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Energetics of Respiration and Oxidative Phosphorylation in Mycobacteria

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Abstract

Mycobacteria inhabit a wide range of intracellular and extracellular environments. Many of these environments are highly dynamic and therefore mycobacteria are faced with the constant challenge of redirecting their metabolic activity to be commensurate with either replicative growth or a non-replicative quiescence. A fundamental feature in this adaptation is the ability of mycobacteria to respire, regenerate reducing equivalents and generate ATP via oxidative phosphorylation. Mycobacteria harbor multiple primary dehydrogenases to fuel the electron transport chain and two terminal respiratory oxidases, an *aa*$_3$-type cytochrome *c* oxidase and cytochrome *bd*-type menaquinol oxidase, are present for dioxygen reduction coupled to the generation of a protonmotive force. Hypoxia leads to the downregulation of key respiratory complexes, but the molecular mechanisms regulating this expression are unknown. Despite being obligate aerobes, mycobacteria have the ability to metabolize in the absence of oxygen and a number of reductases are present to facilitate the turnover of reducing equivalents under these conditions (e.g. nitrate reductase, succinate dehydrogenase/fumarate reductase). Hydrogenases and ferredoxins are also present in the genomes of mycobacteria suggesting the ability of these bacteria to adapt to an anaerobic-type of metabolism in the absence of oxygen. ATP synthesis by the membrane-bound F_1F_O-ATP synthase is essential for growing and non-growing mycobacteria and the enzyme is able to function over a wide range of protonmotive force values (aerobic to hypoxic). The discovery of lead compounds that target respiration and oxidative phosphorylation

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in *Mycobacterium tuberculosis* highlights the importance of this area for the generation of new front line drugs to combat tuberculosis.

Overview of mycobacterial respiration and oxidative phosphorylation

The genus *Mycobacterium* comprises a group of obligately aerobic bacteria that have adapted to inhabit a wide range of intracellular and extracellular environments. A fundamental feature in this adaptation is the ability to respire and generate energy from variable sources or to sustain metabolism in the absence of growth. Early studies on respiration demonstrated that *Mycobacterium tuberculosis* H37Rv, grown in the lungs of infected mice, had high rates of endogenous respiration that were not stimulated by exogenous substrates (e.g. acetate, pyruvate, glucose, glycerol, lactate) (1). In contrast, cells grown *in vitro* respired these substrates at high rates. Fatty acids, however, stimulated the respiration of *in vivo* grown *M. tuberculosis*, suggesting for the first time that *M. tuberculosis* switches to different energy sources in host tissues to fuel respiration. These early studies pointed to the fact that electron donor utilization and respiration are precisely controlled in *M. tuberculosis*, not only in response to growth rate, but also in response to the carbon and energy sources used for growth. The pioneering work of Brodie and colleagues on *Mycobacterium phlei* established much of the primary information on the electron transport chain and oxidative phosphorylation system in mycobacteria (reviewed in (2)). Since these early studies, comparatively few biochemical studies have been performed on the electron transport components and the energetics of respiration in mycobacterial species. With the advent of microbial genome sequencing, sequence analyses have revealed that branched pathways exist in mycobacterial species for electron transfer from many low potential reductants via quinol, including $H_2$to oxygen (Figure 1). Unlike other bacteria, there appears to be little redundancy in the transfer of electrons to oxygen during growth with only two terminal respiratory oxidases present in mycobacteria, an aa_3-type cytochrome *c* oxidase (encoded by *ctaBCDE*) belonging to the heme-copper respiratory oxidase family and cytochrome *bd*-type menaquinol oxidase (*cydABCD*) (Figure 1). Mycobacteria coordinate the expression of these oxidases in response to oxygen supply to achieve maximal efficiency of oxygen utilization. The rationale for this coordinated expression is largely based on the assumption that the mycobacterial aa_3-type cytochrome *c* oxidase and cytochrome *bd*-type have markedly different affinities for oxygen in mycobacteria, but this remains to be experimentally shown. Furthermore, the molecular mechanisms governing the regulation of terminal oxidase expression in mycobacterial species are unknown. In the absence of oxygen, mycobacterial growth is inhibited, even if alternative electron acceptors are present (e.g. nitrate, fumarate). Despite growth being inhibited, mycobacteria are able to metabolize exogenous and endogenous energy sources under low oxygen for maintenance functions. Whether mycobacteria utilize energy spilling or overflow metabolism (3) under these conditions to balance the rate of catabolic versus anabolic reactions remains to be determined. The electron acceptors and mechanisms to recycle reducing equivalents under these conditions are poorly understood. Irrespective of the oxygen concentration or the protonmotive force (PMF), ATP synthesis is obligatorily coupled to the electron transport chain and the F_1F_o-ATP synthase, but the reasons for this remain unexplained. Research into mycobacterial respiration and oxidative phosphorylation

has been energized by the discovery of a new drug (TMC207 – Sirturo - Bedaquiline) that targets the ATP synthase of mycobacteria, suggesting that inhibitors of respiration and ATP synthesis will provide the next generation of front line drugs to combat tuberculosis and nontuberculous mycobacterial disease. The aim of this chapter is to discuss recent advances in understanding the processes of respiration and oxidative phosphorylation in mycobacteria, with a particular focus on the obligate human pathogen *M. tuberculosis*.

Energetics of mycobacteria during growth and non-growth conditions

Mycobacteria generally grow at neutral pH and under these conditions generate a PMF of approximately −180 mV (4). The PMF generated is used to drive proton-coupled energetic processes (e.g. ATP synthesis, solute transport, etc.) (4). The PMF-generating machinery in mycobacteria comprises three energy-conserving steps: complex I ($4H^+/2e^-$), complex III (menaquinol-cytochrome *c* oxidoreductase, $4H^+/2e^-$) and complex IV ($2H^+/2e^-$) suggesting that the overall proton translocation stoichiometry for the transfer of $2e^-$ from NADH to oxygen is $10H^+/2e^-$. Based on a ratio of $3H^+$ utilized by the ATP synthase/ATP synthesized leads to a theoretical maximum P/O ratio (i.e. the number of moles of ADP phosphorylated to ATP per $2e^-$ passing to oxygen) of approximately 3.3 (theoretical maximum). If complex I is bypassed by the non-proton translocating type II NADH:menaquinone oxidoreductase, the P/O ratio would be approximately 2. Electron flow from the menaquinone pool to the cytochrome *bd* oxidase branch (bypassing complex III and IV) would produce a P/O ratio of 0.67. Measured experimental values for mycobacteria oxidizing NADH or succinate yield P/O ratios of 0.52, and 0.36, respectively (5). Variations in theoretical P/O ratios versus those determined experimentally is well accepted and can be explained by pathways that involve proton leakage (i.e. bypass ATP synthase) or the PMF is used to drive reverse electron transport. When succinate oxidation is coupled to menaquinone reduction, the energetics suggest a reverse electron flow from succinate (lower midpoint redox potential E_m = +30 mV) to menaquinone (E_m = −74 mV) resulting in lower P/O ratios.

Growth of mycobacteria is sensitive to compounds that dissipate the membrane potential (e.g. protonophores and valinomycin) and these compounds are bactericidal towards growing and non-growing (aerobic or hypoxic) cells further highlighting the importance of the membrane potential in mycobacterial viability (4, 6). Growth is also sensitive to the ionophore monensin, however, the reasons for this are not clear. Interestingly, some solute transporters in mycobacteria are driven by a sodium-motive force (7), but the mechanism used for the generation of the primary sodium gradient in mycobacteria is not known. As the pH of the growth medium changes and becomes mildly acidic, mycobacteria are able to generate a considerable transmembrane pH gradient (ZΔpH) and maintain a constant PMF (4). While proton translocation *via* the respiratory chain generates the PMF during respiration with oxygen as the terminal electron acceptor, it is not clear how the PMF is established in the absence of oxygen under anaerobic growth conditions. Anaerobic bacteria are able to generate a significant PMF (−100 mV) using their membrane-bound F_1F_O -ATP synthase in the ATP hydrolysis direction (8). The ATPase activity (proton-pumping) of the enzyme is fuelled by ATP produced by substrate level phosphorylation. This mechanism does not appear to operate in mycobacterial cells where the F_1F_O-ATP synthase has been reported to have latent ATPase activity when measured in inverted membrane vesicles (9,

10). Whether the enzyme is also latent in actively growing cells is not known and therefore the potential exists for this enzyme to function as a primary proton pump in the absence of oxygen and a functional respiratory chain to generate the PMF. Rao *et al.* (6) have reported that hypoxic, non-replicating *M. tuberculosis* generate a total PMF of −113 mV, −73 mV of electrical potential (ΔΨ) and −41 mV of ZΔpH. The addition of thioridazine, a compound that targets NDH−2, results in dissipation of the ΔΨ and significant cell death suggesting that NADH is an important electron donor for the generation of the ΔΨ under hypoxic conditions. The addition of TMC207, a specific inhibitor of the F_1F_O-ATP synthase, was bactericidal against hypoxic, non-replicating *M. tuberculosis*, but had no effect on the ΔΨ (6).

Electron donors fueling respiration in mycobacteria

Mycobacterial species use a variety of primary dehydrogenases to deliver electrons from central metabolism into the respiratory chain to generate energy (Figure 1).

