ND       | ND         | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-3880**       | M. smegmatis         | ARK78672    | Menaquinone              | +++               | FDOR-B2      | 5Y4V   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-5030**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 4ZKY   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |
| **MSMEG-3380**       | M. smegmatis         | ARK72164    | Menaquinone              | +++               | FDOR-B1      | 4ZKY   | ++       | -          | ND     | ND     | ND       | ND     | ND              | ND                |

*In vitro activity: +++, ++, +, -, ND.*

**References:**
- Greening et al. (2017)
- Taylor et al. (2010)
- Lapalikar et al. (2012)
- Martin et al. (2020)
- Mathew et al. (2018)
- Mathew et al. (2018)
or oxidation by F420-dependent enzymes, which are recalcitrant to currently available synthetic chemical processes. The wealth of F420-dependent enzymes present in microbial genomes could be screened for enzymes capable of reducing these substrates to some degree. These enzymes could be then subjected to rigorous structural and biochemical characterization, combined with concerted protein engineering efforts, to produce enzymes suitable for the reduction of these substrates on an industrial scale.

**Ramifications and applications of microbial utilization of F420**

In addition to the importance of F420 in microbial physiology and its potential applications for chemical synthesis, our understanding of the diverse role of the cofactor has wider significance for improving human health and sustainability. As discussed below, hydrogenotrophic methanogens that rely on F420 are an important source of global greenhouse gases, while in M. tuberculosis F420 is important for the activation of nitroimidazole prodrugs and plays an insufficiently characterized role in survival in the host. Our recently acquired knowledge on the biosynthesis and roles of F420 can be utilized to develop methanogenesis inhibitors and antitubercular drugs, as well as to predict and alleviate nitroimidazole resistance.

**Methane mitigation through inhibition of F420 dependent enzymes**

F420-dependent hydrogenotrophic methanogens, notably Methanobrevibacter gottschalkii and Methanobrevibacter ruminantium, are core members of the foregut microbiota of ruminants (Leahy et al. 2010; Henderson et al. 2015). Methane emitted by ruminants is a significant driver of global warming, with ruminant methane emissions accounting for approximately 40% of global methane emissions and 5% of global greenhouse gas emissions (Greening et al. 2019). Bacteria and archaea that utilize alternative aceticogenic or respiratory pathways for H2 oxidation, independently of F420, are abundant in the rumen (Greening et al. 2019). Thus, strategies to inhibit or outcompete hydrogenotrophic methanogens should foster a microbial community that produces less methane, reducing the greenhouse gas emissions from livestock farming (Morgavi et al. 2010; Greening et al. 2019). Potent methyl-CoM reductase inhibitors, such as 3-nitroxypropanol, reduce methane production without compromising animal health and productivity (Hristov et al. 2015; Duin et al. 2016). Given hydrogenotrophic methanogenesis requires F420, strategies that inhibit F420 production or its use by F420-dependent enzymes will also inhibit the growth of these archaea. While specific strategies for the inhibition of F420 biosynthesis or dependent enzymes in the rumen have not been reported, several studies indicate this may be possible. The pterin lumazine inhibits methanogen growth and methane formation in pure culture, possibly through its structural similarity to F420 (Nagar-Anthal et al. 1996), although this effect was less significant in mixed culture (Ungerfeld et al. 2004). Recently, in silico screening identified inhibitors of Fno from the methanogen Methanobrevibacter smithii, some of which bind the enzyme with affinity in the nM range. Some of these inhibitors are non-toxic dietary supplements and could be readily investigated for their ability to reduce methane emissions by inhibiting F420-dependent enzymes (Cuccioloni et al. 2020). If effective, inhibitors of F420 dependent pathways could be provided as dietary supplements, possibly in conjunction with other strategies to inhibit the growth of hydrogenotrophic methanogens or promote the growth of other hydrogenotrophs in the rumen.

**Nitroimidazole prodrug activation in M. tuberculosis**

It has been recognized since the late 1980s that compounds containing the nitroimidazole functional group can display potent antitubercular activity (Nagarajan et al. 1989; Liu et al. 2018). Early attempts to develop nitroimidazole-containing molecules for tuberculosis treatment, including the compound CGI-17341 (Ashtekar et al. 1993; Fig. 18A), failed due to toxicity concerns. However, concerted drug development efforts have yielded two compounds with potent antitubercular activities and acceptable safety profiles. These compounds, named delamanid and pretomanid (Fig. 18A), were both recently approved

