Breathing in thin air: structure, function and therapeutic opportunities of bd type oxidases

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Chapter 1

General introduction

The antibiotic resistance crisis

Antibiotics have been among the most transformative achievements in modern medicine, offering powerful tools to combat a wide range of bacterial infections. However, the growing prevalence of antibiotic resistance represents one of the most urgent challenges to global health. The emergence and spread of multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) bacterial strains have rendered many conventional antibiotics ineffective. Recent projections estimate that the number of annual deaths associated with antibiotic resistant bacteria will increase from 4.7 million in 2021 to 8.2 million in 2050[1].

Among the most concerning pathogens is *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), which claims over one million lives annually. Approximately one-quarter of the global population is latently infected with *M. tuberculosis*, creating a substantial population for reactivation and drug resistance[2]. Drug-resistant TB strains have complicated treatment protocols, often necessitating prolonged regimens involving a cocktail of toxic and costly antibiotics[2]. Standard therapy spans 4–9 months of such antibiotic cocktail, yet up to 15% of patients exhibit treatment failure, requiring alternative intervention.

Alarmingly, *M. tuberculosis* acquires antibiotic resistance at an estimated rate of 5% per year, outpacing all other major bacterial pathogens[1]. Despite the World Health Organization's 2015 "End TB Strategy" targeting a 20% reduction in incidence by 2020, only an 11% decline was achieved within the seven years until 2022[2]. Without accelerated intervention, TB alone may account for over 31 million deaths between 2020 and 2050[3]. These trends underscore the urgent need for novel antimicrobials capable of overcoming drug-resistant *M. tuberculosis* and other pathogens. The antibiotic resistance crisis is compounded by a stagnation in antibiotic discovery. Most antibiotics in clinical use today were developed in the mid-20th century, and the discovery pipeline for new compounds remains sparse[4].

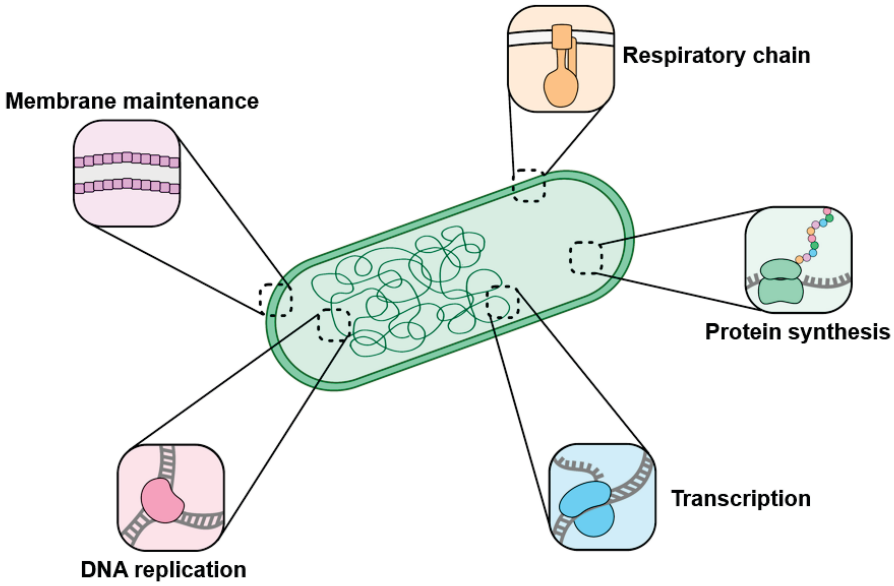

Figure 1.1: Overview of antibiotic classes. Antibiotics can impede membrane maintenance, DNA replication, transcription, protein synthesis, or the respiratory chain.

Traditional targets, such as cell wall synthesis, DNA replication, transcription, and protein translation (Fig. 1.1), have been extensively exploited, and resistance mechanisms against these pathways are now widespread[5]. To overcome this challenge, it is essential to identify new bacterial targets that can be therapeutically exploited.

How bacteria gain resistance

Antibiotic exposure imposes strong selective pressure, driving the evolution of resistant bacteria. Resistance arises from intrinsic bacterial traits, spontaneous genetic mutations, or horizontal gene transfer, enabling resistant subpopulations to thrive in antibiotic-rich environments. Four principal mechanisms underpin bacterial resistance[6]:

- *Drug Modification*: Bacteria produce enzymes that chemically inactivate antibiotics. For example, β-lactamases cleave the β-lactam ring, rendering drugs like cephamycins and carbapenems ineffective [7].
- *Reduced Intracellular Drug Concentration*: Resistance can arise through decreased drug uptake or increased efflux. Alterations in porins, e.g., replacement of OmpK35 with OmpK36, reduce β-lactam permeability, while efflux pumps such as Tet actively expel tetracycline from the cell[8].
- *Target Modification*: Mutations in antibiotic target genes can reduce drug binding. Proteins like TetO and TetM displace tetracycline from the ribosome, and mutations in DNA gyrase elevate fluoroquinolone MIC values[9].
- *Global Cellular Adaptations*: Regulatory systems such as LiaFSR modulate membrane responses to antibiotics like daptomycin, although the precise mechanisms remain under investigation[10].