Proton-pumping type I NADH dehydrogenase and non-proton pumping type II NADH dehydrogenase

The major entry point is the transfer of electrons from NADH oxidation to quinone reduction (e.g. ubiquinone or menaquinone). In bacteria three different types of respiratory NADH dehydrogenases have been identified and characterized on the basis of reaction mechanism, subunit composition and protein architecture (11). These include the proton-pumping type I NADH dehydrogenase (NDH-1, complex I), non-proton pumping type II NADH dehydrogenase (NDH-2) and sodium-pumping NADH dehydrogenase (NQR). Weinstein *et al.* identified genes for two classes of NADH:menaquinone oxidoreductases in the genome of *M. tuberculosis* (12) (Table 1). NDH-1 is encoded by the *nuoABCDEFGHIJKLMN* operon and transfers electrons to menaquinone, conserving energy by translocating protons across the membrane to generate a PMF (Figure 2). The second class is NDH-2, a non-proton translocating type II NADH dehydrogenase that does not conserve energy and is present in two copies (Ndh and NdhA) in *M. tuberculosis* (12) (Table 1). NQR has not been reported in mycobacterial genomes.

NDH-1 is composed of 14 subunits (*nuoA-N*), which represent a 15704 bp operon in *M. tuberculosis* (Rv3145 to Rv3158) (Table 1). NuoB, C, D, E, F, and G are peripheral membrane proteins located in the cytoplasmic side while NuoA, H, J, K, L, M, and N are in the membrane section of the complex with multiple predicted transmembrane domains (from 3 to 16). In contrast, the *nuo* operon has been lost from the genome of the intracellular parasite *Mycobacterium leprae* except for a single remaining *nuoN* pseudogene (13). NDH-1 uses flavin mononucleotide (FMN) and iron-sulfur clusters to transport electrons from NADH to the quinone pool (menaquinone). The release of the two electrons during the NADH oxidation produces enough energy to pump four protons across the membrane to generate a PMF (Figure 2). In *M. tuberculosis*, the *nuo* operon is neither essential for growth nor persistence in an *in vitro* Wayne model (6). *Mycobacterium smegmatis* also contains genes for a type I NADH:menaquinone oxidoreductase (*nuoA-N*). However, *M. smegmatis* NDH-I activity is very low, representing about 5% of the NDH-2 activity (14, 15). Rather,

increased expression (15-fold) of the *nuo* operon in *M. smegmatis* was observed in a carbon-limited chemostat in response to a slow down in growth rate (16). In the slow-growing *M. tuberculosis* and *M. bovis* strains, NDH-1 activity is 28% to 50% lower than the NDH-2 activity when measured in membrane fractions of mycobacteria (Vilchèze and Jacobs, unpublished data.), suggesting that the major NADH oxidizing activity is mediated by NDH-2. This is supported by the observation that NADH oxidation by mycobacterial membranes is relatively insensitive to complex I inhibitors (12). Notwithstanding this, *M. tuberculosis* mutants lacking only one of the subunits of NDH-1, *nuoG*, have an *in vivo* phenotype, where this gene was critical for host macrophage apoptosis inhibition and mouse virulence (17). This implies that *nuoG* and potentially other subunits of NDH-1 are anti-apoptosis factors and are attractive candidates for vaccine development.

Several studies have reported that *nuo* is down-regulated in *M. tuberculosis* during mouse lung infection (18), survival in macrophages (19), in both non-replicating persistence (NRP)-1 (1% oxygen saturation) and NRP-2 (0.06% oxygen saturation) relative to aerated mid-log growth (18), and upon starvation *in vitro* (20). The transcription of NDH-2 (*ndh*) is also down-regulated in *M. tuberculosis* during mouse lung infection, but transcript levels for *ndh* peak during NRP-2 *in vitro* demonstrating that the pattern of *ndh* regulation is different between *in vivo* and *in vitro* conditions (19). These data are in contrast to *Escherichia coli* where NDH-1 is usually associated with anaerobic respiratory pathways (e.g. fumarate) and non-coupling dehydrogenases such as NDH-2 are synthesized aerobically (21).

The non-proton translocating NDH-2 is a small monotopic membrane protein (50–60 kDa) that catalyses electron transfer from NADH via FAD (non-covalently bound redox prosthetic group) to quinone. No tertiary structural information exists for either the bacterial, plant or protist NDH-2 enzymes, but the yeast NDH-2 structure was recently solved by two laboratories (22, 23). NDH-2 is widespread in bacteria, and the mitochondria of fungi, plants, and some protists. In some cases more than one copy is present (24). The role(s) of multiple type II NADH dehydrogenases in prokaryotes, plants and parasites is unclear. In some organisms with multiple type II NADH dehydrogenases, one copy appears more essential than the other pointing to non-redundant functional differences (25, 26). *M. tuberculosis* harbors two copies of NDH-2 (*ndh* Rv1854c and *ndhA* Rv0392c) (12) (Table 1), which are well conserved among slow-growing mycobacterial species. In *M. tuberculosis*, Ndh (1392 bp) and NdhA (1413 bp) share 65% identity, however the FAD and NADH binding motifs, $\underline{G}^{21}S\underline{G}FGG^{26}$ and $\underline{G}^{177}A\underline{G}PT\underline{G}^{182}$ are highly conserved. Both proteins contain one transmembrane domain located at the amino acids 385 to 407 and 387 to 409, respectively. The Ndh and NdhA proteins of *M. tuberculosis* have been shown to be functional NADH dehydrogenases that transfer electrons to the quinone pool via a ping-pong reaction mechanism (27). NdhA is not present in *M. smegmatis*, yet the level of NADH oxidation by *M. smegmatis* NDH-2 is several fold higher than in *M. tuberculosis* or *M. bovis* and represents 95% of the total NADH oxidation measured (15). This might correlate with higher NAD^+ concentrations in *M. smegmatis* compared to *M. bovis* (three times higher as shown in (15)). In addition, when *M. smegmatis* and *M. bovis* were transformed with a replicative plasmid containing *ndh,* the ratio NAD^+/NADH doubled (15) further confirming the involvement of *ndh* in the oxidation of NADH into NAD^+.

Several studies have suggested that *ndh* is essential for growth of *M. tuberculosis* (12, 26, 28, 29) (Table 1). Mutations in the *ndh* gene of *M. smegmatis* result in a pleitropic effect: temperature sensitivity, amino acid auxotrophy and resistance to the first-line anti-TB drug isoniazid (INH) and its analog and second-line anti-TB drug ethionamide (ETH) (14, 15). The *ndh* mutants had decreased NADH oxidase activity and increased NADH concentration (14, 15). Selection for *ndh* mutants grown on rich media (Mueller Hinton) led to the isolation of *ndh* mutants that were auxotrophic for serine and glycine; this auxotrophy was resolved with complementation by a wild-type copy of *ndh*. The correlation between *ndh* mutations and serine/glycine auxotrophy was attributed to the increase in NADH concentration, which might inhibit the first step in serine/glycine biosynthesis (14). The increase in NADH concentration was also responsible for the high resistance to INH and ETH by competitively inhibiting the binding of the INH-NAD or ETH-NAD adduct to the NADH-dependent enoyl-ACP reductase InhA (15). *ndh* mutants in both slow-growing (*M. bovis* BCG) and fast-growing (*M. smegmatis*) mycobacteria had no growth defect at permissive temperature although they had lost up to 90% of their ability to oxidize NADH. Interestingly, in *M. smegmatis* and *M. bovis*, the levels of NAD^+ cofactor stayed relatively constant despite overexpression of *ndh* or mutations in *ndh*, which highly reduced their NADH oxidation capability (15), suggesting that the NAD^+ pool is tightly regulated in mycobacteria to maintain essential biochemical functions.

NDH-2 has not been reported in mammalian mitochondria leading to the proposal that these enzymes may represent a potential drug target for the treatment of human pathogens (6, 12, 27, 30, 31) and intracellular parasites (32). Despite the potential of NDH-2 as drug target, no potent nanomolar inhibitors of NDH-2 are known. Several classes of compounds are proposed to target the enzyme at micromolar concentrations (e.g. phenothiazine analogues, platanetin, quinolinyl pyrimidines) (12, 27, 33), but the mechanism of inhibition remains unknown. Despite poor in vitro activity, drugs of the phenothiazine family (trifluoroperazine, chlorpromazine) have potent activity in vitro against drug-susceptible and drug-resistant *M. tuberculosis* strains (34, 35). A phenothiazine analog was also tested in a mouse model of acute *M. tuberculosis* infection and found to reduce by 90% the *M. tuberculosis* bacterial load in the lungs after 11 days of treatment compared to a 3 to 4-log reduction in colony-forming units (CFUs) with the INH or rifampicin control (12). From a library of microbial products, two new compounds, scopafungin and gramicidin S, were identified as inhibitors of *M. smegmatis* NDH-2 with IC_{50} values better than trifluoperazine (36). There is a crucial need for new drug targets to inhibit *M. tuberculosis* and the electron transport chain is a very attractive avenue. However, reduction in NDH-2 activity has been linked to INH and ETH resistance in both slow- and fast-growing mycobacteria (15) and phenothiazines have been shown to be antagonistic with INH (30). Therefore, the development of NDH-2 inhibitors will have to ascertain that interference with current drug therapy does not occur.

Some interesting questions arise from these observations. Why do mycobacteria use type II NADH dehydrogenases to recycle NADH, when they could continue to use the energy conserving and PMF generating NDH-I? One potential explanation is that because type II NADH dehydrogenases are non-proton translocating, they will not be impeded by a high PMF, which could ultimately slow down metabolic flux due to back-pressure on the system.