![Figure 18. Nitroimidazole prodrugs effective against M. tuberculosis and their activation by Ddn. (A) Structure of nitroimidazole-containing prodrugs developed for tuberculosis treatment. Delamanid and pretomanid were recently approved for the treatment of M. tuberculosis infection, while CGI-17341 was abandoned due to toxicity concerns. The nitroimidazole functional group is highlighted in orange. (B) The complex between pretomanid and its activating enzyme Ddn from M. tuberculosis was generated by molecular docking using AutoDock Vina (Trott and Olson 2010). The proximity between the nitroimidazole group of pretomanid and the hydride transferring C5 carbon of F420 is shown in the inset panel. (C) Proposed products for the breakdown of pretomanid following its reduction by Ddn, full reaction schemes leading to product generation refer to Singh et al. (2008).](image-url)
for the treatment of multidrug-resistant tuberculosis (MDR) as a combination therapy (Liu et al. 2018; Keam 2019). Delamanid was approved in 2014 by the European Medicines Agency for use in an appropriate combination regimen for MDR tuberculosis treatment and is subject to ongoing safety and efficacy studies (Ryan and Lo 2014; von Groote-Bidlingmaier et al. 2019). Pretomanid was approved in 2019 by the U.S. Food & Drug Administration for treatment of MDR or extensively drug-resistant tuberculosis (XDR), as part of a three-drug ‘BPaL’ regime, also including bedaquiline and linezolid (Keam 2019). Safety and efficacy trials indicate pretomanid, administered in combination with bedaquiline, moxifloxacin and pyrazinamide, is also effective in the treatment of MDR and XDR tuberculosis, although this regime is awaiting regulatory approval (Tweed et al. 2019).

Nitroimidazole-containing compounds act as prodrugs in the treatment of tuberculosis and are reductively activated in the M. tuberculosis cell by the aforementioned FDOR-A enzyme Ddn (Fig. 18B; Cellitti et al. 2012; Fujiwara et al. 2018). The activation of delamanid and pretomanid occurs through the promiscuous activity of Ddn, which appears to play a physiological role in the reduction of menaquinone (Lee et al. 2020). The reduction of the nitroimidazole functional group by Ddn is thought to lead to several reaction products, including the release of HNO and HNO2 and the formation of des-nitro forms of the drugs (Fig. 18C; Singh et al. 2008). While the precise details of the mechanism of action of delamanid and pretomanid towards M. tuberculosis remain to be elucidated, evidence supports a role for the release of reactive nitrogen species in their toxicity (Manjunatha et al. 2006; Singh et al. 2008; Manjunatha, Boshoff and Barry 2009). Pretomanid also selectively inhibits the synthesis of ketomycolic acids in the M. tuberculosis, potentially through the direct or indirect inhibition of the F420-reducing dehydrogenase FHMD (Stover et al. 2000; Purwantini and Mukhopadhyay 2013). Further, global metabolomic analysis of pretomanid treated M. smegmatis identified the accumulation of the toxic metabolite methyglyoxal, indicating that metabolic poisoning may also play a role in the antitubercular activity of nitroimidazole drugs (Baptista et al. 2018). As nitroimidazole drugs are effective against M. tuberculosis under both aerobic and anaerobic conditions, and their activation by Ddn leads to several reactive intermediates and reaction products, the mechanism of action of these compounds is likely complex and multifaceted (Singh et al. 2008; Mukherjee and Boshoff 2011).

Resistance to delamanid and pretomanid in M. tuberculosis is imparted by mutations that prevent the production of F420 (via loss of functional FbIA, FbIB, FbIC or FbID) or its reduction to F420ZH2 (via loss of functional Fgd; Choi et al. 2001; Manjunatha et al. 2006; Jing et al. 2019; Lee et al. 2020). Additionally, mutations that result in either the complete loss of Ddn function or loss of its promiscuous activity towards the prodrugs also result in resistance (Lee et al. 2020). The latter finding is of concern for the longevity of delamanid and pretomanid for treatment of tuberculosis. Particularly concerning is that clinical M. tuberculosis isolates with mutations in Ddn that abolish its activity towards delamanid and pretomanid, but not its physiological substrate menaquinone, have been identified (Lee et al. 2020; Rifat et al. 2020). A number of these isolates were from patients that had not been treated with delamanid and pretomanid, suggesting that inherently resistant strains of M. tuberculosis exist (Yang et al. 2018; Lee et al. 2020). Interestingly, some mutations in Ddn result in resistance to pretomanid but not delamanid (Lee et al. 2020), likely due to the differences in their interactions with the Ddn substrate-binding pocket. Based on this observation, a robust understanding of Ddn substrate binding may allow for the deployment of patient-tailored prodrug variants less susceptible to polymorphisms in Ddn. As discussed in the next section, the effect of the loss of F420 on the virulence and transmissibility of M. tuberculosis as a result of nitroimidazole treatment remains uncertain.