A particularly resilient subset of bacteria, known as persister cells, exists in a metabolically inactive state, making them resilient to many conventional antibiotics. These cells reduce antibiotic susceptibility through low adenosine triphosphate (ATP) levels, which downregulate essential cellular processes[11]. Worryingly, current antibiotic development strategies often focus solely on actively replicating cells, overlooking the resilience of persister cells and rendering treatments less effective[12]. These persisters complicate treatment outcomes and highlight the importance of targeting pathways essential in both replicating and dormant states, such as the bacterial respiratory chain.

The *M. tuberculosis* respiratory chain

The *M. tuberculosis* respiratory chain is a series of membrane bound enzymes that are responsible for the majority of cellular energy generation in the form of ATP. This respiratory chain is a highly adaptable system, distinct from its mitochondrial counterpart in humans. It comprises multiple terminal oxidases and dehydrogenases with built-in redundancy that maintain energy production, redox balance, and survival under stress (Fig. 1.2). Targeting this system offers a unique opportunity to develop selective and potent antibiotics, particularly against non-replicating and persistent bacterial populations which rely on respiration to uphold their minimal metabolic activity which makes them resistant to conventional antibiotics.

The respiratory chain generates energy stored as a proton motive force (PMF) or sodium motive force (SMF), which drives ATP synthesis and solute transport. Novel inhibitors targeting the respiratory chain now represent over 30% of all new antimycobacterial drugs in clinical trials, and more than 65% of phase III candidates[13]. Most bactericidal compounds are thought to induce reactive oxygen species (ROS) formation by disrupting metabolic cycles and increasing energy demand[13–15].

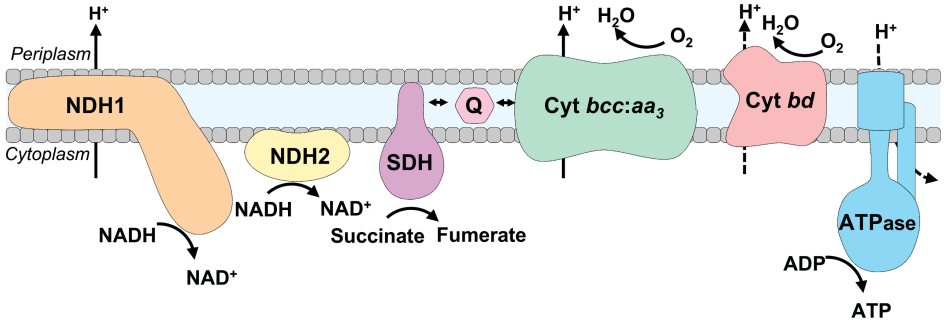

Figure 1.2: The respiratory chain of *M. tuberculosis*. Electrons enter the chain via the dehydrogenases and are shuttled to the terminal oxidases via the quinone pool. The proton motive force created is used the ATP synthase to regenerate ADP to ATP.

In contrast, bacteriostatic agents reduce oxygen consumption, pushing bacteria into a low-energy, non-replicative state[14]. Given the essentiality of various respiratory chain components in *M. tuberculosis* and various other bacteria, often coupled with their absence or divergence in humans, make this pathway a highly attractive drug target with drug development efforts undergoing for each of its members.

Quinones: the central electron carriers

Quinones act as a central electron carrier between the different enzymes of the respiratory chain. These carrier lipids molecules are readily reduced or oxidized to take up or release electrons at the target enzyme[16]. In its reduced form, quinol, the molecule has been dually protonated in conjunction with the uptake of two electrons. These electrons, together with the two protons, are subsequently released upon oxidation at the target enzyme (Fig. 1.3). Different types of quinols are used by different species under specific conditions[16,17]. Depending on the species, ubiquinone or menaquinone predominate, differing in their redox potentials and chemical structure[18]. While *M. tuberculosis* contains only menaquinone, other species like *E. coli* possess multiple quinones that are used under specific conditions. As such, ubiquinone supports aerobic respiration, whereas menaquinone predominates under anaerobic or microaerophilic conditions in *E. coli*.