This mechanism is akin to a “relief valve” that would allow for a higher metabolic flux and ultimately higher rates of ATP synthesis, at the expenses of low energetic efficiency of the respiratory chain. Secondly, why is *ndh* an essential gene when mycobacteria could also use *nuo*? The fact that *ndh* is essential implies that mycobacteria do not have another mechanism to recycle NADH during normal aerobic growth. Alternatively, this is the only NADH dehydrogenase that is operating under these growth conditions and the activity of this enzyme is essential for maintaining an energized membrane. Compounds that target NDH-2 are bactericidal towards hypoxic non-replicating *M. tuberculosis* suggesting that the respiratory chain is essential for the recycling of NADH under these conditions (6).

Multiple succinate dehydrogenases and fumarate reductase: an essential link between central metabolism and respiration in mycobacteria

Succinate dehydrogenase forms complex II of the respiratory chain and couples oxidative phosphorylation to central carbon metabolism by being an integral part of the TCA cycle (Figure 2). This enzyme catalyzes the oxidation of succinate to fumarate wherein two electrons are transferred to quinol. The reverse reaction can be catalyzed by fumarate reductase, which is involved in anaerobic respiration (Figure 3). Fumarate reductase and succinate dehydrogenase are closely related enzymes and the reaction catalyzed cannot be predicted based on the primary sequence alone. Most mycobacteria harbour two annotated succinate dehydrogenases, SDH1 and SDH2 (Table 1). SDH2 has high homology to SDH enzymes from other species and is encoded by four genes *sdhC, sdhD, sdhA* and *sdhB.* The genes *sdhA* and *sdhB* encode for the cytoplasmic part of the enzyme where the succinate to fumarate reaction takes place (SdhA) and electrons are shuttled via three iron-sulfur clusters (SdhB) to the membrane subunits SdhC and SdhD, which catalyze the electron transfer to menaquinone. SdhA (encoded by *Rv0248c*) and SdhB (*Rv0247c*) subunits of SDH1 are similar to the common SDH enzyme. However, the other two genes (*Rv0250c* and *Rv0249c*) in the operon show no homology to membrane-bound components of SDH and fumarate dehydrogenase FRD and are specific to the phylum Actinobacteria. Gene expression data show that all four genes of SDH1 in *M. smegmatis* are expressed in concert (16). As an exception among mycobacteria, *M. tuberculosis* encodes a third complex (*frdABCD*) that is annotated as a fumarate reductase (Table 1) and is absent in all other pathogenic strains like *Mycobacterium avium paratuberculosis, Mycobacterium marinum, Mycobacterium ulcerans and M. leprae*. Succinate dehydrogenase activity has been measured in *M. tuberculosis* as well as many other mycobacterial species (37, 38), but it is still unclear which of the three enzymes or if all are responsible for this activity.

Mycobacteria utilize menaquinone/menaquinol (MQ/MQH_2) as a conduit between electron-donating and -accepting reactions (Figure 1). Menaquinone has a lower midpoint redox potential (E_m = −74 mV) compared to ubiquinone (E_m = +113 mV) and is ideally poised to donate electrons to fumarate during anaerobic conditions (39). This means that the SDH reaction in mycobacteria (succinate oxidation to fumarate) should have an unfavourable free energy due to reverse electron flow and proton uptake to drive this reaction. It is tempting to propose that the unusual subunits of SDH1 could be the result of structural specializations in the transmembrane region to facilitate reverse electron flow from succinate to MQ. In fact,

SDH1 and SDH2 have been shown to be differentially expressed under energy- or oxygen-limiting conditions in *M. smegmatis* (16). Under energy-limiting conditions SDH1 was upregulated 4-fold while SDH2 was downregulated 3-fold. Under oxygen-limiting conditions, SDH1 was downregulated 30-fold while SDH2 was upregulated 2-fold. This indicates that SDH1 could be the dedicated succinate dehydrogenase and SDH2 catalyses fumarate reductase activity, which is important for survival under hypoxia. This hypothesis is supported by several reports in the recent literature. A recent transposon-site hybridization (TraSH) screen in *M. tuberculosis* suggested that SDH1 but not SDH2 was essential for growth under aerobic conditions on standard medium (26). On the contrary, in a TraSH screen selecting for mutants that continue to replicate under hypoxic conditions Baek and coworkers (40) found SDH2 mutants overrepresented. These results suggest that SDH2 has a pivotal role in the transition of *M. tuberculosis* from aerobic to hypoxic conditions and supports the hypothesis of it being a fumarate reductase. In fact, it has been proposed that fumarate may be an important endogenous electron acceptor for energy production and maintenance of redox balance (oxidation of NADH to NAD^+) in hypoxic non-replicating mycobacteria (6). Interestingly, the use of fumarate as an electron acceptor in *E. coli* requires complex I, and expression of the *nuo* operon is stimulated by the presence of fumarate (41). This stands in direct contrast to *M. tuberculosis*, where the *nuo* operon seems to be silent under anaerobic conditions (18). In a recent study it was shown by ^{13}C flux analysis that *M. tuberculosis* grown under hypoxia metabolizes glucose through a reverse TCA cycle to generate succinate as an excreted fermentation end product (42). However the metabolic flux from fumarate to succinate was unchanged in an *M. tuberculosis* FRD deletion mutant, suggesting that one of the remaining putative succinate dehydrogenases (most likely SDH2) could catalyze this reaction. A more recent study suggests that the glyoxylate shunt, and not the reverse TCA, is used to metabolize both glycolytic and fatty acid carbon sources in response to oxygen limitation and this route also produces succinate as its metabolic end product (43). The authors propose that during oxygen limitation large amounts of succinate are produced by this pathway that are used to sustain the membrane potential, ATP synthesis and TCA cycle precursors akin to a “metabolic battery” (43). Moreover, because of the near neutral mid-point potential of succinate/fumarate redox couple (+30 mV), succinate is able to bridge both oxidative and fermentative metabolic schemes depending on electron acceptor availability (43).

The role of the *frdABCD* operon in the *M. tuberculosis* complex is not clear. Increased expression of this operon has been shown during carbon starvation (20), oxygen depletion (44) and in macrophages (19) suggesting a role for this enzyme in persistence.

Alternative electron donor utilization during starvation and hypoxia

During carbon starvation and slow growth, mycobacteria switch to alternative electron donors (16, 20, 45) (Figure 2). Proline dehydrogenase is upregulated under both energy-limiting conditions and hypoxia (7, 16) and is increasingly being recognized as a critical amino acid in cellular bioenergetics and redox control (46). Proline can be utilized as an electron donor as well as a carbon and nitrogen source (Figure 2). The degradation of proline occurs by means of two enzymes: proline dehydrogenase (PRODH) and pyrroline-5-carboxylate dehydrogenase (P5CDH). These two enzymes catalyze the oxidation of proline

to glutamate with four electrons transferred to the respiratory chain (46). In the first step FAD is reduced to $FADH_2$ while in the second step NAD^+ is reduced to NADH. In some bacteria, PRODH and P5CDH are monofunctional enzymes but in the majority of bacterial species they are fused into one protein called proline utilization A flavoenzyme PutA (47).

In mycobacteria, PRODH and P5CDH are predicted to be monofunctional enzymes (46). The genes encoding PRODH (*putB, Rv1188*) and P5CDH (*putA, rocA, Rv1187*) are expressed as part of an operon (7). It has been shown that *M. smegmatis* can grow on proline as the sole carbon and energy source and also that PRODH is an important electron donor, under both energy-limiting conditions and hypoxia (7, 16). The same authors show that proline metabolism in mycobacteria is regulated by a unique membrane-associated transcriptional regulator called PruC (Rv1186c), encoded upstream of *pruA*. The *pruAB* operon with its upstream regulator *pruC* is highly conserved in mycobacteria with the exception of *M. leprae*. Recent proteomics data on *M. avium paratuberculosis* (MAP) show that protein levels of RocA (PutA) are elevated in the intestinal tissues of cows (48). The authors propose that MAP has adapted to utilize proline as a carbon and nitrogen source due to the high abundance of this amino acid in the plant material that is eaten by such ruminants. Mycobacteria also encode pyrroline-5-carboxylate reductase (encoded by *proC*) that catalyses the reverse reaction of *putA* converting pyrroline-5-carboxylate to proline. Interestingly, a *proC* mutant of *M. tuberculosis* was avirulent in immunocompetent mice, however this was not due to its inability to proliferate intracellulary because bacterial loads increased in the mouse lungs after 20 days post infection (49).