**Targeting F420 for the development of antitubercular drugs**

The role of F420 in activating pretomanid and delamanid has rekindled interest in the role of F420 in mycobacterial physiology (Cellitti et al. 2012; Haver et al. 2015; Fujiwara et al. 2018; Lee et al. 2020; Rifat et al. 2020). However, despite recent progress in this area, we lack a comprehensive understanding of the importance of F420 in mycobacterial physiology. This is especially true for mycobacterial pathogens like M. tuberculosis, in part due to the difficulty in working with this slow-growing and highly pathogenic bacterium (Cole et al. 1998). However, phenotypes associated with the loss of F420 production discussed above strongly suggest that the cofactor plays a role in the ability of M. tuberculosis to cause disease (Gurumurthy et al. 2013; Jirapanjawat et al. 2016; Lee et al. 2020; Rifat et al. 2020). These observations, together with the ubiquity of F420 in mycobacteria, the abundance of F420-dependent enzymes in M. tuberculosis and the absence of the cofactor from human cells, make the processes that produce or use F420 potential targets for the development of antimicrobial compounds. One method of targeting F420 would be the development of compounds that inhibit the enzymes responsible for its biosynthesis (i.e. FbIA, FbIB, FbIC and FbID) or reduction (Fgd; Bashiri et al. 2008, 2019). Crystal structures are available for the majority of these enzymes, facilitating inhibitor design (Bashiri et al. 2008, 2019; Grinter et al. 2020). An alternative approach would be the development of F420 analogs that inhibit F420-dependent enzymes (Eirich, Vogels and Wolfe 1978). However, to make the considerable effort required for the identification and optimization of F420 biosynthesis inhibitors attractive, a better understanding of the importance of the cofactor for virulence is required.

The role of F420-dependent enzymes in reductively detoxifying antimicrobial compounds is also of interest for M. tuberculosis treatment (Jirapanjawat et al. 2016; Rifat et al. 2020). Profiling the antibiotic sensitivity of F420-deficient mutants of mycobacterial pathogens may identify antibiotics that selectively display activity against these strains. There is some evidence that loss of F420 leads to a heightened sensitivity to the antibiotics isoniazid, moxifloxacin and clofazimine in M. tuberculosis (Gurumurthy et al. 2013; Rifat et al. 2020). Given that loss of F420 production is known to mediate resistance to pretomanid and delamanid (Haver et al. 2015; Jing et al. 2019; Lee et al. 2020), antibiotics that are more effective against F420 deficient mutants may be useful in combination with these drugs to reduce or mitigate the development of resistance. Preliminary analysis of F420-dependent LLHTs in pathogenic mycobacteria demonstrates they play an important role in outer-envelope lipid biosynthesis. Outer envelope lipids, like PDIMs and ketomycolic acids, are important for mycobacterial virulence (Purwantini and Mukhopadhyay 2013; Purwantini, Daniels and Mukhopadhyay 2016). Growing evidence indicates these outer envelope lipids constitute a second outer membrane in mycobacteria (Hoffmann et al. 2008; Bansal-Mutalik and Nikaido 2014). In M. tuberculosis this membrane contains high concentrations of PDIM, which contribute to the impermeability of this barrier and the antibiotic resistance of this species (Wang et al. 2020). As such, a systematic understanding of the role of F420 dependent enzymes in outer-envelope lipid biosynthesis will inform future efforts to combat pathogenic mycobacteria through inhibition of this process.
OUTLOOK
When we first reviewed this topic five years ago (Greening et al. 2016), we noted multiple knowledge gaps that have since been addressed. These included: resolving the chemical steps and structural basis of F420 biosynthesis (Bashiri et al. 2019; Braga et al. 2019; Grinter et al. 2020); surveying the distribution of F420 across different taxa and ecosystems (Ney et al. 2017a); investigating the chemistry of the F420 headgroup and tail to catalysis (Mohamed et al. 2016a; Ney et al. 2017b); and enabling F420− dependent industrial biocatalysis through achieving heterologous cofactor production (Bashiri et al. 2019; Braga et al. 2019; Ney 2019) and characterizing promising F420− dependent oxidoreductases (Greening et al. 2017; Mascotti et al. 2018; Mathew et al. 2018; Drenth, Trajkovic and Fraaije 2019; Martin et al. 2020).

Some of these lines of investigation resulted in unexpected findings, most notably that F420 biosynthesis genes are extremely widely distributed (Ney et al. 2017a; Table S2, Supporting Information), the biosynthesis pathway has multiple variants and was misannotated in bacteria (Bashiri et al. 2019; Braga et al. 2019, 2020; Grinter et al. 2020) and some bacteria produce entirely novel variants of this cofactor (Braga et al. 2019).

Despite these important insights, we still lack a systematic understanding of the physiological role of the cofactor. Unanswered questions include why mycobacteria encode a multitude of predicted FDORs and LLHTs, and why newly identified F420 producers such as Proteobacteria and Chloroflexi synthesize this cofactor. Further research is needed to resolve whether lineages such as the TACK and Asgard archaea, Firmicutes and Tectomicrobia do produce F420 as predicted, and if so, which variants do they make and through which pathways? Other knowledge gaps that could be addressed in coming years include a more detailed understanding of the evolution of the F420 biosynthetic pathway, structural resolution of F420 biosynthesis and newly discovered F420− dependent methanogen enzymes, as well as the long-standing question of which enzyme mediates 2-phospholactate production in methanogens. As we detailed in the final section of this review, there is also ample potential to translate this fundamental knowledge to address medical, environmental and industrial challenges to improve human health and sustainability. Exactly 50 years since its discovery by Wolfe and colleagues, it is increasingly clear that F420 is a widespread and versatile cofactor, fundamental to the physiology of many bacteria and archaea.

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