Drug development efforts that target the quinone pool have resulted in the drug candidates SQ109 and Lysocin E. SQ109 inhibits the synthesis of new quinols by targeting the enzymes MenA and MenG[19], whereby the decrease in quinol concentration impairs the shuttling of electrons within the respiratory chain. Lysocin E on the other hand, binds directly to the menaquinone, blocking its redox cycling and collapsing the membrane potential[20]. Lysocin E has been shown to attenuate both *Mycobacterium smegmatis* [21] and *Staphylococcus aureus* [20] although their efficacy against clinically relevant *M. tuberculosis* strains and *in vivo* infection models warrants further evaluation.

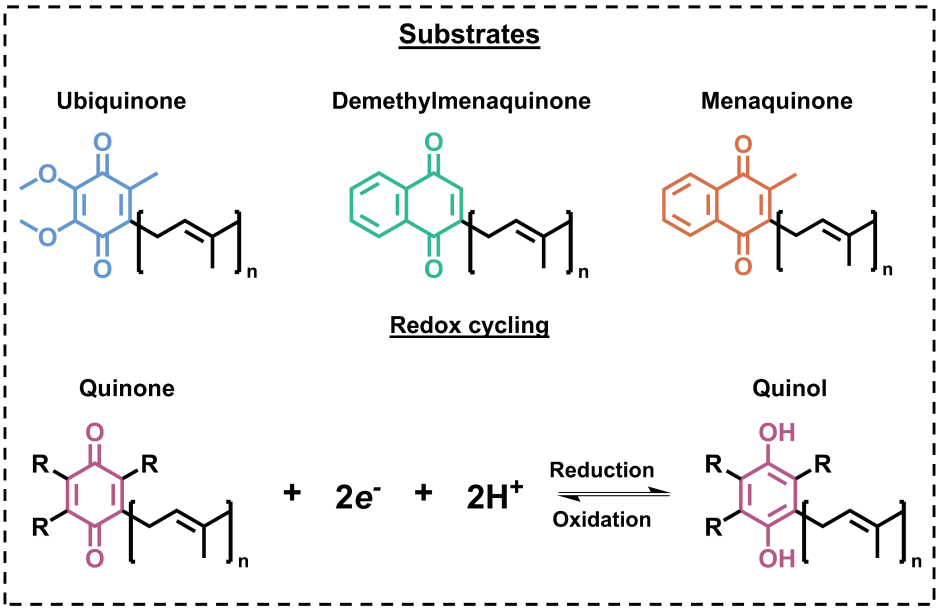

Figure 1.3: Quinone and their redox chemistry. Different quinone subtypes found across various bacterial species. All three quinone subtypes are found in *E. coli*, while *M. tuberculosis* is restricted to menaquinone. These quinones undergo reduction by the dehydrogenases to become quinol, followed by oxidation at the terminal oxidases back to quinone.

Respiratory dehydrogenases

NADH dehydrogenases

The NADH dehydrogenases (NDH) function in the reduction of the quinone pool, coupled to the oxidation of NADH to NAD^+[13]. NDH exists in two subtypes, the proton pumping NDH-1 (Nuo) and the non-proton pumping NDH-2 (Ndh and NdhA)[22]. In *M. tuberculosis*, Nuo and NdhA are individually dispensable, while Ndh deletion causes growth defects[22,23]. Conversely, the inhibition of all three NADH dehydrogenases seems to be an effective antimycobacterial strategy[24]. This need for simultaneous inhibition underscores the functional redundancy in the respiratory chain of *M. tuberculosis*.

To study the potential of pharmaceutical intervention of the NADH dehydrogenases, two types of drugs have been repurposed; phenothiazines, and riminophenazines[13], although phenothiazines likely have a multi-target mechanism including a decrease in PMF [25–28]. Of these drug subtypes, Thioridazine[29,30] and Clofazimine[31] have been deemed most effective. Thioridazine is proposed to reinstate antibiotic susceptibility of bacteria by inhibiting the overexpressed efflux pumps that cause resistance[13], while Clofazimine competes with menaquinone as an electron acceptor, generating ROS that kill the bacteria[32]. Additional research is needed to fully understand the mechanism of Thioridazine[13], and decrease the side effect of Clofazimine[33] before they can become an important tool in the battle against antibiotic resistance.