Hydrogenases catalyze the reversible oxidation of molecular hydrogen: $2H^+ + 2e^- \rightarrow H_2$ and play a central role in energy metabolism of bacteria, archaea and eukarya (50). Under physiological conditions, hydrogenases couple H_2 oxidation to respiration (Knallgas reaction) or reduce protons as a way to dispose of surplus reducing equivalents. Four different types of hydrogenases are found in mycobacteria and are annotated to be of the NiFe type. *M. smegmatis* harbours three (designated Hyd1, 2 and 3) of the four hydrogenase complexes (16), Hyd1 aligns closely with Group 2a uptake hydrogenases of the cyanobacteria such as *Nostoc*, indicating it oxidises H_2 (51). In contrast, Hyd3 is closely related to the Group 3 cytoplasmic bidirectional hydrogenases. Hyd2 appears to be a founding member of the Group 5 high-affinity hydrogenases (52, 53). Hyd1, Hyd2 and Hyd3 are all soluble hydrogenases and found in mycobacteria of the slow-growing and fast-growing type, as well as pathogenic and non-pathogenic mycobacteria (16). However, the fourth putative hydrogenase is only found in pathogenic mycobacteria (including *M. tuberculosis* complex) and seems to be restricted to slow-growers (Table 1). It shows homology to Group 4 membrane-bound H_2 evolving hydrogenases. It has been shown that *M. smegmatis*, among other mycobacterial species, can oxidize molecular hydrogen in the presence of carbon monoxide (CO), implying that *M. smegmatis* expresses a functional hydrogenase (54). To date no studies have reported on the ability of mycobacteria to produce hydrogen. Gene expression studies suggest that Hyd1 and Hyd2 are used during nutrient starvation as an alternative electron source (Figure 2) while Hyd3 and the membrane-bound hydrogenase are more likely to have a function in disposing electrons under anaerobic conditions (16) (Figure 3). A knockout mutant of Hyd2 in *M. smegmatis* showed reduced biomass production when grown on complex medium under atmospheric

conditions (16) and its homolog in *Streptomyces* sp. was shown to facilitate hydrogen oxidation (53). These data suggest that Hyd2 oxidizes hydrogen at very low concentrations, which fits with its purported role as a high-affinity hydrogenase (52).

Carbon monoxide dehydrogenase (CO-DH) is responsible for the oxidation of CO to carbon dioxide (CO_2) in carboxydobacteria, which grow on CO as a sole source of carbon and energy (55). Carboxydobacteria catalyze the oxidation of CO to CO_2 by the following reaction: $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$. Several pathogenic and nonpathogenic mycobacteria including *M. tuberculosis* are known to possess CO-DH genes. It has been shown that *M. tuberculosis* H37Ra, which possesses CO-DH activity, can grow on CO as a sole source of carbon and fuel for energy generation (56) (Figure 2).

Glycerol-3-phosphate dehydrogenase catalyzes the oxidation of glycerol-3-phosphate to dihydroxy-acetone phosphate and reduces either quinone or NADP (57). In *E. coli*, glycerol 3-phosphate is either used as precursor in the biosynthesis of phospholipids or as a carbon source for energy supply (58). *M. tuberculosis* possesses genes for four predicted glycerol-3-phosphate dehydrogenases, but their role and function in mycobacterial respiration remain unknown (Table 1).

The membrane-associated malate quinone oxidoreductase (MQO) oxidizes malate to oxaloacetate and transfers the reducing equivalents to menaquinone (MK) (59, 60) (Figure 3). *M. tuberculosis* harbors a copy of MQO and a cytoplasmic NAD^+-dependent malate dehydrogenase (MDH) (61, 62). The function and role of MQO in mycobacterial respiration is unknown. *M. smegmatis* lacks an MDH homologue and it was shown that MQO responded to low oxygen concentration with 4-fold increase in gene expression (16). Mutants of *Corynebacterium glutamicum* defective in NDH-2 activity are able to grow despite the loss of all membrane-bound NADH dehydrogenase activity (59). The authors propose a reaction scheme whereby electron transfer from NADH to MK is catalyzed by the sequential action of malate dehydrogenase (MDH) and malate:quinone oxidoreductase (MQO) (membrane-bound). MDH can reduce oxaloacetate with NADH to malate, and then malate is reoxidized to oxaloacetate by MQO using MK as an electron acceptor (59, 60). Support for this model comes from the observation that a Δ*mqo/ndh* double mutant failed to grow under conditions where the Δ*ndh* mutant grew. Furthermore, *M. smegmatis ndh* mutants could be complemented by *M. bovis* BCG *mdh* suggesting that such a reaction scheme might operate in mycobacteria (64).

Terminal Electron Acceptor Utilization

During aerobic respiration, energy is conserved by the generation of a PMF across a proton-impermeable membrane. The electron transport chain components are membrane-bound and asymmetrically arranged across the membrane to achieve net consumption of protons from the cytoplasm and net release of protons on the outside of the cell. An important part of all electron transport chains are the terminal respiratory oxidases. In order for mycobacteria to utilize oxygen efficiently and obtain the maximum growth yield on a particular carbon and energy source, there must be co-ordinate regulation of terminal respiratory oxidase expression/activity. For example, in *E. coli*, cytochrome *bo* (K_m for oxygen in the

micromolar range) and cytochrome *bd* (K_m for oxygen in the nanomolar range) (63, 64) are coordinately regulated by the ArcBA system and transcriptional regulator FNR (65). Cytochrome *bo* is synthesized at high oxygen tension (optimal between 15 to 100% air saturation) and repressed as the oxygen concentration decreases (66). This coincides with the induction of cytochrome *bd* at 7% air saturation, which is turned off (FNR-mediated repression) once the cells enter anaerobiosis (65, 66). Mycobacteria adopt regulation of oxidase expression to match oxygen supply. Under conditions of low oxygen tension (ca. 1 % air saturation), cytochrome *bd* is induced in *M. smegmatis* as the transition to anaerobiosis is approached (67), a value that is 10-fold lower than that observed in *E. coli* (ca. 10% air saturation). In *M. tuberculosis*, cytochrome *bd* is upregulated in the early stages of NRP-1 (i.e. decreasing oxygen) (68), in NRP-2 (18) and in response to nitric oxide (NO) (18). Intriguingly mRNA levels of *cydA* increased transiently about 7-fold after 30 days in mouse lungs (18). Mycobacteria appear to adopt a strategy whereby a down-regulation or a slowing of metabolism occurs as cells enter NRP-1 and NRP-2. Transcriptional analysis of *M. tuberculosis* in the macrophage (phagosomal environment) has revealed that NDH-1, menaquinol-cytochrome *c* oxidoreductase and the ATP synthase are all down-regulated when compared to cells growing exponentially, suggesting that there is a reduced need for energy generation during bacteriostasis i.e., the growth state of intraphagosomal *M. tuberculosis* (19). Consistent with these observations is the repression of these operons during starvation. In contrast, fumarate reductase, nitrate reductase and NDH-2 are all upregulated under these conditions (19). Whilst these proteins do not appear to contribute to increased energy conservation, it has been suggested that they may play a pivotal role in the recycling of NAD^+ as a result of β-oxidation of fatty acids (19).

Complex III and IV of the M. tuberculosis electron transport chain

M. tuberculosis harbours genes for a cytochrome *c* pathway consisting of a menaquinol-cytochrome c oxidoreductase termed the bc_1 complex (encoded by the *qcrCAB* operon, complex III) and an aa_3-type cytochrome c oxidase (encoded by the genes *ctaBCDE*, complex IV) belonging to the heme-copper respiratory oxidase family (69–71) (Table 1). The bc_1 complex (menaquinol-cytochrome c oxidoreductase) consists of redox groups comprising a 2Fe/2S centre, located on a Rieske protein (QcrA); two *b*-type haems (low and high potential) located on a single polypeptide (QcrB); and the haem of cytochrome c_1 (QcrC). For every two electrons passing from quinol to cytochrome *c*, complex III releases four protons into the periplasmic side of the membrane ($4H^+/2e^-$) (Figure 2). The iron sulfur subunit QcrA has three transmembrane helices and sequence motifs characteristic of 2Fe-2S Rieske iron-sulfur proteins (viz. CSHLGC and CPCH). In contrast to bovine heart cytochrome *b*, QcrB from *M. tuberculosis* has a 120 amino acid extension at the C-terminus as noted for *C. glutamicum* and *Streptomyces* species (72). The QcrB subunit has been recently shown to be the target of novel imidazo[1,2-*a*]pyridine inhibitors that are active against *M. tuberculosis* at low micromolar concentrations (73). As first reported by Niebisch and Bott (72) the QcrC subunit (cytochrome c_1) harbours two haem-binding signatures (CXXCH motifs) for *c*-type cytochromes (viz. CVSCH and CASCH) suggesting a covalent di-haem (*bcc*) configuration. Mycobacterial genomes do not appear to harbor genes for either a soluble or membrane-bound cytochrome *c* suggesting the *bcc* complex interacts

directly with the *aa*$_3$-type cytochrome *c* oxidase to achieve electron transfer and function as a supercomplex. Niebisch and Bott (72, 74) suggest a supercomplex mechanism for *C. glutamicum* in which the haem group of QcrC takes over the function of a separate cytochrome *c* in electron transfer to cytochrome *aa*$_3$ oxidase forging a close contact relationship between cytochrome *c*$_1$ and the Cu_A site in the subunit II of cytochrome *aa*$_3$ oxidase. Supercomplexes between *bc*$_1$ and *aa*$_3$ have been reported for *C. glutamicum* (72), *Paracoccus denitrificans* (75) and thermophilic strain PS3 (76). A functional association between *bcc* and *aa*$_3$ has been shown for the complex in *M. smegmatis* family (71).

The *aa*$_3$-type cytochrome *c* oxidase in *M. tuberculosis* appears to be encoded by four genes *ctaBCDE* (Table 1). The *ctaE* gene is located immediately upstream of the *qcrCAB* operon, but the other three genes are located in three different locations on the chromosome and in some cases appear to be in operons with other genes (Table 1). The four subunits of the *M. tuberculosis aa*$_3$-type cytochrome *c* oxidase include: CtaB (a cytochrome c oxidase assembly factor); the catalytic subunits CtaD (cytochrome *c* oxidase subunit I containing haem *a, a*$_3$, and Cu_B) and CtaC (cytochrome *c* oxidase, subunit II location of copper A (Cu_A), and CtaE (subunit III). For every two electrons passing through complex IV, four protons are taken up from the cytoplasm and two are released into the periplasm. The oxidase acts as a proton pump with a stoichiometry of $2H^+/2e^-$ (Figure 2).

The *bc*$_1$*-aa*$_3$ pathway appears to be the major respiratory route in mycobacteria under standard aerobic culturing conditions (70). Matsoso et al. (70) have demonstrated that disruption of this pathway in *M. smegmatis* is accompanied by a constitutive upregulation of the cytochrome *bd*-type menaquinol oxidase. In *M. tuberculosis*, the *bc*$_1$*-aa*$_3$ pathway is essential for growth suggesting an inability of this bacterium to adapt in a manner analogous to *M. smegmatis* (70). The *aa*$_3$ branch is proposed to contain three *ctaD* alleles in *M. smegmatis* versus the one in *M. tuberculosis*, suggesting the existence of alternate isoforms of cytochrome *c* oxidase in *M. smegmatis* (67).

Cytochrome bd-type oxidase

M. tuberculosis and other mycobacterial species harbour genes for the cytochrome *bd*-type menaquinol oxidase (*cydAB*) (67) (Table 1). The cytochrome *bd* branch is bioenergetically less efficient (non-proton translocating) and is synthesized at low oxygen tensions in mycobacteria (67). In addition to cytochrome *bd*, the *M. smegmatis* respiratory chain has been proposed to contain a third possible respiratory branch terminating in the YthAB (*bd*-type) menaquinol oxidase (67). The existence of two cytochrome *bd*-type oxidases (I and II) is not unprecedented in bacteria (77), and recent work had reported that cytochrome *bd*-II in *E. coli* is able to generate a PMF with a H^+/e^- ratio of 1.0 (78). Cytochrome *bd* mutants of *E. coli* have been shown to have a pleiotropic phenotype: they are sensitive to H_2O_2nitric oxide, temperature, and iron (III) chelators; and are unable to exit from stationary phase and resume aerobic growth at 37°C (77). A *cydA* mutant of *M. smegmatis* has been generated, but the phenotypes are very subtle compared to *E. coli*. The *M. smegmatis cydA* mutant showed a reduced growth rate at 0.5–1 % air saturation and also a 10-fold difference in CFU after 140 h of growth in the presence of cyanide at 21% air saturation when co-cultured with wild-type *M. smegmatis* (67).

The *cydAB* genes appear to be in an operon with *cydDC* in mycobacterial species (Table 1), an arrangement similar to that found in *Bacillus*. In *E. coli, cydAB* and *cydDC* form two discrete operons and c*ydDC* mutants are defective in cytochrome *bd* assembly and the periplasmic space is more oxidized in the mutant versus wild-type (79). In *E. coli*, the CydDC protein (ABC transporter) has been reported to pump glutathione and cysteine into the periplasm to maintain redox homeostasis (80). The role of the *cydDC* genes in mycobacteria is unknown, however, evidence exists that CydDC plays a role during mycobacterial persistance *in vivo*. A *cydC* mutant of *M. tuberculosis* showed reduced ability to survive the transition from acute to chronic infection in mice (18) and Dhar and McKinney have reported that CydC plays a role in mycobacterial persistence in isoniazid-treated mice (81).

Electron acceptor utilization under hypoxia

In the absence of oxygen, alternative electron acceptors (e.g. nitrate and fumarate) are available for mycobacterial metabolism, but none of these electron acceptors have been shown to support growth of mycobacterial species. These potential electron acceptors may play an important role in the disposal of reducing equivalents in the absence of oxygen.

NO is generated in large amounts within the macrophages and restricts the growth of *M. tuberculosis.* Nitrate can be produced by oxidation of NO and is an alternative source of nitrogen for bacteria within the human host. Early work in *E. coli* had suggested that *narK* was involved only in nitrite export (82), and so the homologous *narK2* in *M. tuberculosis* was annotated as a “nitrite extrusion protein”. More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (83, 84). In *M. tuberculosis*, four genes, *narK1* through *narK3* and *narU* are homologous to *narK* and *narU* (Table 1). Since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must be exported out of the cell. The *M. tuberculosis narK2* was shown to complement this *E. coli* double mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell (85). Nitrate reduction by *M. tuberculosis* is regulated by control of nitrate transport into the cell by NarK2. It is proposed that NarK2 senses the redox state of the cell, possibly by monitoring the flow of electrons to cytochrome oxidase, and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing conditions (86). Inhibition of nitrate transport by oxygen has been documented in other bacteria (87). It is intriguing that *M. tuberculosis*, classified as an obligate aerobe, should have such intricate control of an anaerobic enzyme system. Transcription of *narK2* is controlled by DosR/DevR, which responds to O_2NO, and CO (88–90). Both the transcription of the *narK2* gene and the activity of NarK2 are controlled by similar signals (86).

M. tuberculosis contains genes (*narGHJI, Rv1161–1164*) that code for a putative membrane-bound molybdenum-containing nitrate reductase complex similar to the corresponding *narGHJI* operon of *E. coli* (Table 1). Moreover, the *narGHJI* operon of *M. tuberculosis* is able to functionally complement a *nar* mutant of *E. coli* to grow on glycerol and reduce nitrate anaerobically (85). Importantly however, the expression of the *narGHJI* operon in *M. tuberculosis* is not upregulated in response to either hypoxia or stationary phase (85).

Sohaskey and Wayne demonstrate that overexpression of recombinant *M. tuberculosis* nitrate reductase in either *M. tuberculosis* or *M. smegmatis* (low nitrate reductase activity) does not confer the ability of these cells to grow anaerobically i.e. no growth of either species is observed with nitrate anaerobically even though the nitrate reductase activity of whole cells increases (85). The genome of *M. tuberculosis* also lacks orthologs of transcription regulator FNR which, in combination with NarL, are responsible for the transcriptional activation of the *narGHJI* operon by anaerobic conditions in *E. coli* (21). A putative NarL (Rv0884c) has been indentifed in *M. tuberculosis* but the promoter of the *narGHJI* lacks consensus-like binding sites for this regulatory protein. Based on these observations, it is apparent that this enzyme does not support anaerobic growth of mycobacteria and therefore the role of this enzyme in the physiology of mycobacteria is unclear. Given the proposed membrane-bound location of the enzyme and the proton-translocating activity *via* a redox loop of the *E. coli* enzyme, perhaps the primary role of the mycobacterial enzyme is to generate a PMF when the concentration of oxygen is low, and hence its activity increases but not its expression.

An alternative role for nitrate reductase may be maintaining the redox balance of the cell during conditions of hypoxia. Sohaskey (91) has reported that exogenously supplied nitrate has no effect on long-term persistence during gradual oxygen depletion, but played an important role during rapid adaptation to hypoxia (< 18 h). This effect required a functional nitrate reductase, suggesting that nitrate reduction may play a role in protecting cells during sudden changes in oxygen concentration leading to disruption of aerobic respiration. The same author (86) has proposed a role for NarK2 in sensing the redox state of the cell such that nitrate is transported into the cell under reducing, but not oxidizing conditions. Lastly, Tan et al. (92) propose a role for nitrate reductase in protecting *M. tuberculosis* from mild acid challenge under hypoxic conditions.

M. tuberculosis is unable to reduce nitrite and the genome was proposed to harbor genes for a putative ferredoxin-dependent nitrite reductase (NirA) and a NAD(P)H nitrite reductase (NirBD). However, a subsequent study has reported that the *nirA* gene is misannotated and is in fact *sirA*, a sulfite reductase shown to be essential for growth on sulfate or sulfite as the sole sulfur source (93) (Table 1). The *nirBD* genes are operonic and non-essential for growth, and unlike the *narGHJI* genes, no role in resistance to acid stress under hypoxia was noted for NirBD (92). These data support the proposal that nitrite produced by nitrate reductase is not converted to ammonium, but instead is exported from the cell by NarK2.

The evolution of H_2 is a mechanism commonly employed by anaerobic bacteria to recycle reducing equivalents obtained from anaerobic degradation of organic substrates. However, even strictly aerobic bacteria have been shown to produce H_2 under anaerobic conditions (94). In the family of *Mycobacteriaceae,* two potential hydrogen-evolving hydrogenases are present. One belongs to Group 2 cytoplasmic bidirectional hydrogenases and can be found in some slow-growing (e.g. *M. marinum* and *M. kansasii*) and fast-growing (e.g. *M. smegmatis*) mycobacteria. In *M. smegmatis,* this enzyme is termed Hyd3 and has been shown to respond strictly to oxygen-limiting conditions with up to 50-fold increase in gene expression (16). The second type is a yet uncharacterized enzyme that shows homology to membrane-bound Group 4 hydrogenases. This putative formate-hydrogen lyase is enocoded

in *M. tuberculosis* (viz. Rv0082 to Rv0087) and is upregulated during infection of human macrophage-like THP-1 cells (95). Moreover, the transcription of the early genes of the *hycP/hycQ* containing operon was shown to be upregulated during anaerobic adaptation in several studies (44, 68, 90, 96, 97). This gene cluster shows homology to components of hydrogenase 4 and 3 complexes of *E. coli*, the latter of which has been shown to catalyze hydrogen evolution at acidic pH (98). Upon closer inspection, the predicted [NiFe]-hydrogenase of *M. tuberculosis* appears to form part of an Ehr (Ech hydrogenase-related) complex (99, 100). Ehr enzymes lack the cysteine residues needed to ligate a [NiFe] center, which is the catalytic center of hydrogenases. Additionally, no maturation factors to synthesize these complex centers are present in *M. tuberculosis*. Recently, it was shown that the operon Rv0081-Rv0087 is positively regulated by the two two-component signal transduction systems DosRS-DosT and MprAB and negatively regulated by Rv0081 a member of the ArsR/SmtB family of metal-dependent transcriptional regulators (101). DosRS-DosT is a redox sensing regulatory system that is important during the adaptation to hypoxic conditions. No function has yet been proposed for Ehr enzymes, but its homology to hydrogenases and to proton-pumping type I NADH dehydrogenases points to a role in energy metabolism.