Succinate dehydrogenase

Similar to the NADH dehydrogenases, succinate dehydrogenases (SDH) function to reduce quinones to quinols. SDH links the tricarboxylic acid cycle to respiration, oxidizing succinate to fumarate while reducing the quinone pool[34]. This enzyme holds similar pharmaceutical potential as the NADH dehydrogenases to inhibit the reduction of the quinone pool, effectively shutting down the electron injection into the respiratory chain. Drug development efforts, however, have been sparse, as hesitance is in place due to the presence of human counterparts to this enzyme[13]. The off-target effect of the potential antibiotic on the human counterpart enzyme could have detrimental effects on human energy generation, thereby killing our own cells. Although concerns about homology to human SDH have tempered development efforts, selective inhibitors targeting divergent bacterial residues hold promise[35].

One of the more promising succinate dehydrogenase inhibitors is Siccanin[36], a quinone mimicking molecule. It should be noted, however, that the Siccanin effectivity against most pathogens remains to be tested. Alternatively, 3-nitropropionate has been found as a potential inhibitor, however, little research has been conducted[37].

The terminal oxidases

Cytochrome *bcc:aa*$_3$

After reduction of the quinone pool by the dehydrogenases, the quinols shuttle the acquired electrons to the terminal oxidases. The primary terminal oxidase under aerobic conditions differs per species and consists of enzymes such as the cytochrome *bcc:aa*$_3$ super complex in *M. tuberculosis*. These terminal oxidases oxidize quinols back to quinones and use the associated released electrons to reduce O_2 to H_2O. In addition to the chemical protons that are taken up in the cytosol and released in the periplasm during the turnover cycle, these terminal oxidases pump protons into the periplasm that add to the PMF. In total, cytochrome *bcc:aa*$_3$ adds six protons to the PMF per quinol oxidized[38].

Early studies found that cytochrome *bcc:aa*$_3$ is the primary terminal oxidase used and deemed it essential as no mutant could be obtained when they were deleted[39]. More recent studies, however, have shown that knockout strains can be obtained, although with severe growth defects, while glycerol is present [24].

Due to its prominent function in the respiratory chain, several drug development efforts have led to specific inhibitors. The most promising inhibitor is Q203, also called Telabec[40], and is currently in phase 2 clinical evaluation. This compound inhibits cytochrome *bcc-aa*$_3$ through the binding of subunit QcrB, where the quinols bind for oxidation[41,42]. It has been reported however, that the use of Q203 alone is bacteriostatic in *M. tuberculosis*, highlighting the adaptability of the respiratory chain[43].

Additionally, the FDA-approved drug Lanzoprazol has been found in a drug screen to be repurposed from the treatment of gastroesophageal reflux disease to the inhibition of the cytochrome *bcc:aa*$_3$ complex[44]. It was shown that lansoprazole act as a prodrug that after conversion to lansoprazole sulfide binds to QcrB[44].

Cytochrome *bd*

An alternative terminal oxidase present in *M. tuberculosis*, and most other bacteria, is cytochrome *bd*. Cytochrome *bd* mainly functions as the alternative terminal oxidase once cytochrome *bcc:aa*$_3$ is unable to function, increasing the adaptability of the respiratory chain. This happens once bacteria enter a hostile environment, such as low oxygen tensions and increased reactive oxygen species (ROS) concentrations[45]. Due to the higher oxygen affinity of cytochrome *bd* [46,47], it can reduce O_2 to H_2O under low oxygen tension to maintain the formation of the proton motive force required to generate ATP. Additionally, cytochrome *bd* acts as a defense against nitric oxide[48], hydrogen peroxide[49,50], ammonia[51], nitrite[52], peroxynitrite[53], cyanide [54,55], chromate[56], sulfide[56,57], as well as antibiotics[58–60]. Recent research has shown that cytochrome *bd* is an essential enzyme under infectious conditions, making it a promising target for pharmaceutical intervention[61]. Additionally, dual inhibition of cytochrome *bcc:aa*$_3$ and cytochrome *bd* showed bactericidal against *M. tuberculosis*, further underlining its therapeutic value[43].

ATP synthase

The final protein in the respiratory chain required for ATP synthesis is the ATP synthase. This protein consumes the proton motive force created by the respiratory chain to turn ADP into ATP[62]. As this is the protein responsible for ATP synthesis, it has been a major target for drug discovery. Hence, the very first respiratory chain antibiotic that was approved by the FDA, bedaquiline (BDQ), also known as Sirturo, targets this synthase[63–65]. BDQ has shown its promise in the treatment of tuberculosis by its bactericidal activity against dormant cells[66], which most antibiotics cannot[13]. BDQ inhibits the ATP-synthase by binding to the C and ε subunit[67,68], rendering it inactive. Unfortunately, BDQ has a delayed bactericidal activity *in vitro and in vivo* [69], where it takes between 2 to 5 days to exterminate the target bacteria. Regardless, BDQ remains a potent antibiotic as seen by shortened treatment times in comparison to other TB antibiotics.