ATP Synthesis by the F_1F_o ATP Synthase

In *M. tuberculosis* and other mycobacterial species, ATP is synthesized via substrate level phosphorylation and oxidative phosphorylation using the membrane-bound F_1F_o-ATP synthases (encoded by the *atpBEFHAGDC* operon, Rv1304–1311) (Table 1). In *M. tuberculosis*, the *atp* operon is downregulated during growth in macrophages (19), the mouse lung and in cells exposed to NO or hypoxia (18). The *atp* operon of *M. bovis* BCG and *M. smegmatis* is downregulated in response to slow growth rate (16, 45). When slow growing cells of *M. smegmatis* (70 h doubling time), with low levels of *atp* operon expression, are exposed to hypoxia (0.6% oxygen saturation), the *atp* operon is upregulated 3-fold suggesting an important role for this enzyme during adaptation to hypoxia (16).

The F_1F_o-ATP synthase catalyzes ATP synthesis by utilizing the electrochemical gradient of protons to generate ATP from ADP and inorganic phosphate (P_i) and operates under conditions of a high PMF and low intracellular ATP. The enzyme is also capable of working as an ATPase under conditions of high intracellular ATP and an overall low PMF (102). As an ATPase, the enzyme hydrolyzes ATP, while pumping protons from the cytoplasm to the outside of the cell. The ATP synthase of mycobacteria has been studied in detail at a biochemical level in *M. phlei* and shown to exhibit latent ATPase activity (10). ATPase activity could be activated by trypsin treatment and magnesium ions, but the mechanism of activation was not elucidated. Recent experiments with inverted membrane vesicles of *M. bovis* BCG and *M. smegmatis* demonstrate latent ATPase activity that could be activated by methanol and the PMF, suggesting regulation by the epsilon subunit and ADP inhibition (9). The reason for the extreme latency in ATP hydrolysis of the mycobacterial ATP synthase is unknown, but may represent an adaptation to function at low PMF and under hypoxia. Hypoxic non-replicating cells of *M. tuberculosis* generate a PMF in the order of −100 mV and the ATP synthase inhibitor TMC207 is bactericidal towards these cells demonstrating that the ATP synthase still continues to function at relatively low PMF (6).

The F_1F_o-ATP synthase in *M. tuberculosis* and *M. smegmatis* has been shown to be essential for optimal growth (29, 103). In other bacteria, the F_1F_o-ATP synthase is dispensable for growth on fermentable carbon sources (104, 105), where increased glycolytic flux can compensate for the loss of oxidative phosphorylation. This strategy does not appear to be exploited by *M. smegmatis*: the F_1F_o-ATP synthase is essential for growth even on fermentable substrates, suggesting that ATP production from substrate level phosphorylation alone, despite increased glycolytic flux, may be insufficient to sustain growth of these bacteria (103). This may be due to an extraordinarily high value for the amount of ATP required to synthesize a mycobacterial cell, a possibility that requires further investigation (106). Alternatively, in conjunction with a high ATP demand for growth, the ATP synthase may be an obligatory requirement for the oxidation of NADH by providing a sink for translocated protons during NADH oxidation coupled to oxygen reduction (103). Such strict coupling would imply that mycobacteria do not support uncoupled respiration; either they lack a conduit for proton re-entry in the absence of the F_1F_o-ATP synthase or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. In this context, the cytoplasmic membrane of *M. smegmatis* has been shown to be extremely impermeable to protons (107). The ATP synthase of the close mycobacterial phylogenetic relative *C. glutamicum* is non-essential for growth on fermentable carbon sources and Δ*atp* mutants of this bacterium show enhanced rates of glucose uptake, oxygen consumption and excretion of pyruvate into the growth medium, suggesting that substrate level phosphorylation alone can sustain growth of this bacterium (108).

Several new anti-tubercular compounds have been reported that target oxidative phosphorylation in mycobacteria (12, 109, 110). The most promising compounds clinically, the diarylquinolines, have been shown to target the F_1F_o-ATP synthase and inhibit ATP synthesis (109, 111, 112). Genome sequencing of both *M. tuberculosis* and *M. smegmatis* mutants that are resistant to diarylquinolines (i.e. TMC207) revealed that the target of these compounds is the oligomeric *c* ring (encoded by *atpE*) of the enzyme (109, 113, 114). The purified *c* ring from *M. smegmatis* binds TMC207 with a K_D of 500 nm, and modelling/docking and kinetic studies suggest that TMC207 blocks rotary movement of the c-ring during catalysis by mimicking key residues in the proton transfer chain (115, 116). Further investigations with inverted membrane vesicles of *M. smegmatis* and TMC207 have revealed that TMC207 acts independently of the PMF, and that electrostatic forces play an important role in the interaction of the drug with the ATP synthase (116).

When mycobacterial cells (growing or non-growing) are treated with TMC207, time-dependent (not dose-dependent) killing is observed (109). The mechanism of killing is not clear, but does not involve the dissipation of the membrane potential, which is lethal to all living cells. A dose-dependent decrease in intracellular ATP has been observed when *M. tuberculosis* cells are treated with TMC207 (111, 112), but these data do not explain cell death because mycobacterial cells can be depleted of ATP and yet remain viable (117). TMC207 is bactericidal towards most species of mycobacteria (109), but is only bacteriostatic against *M. avium* (118) and *M. smegmatis* (Berney and Cook, unpublished data). Even when *M. smegmatis* is grown at a doubling time of 70 h in glycerol-limited continuous culture, TMC207 was bacteriostatic (Berney and Cook, unpublished data). The

identification of the mechanisms underlying this sensitivity will be important in understanding how TMC207 exerts its inhibitory effects on mycobacterial cells.

Control of electron transport chain expression and oxidative phosphorylation

Mycobacteria are obligate aerobes and as such have to possess mechanisms to detect the ambient oxygen tension to enable them to adapt to changes in oxygen availability by adjusting their metabolism accordingly. Furthermore, gradual depletion of oxygen has been implicated in entry of *M. tuberculosis* into non-replicating persistence and latency (119). The mechanisms by which mycobacteria sense oxygen have been extensively studied, and the two major sensory systems known to date are the DosT/DosS/DosR system and WhiB3.

The dormancy survival, or Dos, system consists of the three proteins DosR (or DevR), DosS (or DevS) and DosT. DosR has been shown to act as a transcriptional regulator, which is responsible for the induction of nearly all hypoxia-induced genes of *M. tuberculosis* (96). In addition to hypoxia, the Dos-regulon has also be shown to be activated by exposure of *M. tuberculosis* to NO or CO (88, 90, 120).

WhiB3 has been implicated in sensing of oxygen and redox state by mycobacteria. Singh and colleagues report that WhiB3 contains a 4Fe-4S cluster, which binds NO (121). Furthermore, in the presence of oxygen, the WhiB3 $[4Fe\text{-}4S]^{2+}$ cluster is degraded first to a $[3Fe\text{-}4S]^{+}$ cluster, then a $[2Fe\text{-}2S]^{2+}$ and subsequently lost altogether, in a mechanism reminiscent of the one found in the *E. coli* oxygen sensor FNR (121, 122). Apo-WhiB3 was shown to have protein disulfide reductase activity, and it has been proposed that loss of the Fe-S cluster is required to gain this activity (123). WhiB3-mediated response to the presence of oxygen therefore may occur through direct control of the activity of metabolic proteins or through modification of transcriptional regulators (123). A role of WhiB3 in regulation of the transcriptional machinery in mycobacteria may be supported by the finding that WhiB3 interacts with the major sigma factor, SigA (RpoV) (124), but the effect of this interaction on SigA activity is not known, and further study is required to understand the precise role of WhiB3 in mediating any adaptation of mycobacteria to changes in oxygen tension.