The success of respiratory chain antibiotics

The success of BDQ is highlighted in the NIX-TB trial, a clinical trial run in south Africa that recruited patients burdened with multi- or extreme- drug resistant tuberculosis, unresponsive to second line treatments. In this extremely difficult to treat patient group, an antibiotic mix of pretomanid, linezolid and bedaquiline resulted in patients reaching culture negative status within 16 weeks, curing 90% of the patient cohort[70].

Unfortunately, since the introduction of BDQ in 2012, the first cases of antibiotic resistance have been reported, rendering this treatment ineffective in these patients[71,72]. This resistance is caused by an efflux pump that actively removes BDQ from the bacterial cell, making it unable to bind to the ATP synthase. While many efforts are currently ongoing to avoid the effects of this efflux pump, either through direct inhibition or alteration of the bedaquiline structure, none of these have reached patient care.

A worrying study showed that the affinity of bedaquiline for the mycobacterial ATP-synthase is equally high for the human ATP-synthase[73]. Simultaneously this has not shown any detrimental side effects on intact cells, potentially due to relative selectivity to the *M. tuberculosis* ATP synthase due to either lower affinity or accessibility[74–76]. Regardless, it shows that careful design of these respiratory chain inhibitors can yield potent treatment options that prevent off target effects to the homologues human counterparts.

One of the more promising approaches of respiratory chain inhibition against *M. tuberculosis* is the dual inhibition of the two terminal oxidases[77], cytochrome *bcc:aa*$_3$ and cytochrome *bd*, which have no human homology. While potent drug candidates are available against cytochrome *bcc:aa*$_3$, cytochrome *bd* lacks behind.

Cytochrome *bd* as a drug target

Cytochrome *bd* is the terminal oxidase which plays a critical role in cellular adaptation to both infection and stress conditions. This heme-containing enzyme, found predominantly in many gram-negative bacteria, is part of an alternative oxidase system that functions under low-oxygen or hypoxic conditions[47]. During infection, pathogens encounter fluctuating oxygen availability within the host, and cytochrome *bd* becomes essential for maintaining cellular energy production by facilitating efficient electron transfer in micro-aerobic environments[61,78]. Additionally, cytochrome *bd* is involved in the adaptation to oxidative stress, often present during host immune responses. This enzyme helps protect bacterial cells by reducing the production of reactive oxygen species (ROS), thus minimizing oxidative damage to cellular components[79]. Furthermore, cytochrome *bd* contributes to biofilm formation, a common feature in chronic infections, which enhances bacterial survival under adverse conditions[59]. Overall, cytochrome *bd's* ability to sustain ATP synthesis, mitigate oxidative stress, and support biofilm development underscores its pivotal role in bacterial pathogenesis and survival during infection and stress.

The molecular structure of cytochrome *bd*

In the last few years, the protein structure of cytochrome *bd* from different species have been elucidated by cryogenic electron microscopy (Fig. 1.4A,B) [80–87]. The core structure of cytochrome *bd* consists of two subunits, CydA and CydB, each comprising of 9 transmembrane helices, and exist in a two-fold rotational pseudo-symmetric conformation[85].

CydA contains a hydrophilic periplasmic loop between the 6^{th} and 7^{th} trans membrane helix that had been proposed to bind the quinols for oxidation, termed the Q-loop. Despite this long-standing hypothesis, structural studies have yet to determine the structure of any cytochrome *bd* in the substrate-bound state[83]. Cytochrome *bd* oxidases have been phylogenetically classified in the qOR, OR-C and OR-N subfamilies[88]. The quinol binding *bd*

oxidases, qOR, are further subdivided in qOR1, qOR2, qOR3, and qOR4a, where qOR1 forms the predominant subfamily. These qOR *bd* oxidases can be further subdivided based on the length of the Q-loop, Firstly, the short Q loop variants, as found in *Geobacillus thermodenitrificans* [81], contain only the flexible N-terminal insert of the loop. The long Q-loop variants, as found in *E. coli*, however, are characterized by an additional rigid C terminal part of approximately 60 amino acids that seems to have a structural role[89]. Importantly, the Q-loop from mycobacterial cytochrome *bd*, such as *M. smegmatis* and *M. tuberculosis*, classically referred to as the short Q-loop family, contains an additional eight amino acid insert[82], which means they consist of neither the long or short Q-loop family (Fig. 1.4C). In addition, the N-terminal portion of the mycobacterial Q-loop contains a unique disulfide bond, reducing the characteristic flexibility found in other *bd* variants[82]. Furthermore, exchanging the Q-loops from different species renders cytochrome *bd* inactive, showing a crucial, species-specific role of the Q-loop layout[89,90].

The CydA subunit contains all three heme cofactors, b_{558}, b_{595}, and *d*, to transfer the electrons gained from quinol oxidation towards the heme *d* oxygen reduction site (Fig. 1.3D). These heme groups are orientated in a triangular fashion with iron-iron distances varying between 11 and 19 Å[83]. This distance significantly differs from the heme-copper oxidases, where the binuclear heme iron to copper distance is approximately 4 to 5 Å[85].

Despite the structural similarity between CydA and CydB, CydB does not contain any heme groups. Instead, CydB often carries a ubiquinone-8 or demethylmenaquinone-8 bound in the positions otherwise occupied by the heme groups[91]. Due to the position of these quinones, it is suggested that they serve a stabilizing function. Additionally, mutations in the quinone binding groove of CydB rendered the enzyme inactive[83]. In Mycobacteria, however, the quinone binding site is replaced by a series of tryptophan and phenylaniline residues that reconstitute the stabilizing abilities of the quinone[82,84].

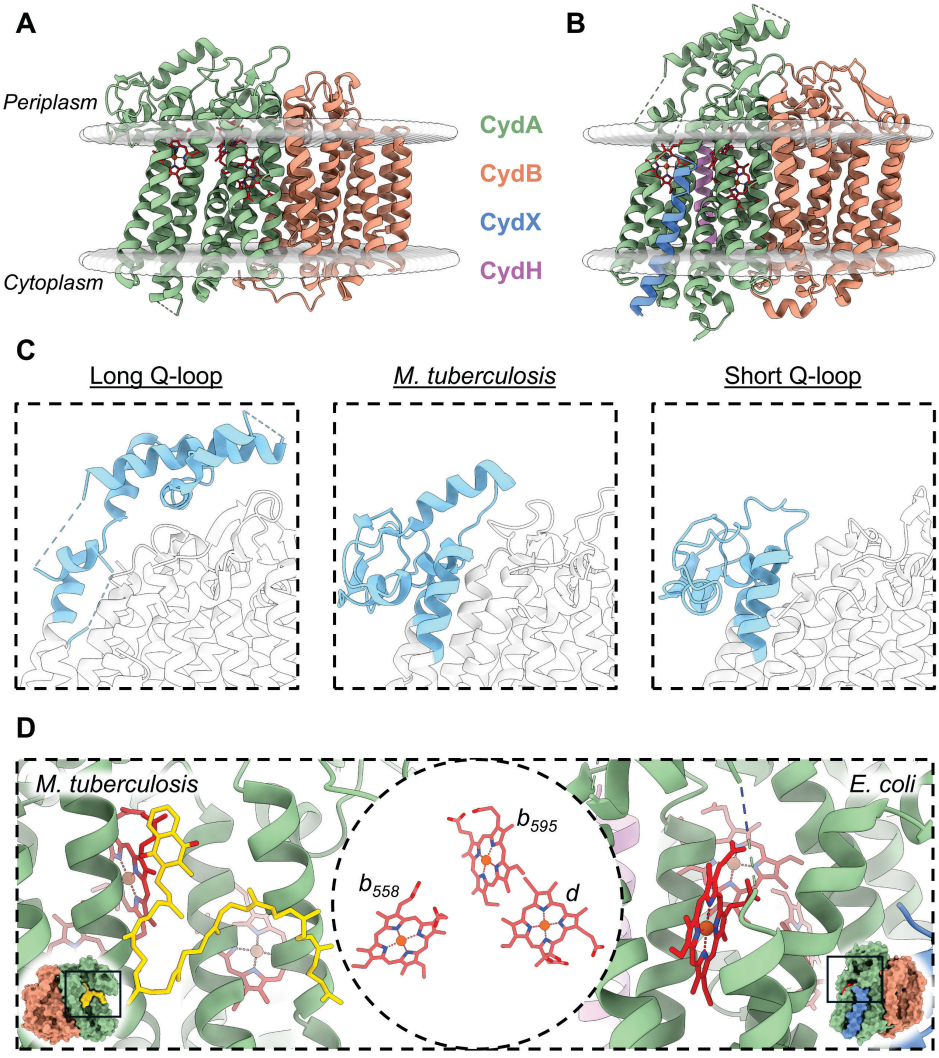

Figure 1.4: Structural features of the *bd* type oxidases. (**A**) Overview of the *M. tuberculosis bd* oxidase. (**B**) Overview of the *E. coli bd* oxidase. (**C**) structural differences between the different Q-loop families. (**D**) Proposed active sites in *M. tuberculosis* and *E. coli* cytochrome *bd*. The insert shows the triangular arrangement of the three heme groups.