A striking observation is that neither Dos nor WhiB3 controls any component of the electron transport machinery in *M. tuberculosis*. While the Dos system and WhiB3 are the most studied regarding the perception of oxygen tension by mycobacteria, they are likely not the only systems used by these bacteria. For example, DosR of *M. tuberculosis* H37Rv was shown not to be strictly required for survival of hypoxia *in vitro* (125). The authors further found that expression of Dos regulon genes in response to hypoxia was transient, with expression of about half of the 50 DosR-dependent genes returning to baseline levels within 24 h of hypoxia. In contrast, a set of 230 genes was significantly up-regulated at four and seven days of hypoxia, but not initially, and were termed the enduring hypoxic response (EHR) (125). Induction of EHR was independent of DosR, suggesting that other sensory and regulatory mechanisms must exist to signal prolonged exposure to hypoxia. Strikingly, the EHR genes contained an unusually high number of regulatory genes (FurA, FurB, PhoP, three WhiB family members and two ECF sigma factors, SigH and SigE), but it is not yet

known which of these, if any, are involved in entry into EHR (125). A study into the effect of addition of cAMP to growing cultures of *M. bovis* BCG or *M. tuberculosis* H37Rv found that the number of genes affected by cAMP was larger under low oxygen, CO_2-enriched conditions than under ambient air (126). The authors proposed that cAMP may be used by mycobacteria as a signaling molecule in response to hypoxic conditions. However, the signal leading to cAMP synthesis or which adenylyl cyclase might catalyse this reaction under hypoxia remains unknown. The regulator of *cydABDC* expression in mycobacteria has not been identified. Several regulators have been implicated in controlling the expression of *cyd* expression i.e. SenX3-RegX3 (127) and cAMP receptor protein (CRP) (128), but the signal transduction pathway or signals sensed remain to be elucidated.

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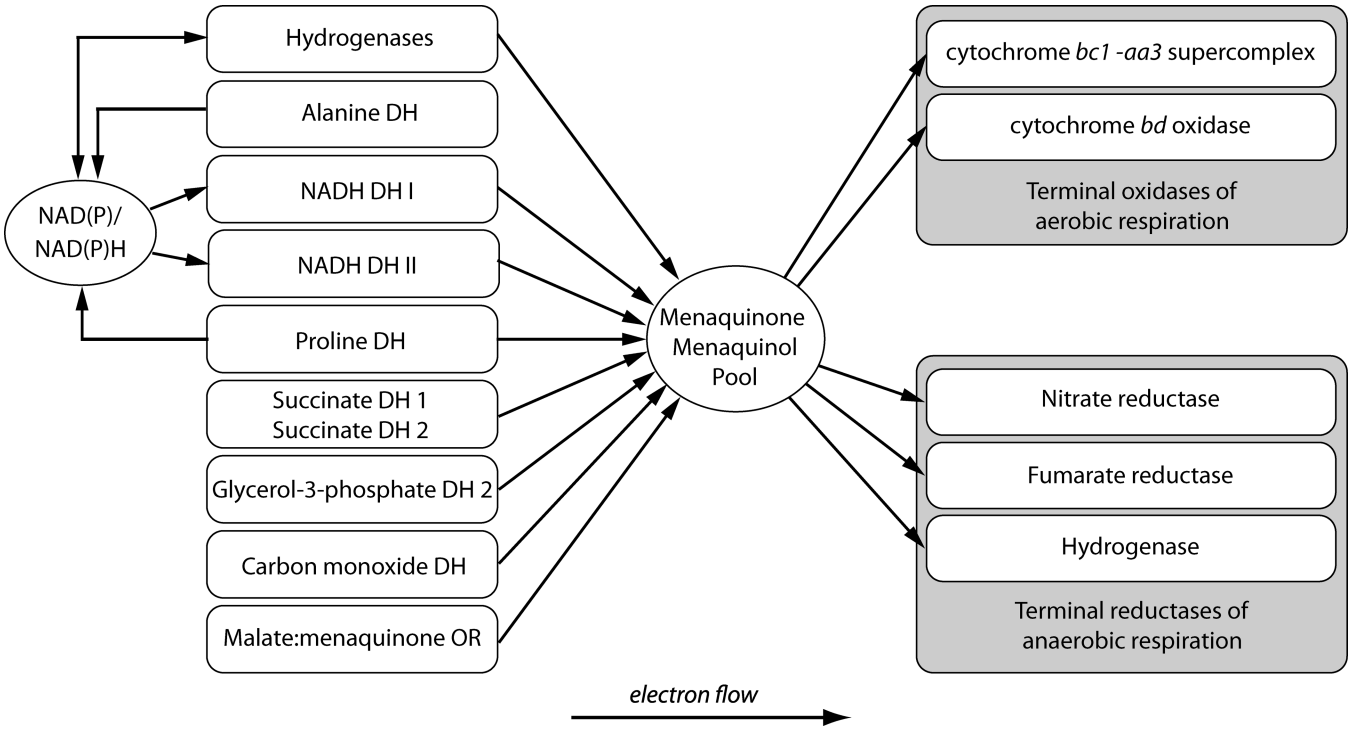

Figure 1.
Organization and components of the electron transport chain in mycobacteria.

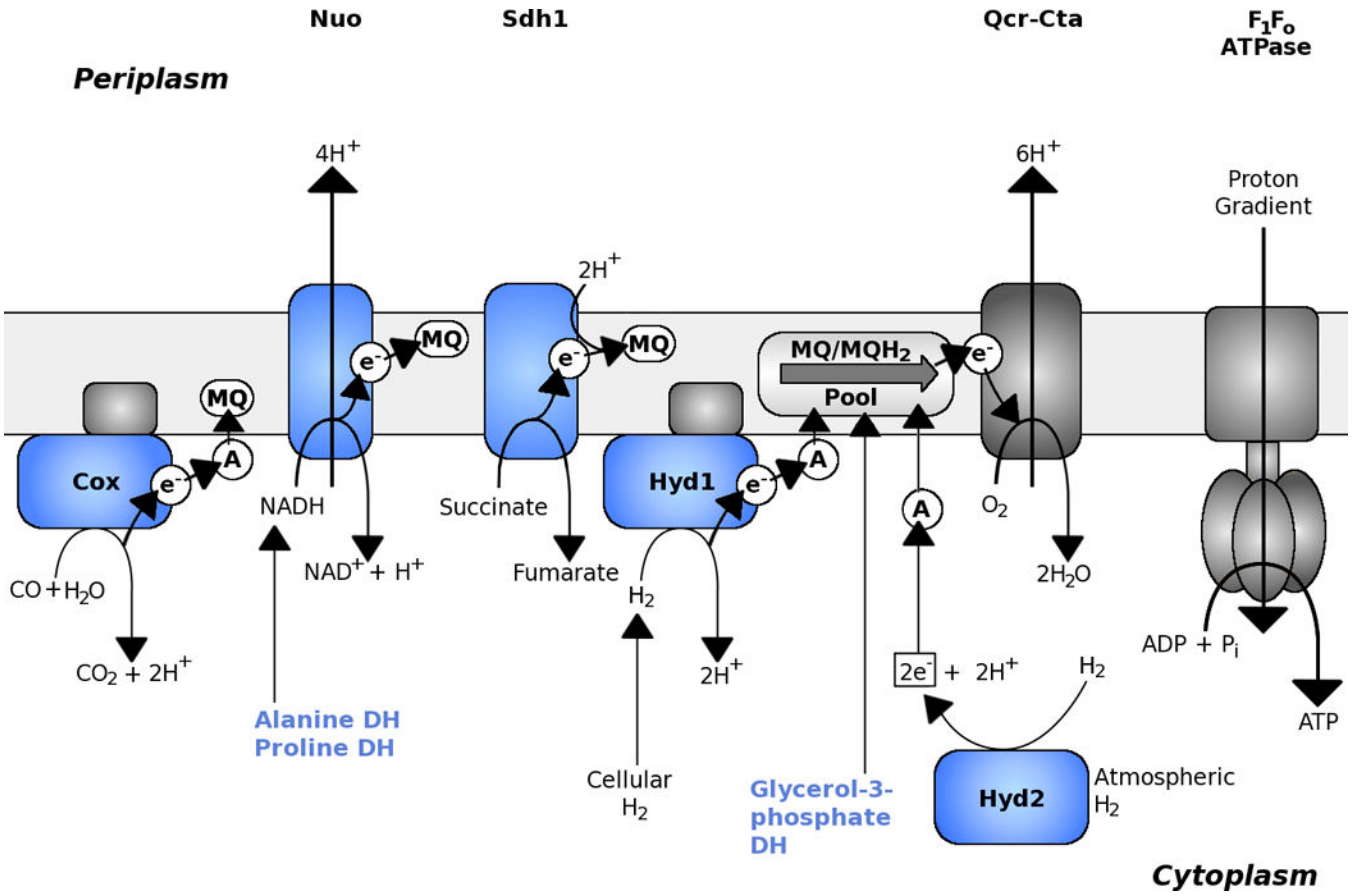

Figure 2.

The core respiratory chain of Mycobacteria and components upregulated under energy-limiting conditions. During *in vitro* exponential growth, Mycobacteria use a classical respiratory chain composed of a Type I NADH:Menaquinone oxidoreductase (Nuo), Succinate:Menaquinone oxidoreductase 1 (SDH1), Cytochrome aa_3-*bc* supercomplex (Qcr-Cta) and F_1F_o ATPase. Menaquinone (MQ) is the only quinone present in mycobacterial membranes and reverse electron transport driven by the proton motive force is proposed to facilitate the function of SDH1 and similar enzymes (see text). Components coloured in light blue are upregulated in response to energy-limiting conditions (6). Catalysis and electron flow are indicated by arrows. Other acronyms: Cox – Carbon monoxide dehydrogenase; Hyd – Hydrogenase; DH – Dehydrogenase; A – Unidentified electron acceptor.