Whereas the structure of cytochrome *bd* in *M. smegmatis* and *M. tuberculosis* is contained to the core components CydA and CydB[84], several different bacterial species have shown to have additional subunits. In *E. coli* for instance, the subunits CydX[83] (termed CydS in *G. thermodenitrificans* [81]) and CydH [83] (also termed CydY[86]) have been identified. Whereas CydX has been proposed to have a structural function[92], CydH is proposed to block a second, putative, oxygen channel reaching heme b_{595} [83]. It remains unclear whether cytochrome *bd* is otherwise functional and whether molecular oxygen can be reduced at two separate hemes in the absence of CydH. Potentially, however, oxygen can still be blocked from entering, regardless of CydH presence, by a conserved isoleucine residue[85]. Regardless, recent research has shown that cytochrome *bd* remain active despite knockout of CydH[93]. Interestingly, cytochrome *bd* from *M. tuberculosis* showed to have a bound menaquinone on the position of the oxygen channel that CydH covers in *E. coli* (Fig. 1.4D), which indicates a stabilizing function[90]. Conversely, cytochrome *bd* from *M. smegmatis* does not contain the bound menaquinol and has been proposed to have a second oxygen channel[84], although this is yet to be supported by biochemical analysis. The structure indicates that in mycobacteria, the open access to heme b_{595} is used as an alternative electron pathway that bypasses heme b_{558}[82].

Maturation of cytochrome *bd* by the ABC transporter CydDC

The assembly and maturation of cytochrome *bd* oxidase requires the incorporation of the heme cofactors into the CydA subunit. The necessity of this step potentiates the indirect inhibition of cytochrome *bd* by impairing its maturation. This process is facilitated by the ATP-binding cassette (ABC) transporter CydDC, which exports heme and thiol compounds from the cytoplasm to the periplasm[94–96]. Genomically, the ABC transporter is located directly downstream of the cytochrome *bd* operon, providing concerted expression (Fig. 1.5A).

CydDC is composed of two subunits: CydD and CydC. It functions as a heterodimeric transporter that binds heme laterally from the membrane and translocates it across the inner membrane[94,97]. Structural studies have revealed the structural changes that CydDC undergoes during heme binding and transport, and highlight that mutations in CydD or CydC lead to defective cytochrome *bd* assembly, and thereby oxidase activity[94].

Similar phenotypes have been observed in *M. tuberculosis*, where CydDC is essential for cytochrome *bd* function and resistance to respiratory inhibitors[98]. The transporter also plays a role in maintaining redox balance by exporting glutathione and cysteine, which are important for oxidative stress tolerance[99].

How CydDC results in heme incorporation in CydA remains to be determined, although the current consensus points towards a presently unknown periplasmic partner protein that forms the missing link, shuttling the hemes between the two protein complexes (Fig. 1.5B). Given its central role in cytochrome *bd* maturation and stress resistance, CydDC represents a novel target for antimicrobial intervention. Inhibitors of CydDC could disrupt heme trafficking, thereby directly impairing cytochrome *bd* activity, and sensitizing bacteria to host defenses and antibiotics.

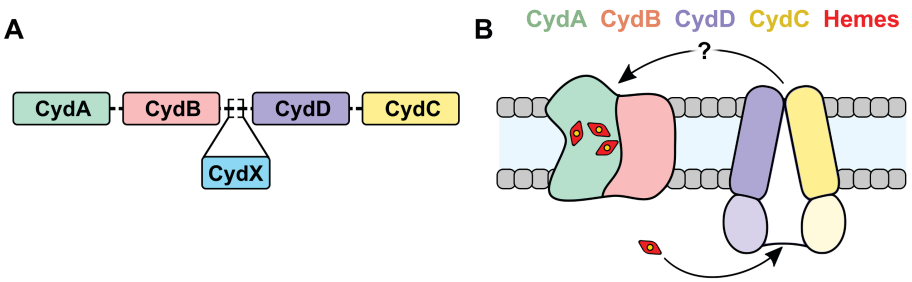

Figure 1.5: Heme maturation of cytochrome *bd* by the CydDC ABC transporter. (**A**) Genomic organization of the cytochrome *bd* – CydDC operon. CydX is only present in a subset of all species. (**B**) Cartoon of the proposed mechanism of heme incorporation into cytochrome *bd* by CydDC.