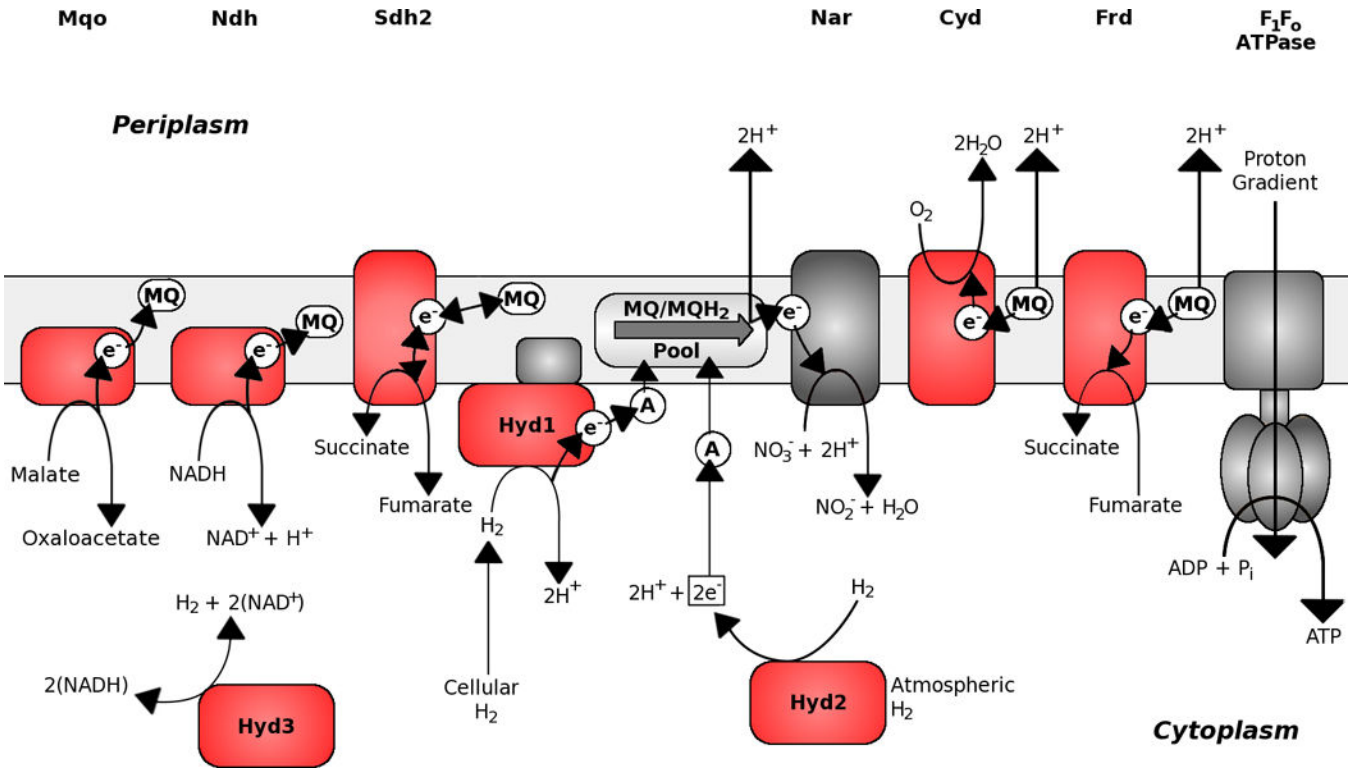

Figure 3.

The preferential respiratory chain of an oxygen-limited mycobacterial cell. Under low oxygen-conditions a diverse response utilizing alternate electron donors and acceptors, energy-conserving enzymes and a high affinity terminal oxidase permit survival under hypoxic conditions. Components coloured in red are upregulated under microaerobic conditions (6). Catalysis and electron flow are indicated by arrows. The possible PMF-driven reverse electron flow of Sdh2 is not shown for clarity. Acronyms: Mqo – Malate:Menaquinone oxidoreductase; Ndh – Type II NADH:Menaquinone oxidoreductase; Sdh2 – Succinate:Menaquinone oxidoreductase 2; Nar – Nitrate reductase; Cyd – Cytochrome *bd* oxidase; Frd – Fumarate reductase; Hyd – Hydrogenase; MQ – Menaquinone; A – Unidentified electron acceptor.

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Table 1

Electron transport chain components and energy generating machinery of mycobacteria

Operon/ Gene[1]	***Subunits***	**Rv #**	**Enzyme Name**	***In vitro* Essentiality**	
				Griffin *et al*[2]	**Zhang *et al*[3]**
nuo	*nuoABCDEFGHIJKLMN*	Rv3145–Rv3158	Type I NADH:Menaquinone oxidoreductase	*nuoDFGHI* = yes	*nuoDFGL* = yes
ndh	-	Rv1854c	Type II NADH:Menaquinone oxidoreductase	yes	no
ndhA	-	Rv0392c	Type II NADH:Menaquinone oxidoreductase	no	no
sdh1	*sdh1CD* *sdh1A* *sdh1B*	Rv0249c Rv0248c Rv0247c	Succinate:Menaquinone oxidoreductase I	yes	Only *sdh1A*
sdh2	*sdh2C* *sdh2D* *sdh2A* *sdh2B*	Rv3316 Rv3317 Rv3318 Rv3319	Succinate:Menaquinone oxidoreductase II	no	no
mqo	-	Rv2852c	Malate:Menaquinone oxidoreductase	no	no
pru	*pruA* *pruB*	Rv1187 Rv1188	Proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase	yes	yes
gpdA1	-	Rv0564c	Glycerol-3-phosphase dehydrogenase A1	no	no
gpdA2	-	Rv2982c	Glycerol-3-phosphase dehydrogenase A2	no	no
glpD1	- -	Rv2249c (Rv2250c)	Glycerol-3-phosphase dehydrogenase D1	no	yes
glpD2	*glpD2* *ldpA*	Rv3302c Rv3303c	Glycerol-3-phosphase dehydrogenase D2 Dihydrolipoamide dehydrogenase	yes	yes
cox	*coxCMSLDEFG*	Rv0376c–Rv0368c	Carbon monoxide dehydrogenase	*coxLEF* = yes	*coxCL* = yes
ald	-	Rv2780	L-Alanine dehydrogenase	no	no
lldD1	*?* *pqqE* *lldD1*	Rv0692 Rv0693 Rv0694	L-Lactate dehydrogenase 1	no	*lldD1* = yes
lldD2	*lldD2* - -	Rv1872c Rv1871c Rv1870c	L-Lactate dehydrogenase 2	no	no
Rv0843	-	(Rv0842) Rv0843	Pyruvate dehydrogenase E1 component alpha subunit	no	no
pdh	*pdhA* *pdhB* *pdhC*	Rv2497c Rv2496c Rv2495c	Pyruvate dehydrogenase	*pdhB* = yes	*pdhC* =yes

Operon/ Gene[1]	*Subunits*	Rv #	Enzyme Name	*In vitro* Essentiality	
				Griffin *et al*[2]	Zhang *et al*[3]
ace	-	Rv2241	Pyruvate dehydrogenase E1 component	yes	yes
lpd	-	Rv0462	Dihydrolipoamide dehydrogenase	yes	yes
hyc	Rv0081 Rv0082 Rv0083 *hycDPQE* Rv0088	Rv0081-Rv0088	Formate hydrogenlyase OR Energy-converting hydrogenase related complex (Ehr) (a.k.a HydTB)	*hycE* and Rv0088 = yes	*hycQE* = yes
qcr	*qcrC* *qcrA* *qcrB*	Rv2194 Rv2195 Rv2196	Cytochrome bc_1	yes	yes
ctaB	-	Rv1451	Cytochrome c oxidase assembly factor	yes	yes
ctaC	*ctaC* -	Rv2200c *(Rv2199c)*	Transmembrane Cytochrome C subunit II	yes	Rv2200c = yes
ctaD	-	Rv3043c	Cytochrome C oxidase polypeptide I	yes	yes
ctaE	-	Rv2193	Cytochrome C oxidase subunit III	yes	yes
cyd	*cydA* *cydB* *cydD* *cydC*	Rv1623c Rv1622c Rv1621c Rv1620c	Cytochrome *bd* oxidase	yes	*cydBC* = yes
nar	*narG* *narH* *narJ* *narI*	Rv1161 Rv1162 Rv1163 Rv1164	Menaquinone:Nitrate reductase	no	no
sirA	*sirA* *cysH* *che1*	Rv2391 Rv2392 Rv2393	Sulfite reductase APS reductase ferrochelatase	yes	Rv2391 and Rv2392 = yes
nirBD	*nirB* *nirD*	Rv0252 Rv0253	Nitrite reductase [NAD(P)H]	no	no
frd	*frdA* *frdB* *frdC* *frdD*	Rv1552 Rv1553 Rv1554 Rv1555	Menaquinone:Fumarate reductase	no	*frdA* = yes
atp	*atpIBEFHAGDC* and Rv1312	Rv1303-Rv1312	F_1F_o ATP synthase operon	yes	yes except *atpI* and Rv1312

[1] In the absence of operons confirmed by previous publications, the TB Database operon prediction algorithms (www.tbdb.org) were used to identify potential operons. Rv loci enclosed in parentheses signify weak operon predictions.

[2] Griffin *et al.* (26)

[3] Zhang *et al.* (129). N.B. Genes identified as containing both essential and non-essential regions (a "D call") are referred to as essential here.