Drug discovery and inhibition strategies for cytochrome *bd*

Recent advances in structural biology and infection-adapted screening approaches have strengthened efforts to identify potent cytochrome *bd* inhibitors and accelerate their translation into clinical applications.

Several inhibitor classes have now established the druggability of cytochrome *bd*. Quinone analogs, such as Aurachin D derivatives, competitively bind the quinol oxidation site in CydA, preventing quinol binding and oxidation[100]. Synthetic molecules such as ND-011992 exhibit potent nanomolar activity against multiple species and demonstrate synergy with *bcc:aa*$_3$ inhibitors, effectively collapsing respiratory flexibility[77,101]. Peptide-based inhibitors, exemplified by microcin J25[102,103], induces superoxide formation, offering an alternative scaffold distinct from classical small molecules (Fig. 1.6). Emerging approaches in drug discovery are addressing the challenges inherent in targeting the *bd* oxidase, which is dispensable under standard aerobic culture conditions[77]. Hypoxia-mimicking assays now replicate infection-relevant environments, selectively inducing *bd*-dependent respiration and enabling context-specific inhibitor screening[104,105]. High-throughput phenotypic platforms further integrate low-oxygen and nitric oxide stress to identify compounds active under host-like conditions[106].

Parallel advances in cryo-electron microscopy have yielded high-resolution structures of *bd* facilitating *in silico* docking and fragment-based drug design tailored to species-specific features of the Q-loop binding site[107,108]. Moreover, combination screens pairing *bd* inhibitors with *bcc:aa*$_3$-targeting agents have revealed synergistic interactions that accelerate bacterial killing and overcome tolerance[77,107,109].

Collectively, these advances exemplify the growing recognition of respiratory pathways as critical vulnerabilities in bacterial physiology. Targeting cytochrome *bd* not only exploits its infection-stage essentiality but also complements a broader shift toward respiratory chain inhibition as a strategy to eliminate both replicating and persistent bacterial populations.

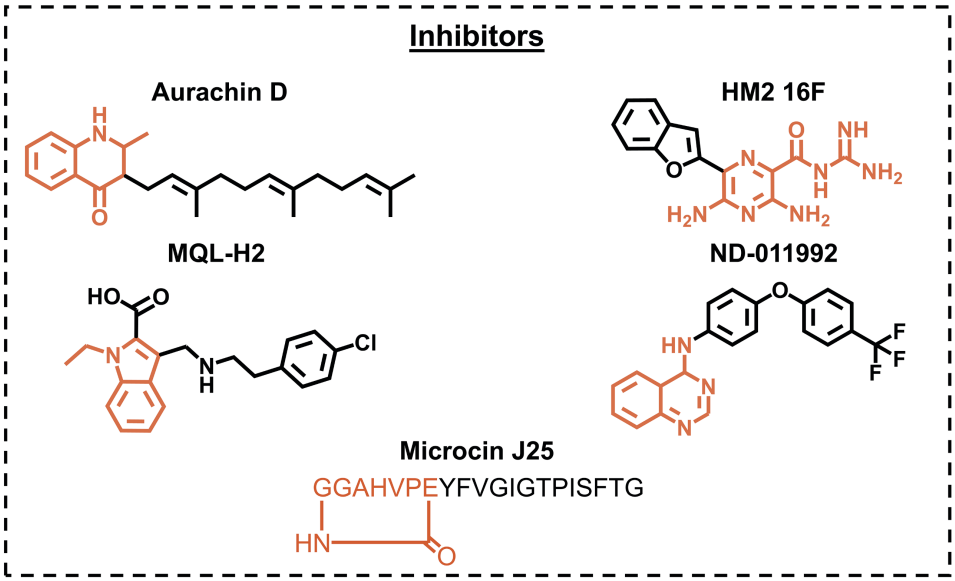

Figure 1.6: Overview of bd-type oxidases inhibitors, with the proposed interactive headgroup highlighted

By integrating structure-guided design, physiologically relevant screening, and rational combination therapies, *bd* inhibition represents a powerful addition to the emerging antimicrobial arsenal poised to address the escalating crisis of antibiotic resistance.

Outline of this thesis

The aim of this thesis is to study the molecular mechanism of cytochrome *bd*, with a specific aim of antibiotic development against *M. tuberculosis*. This chapter (**Chapter 1**) serves as a general introduction to the antibiotic resistance crisis and the respiratory chain as an antibiotic target space with an emphasis on the terminal oxidase cytochrome *bd*. **Chapter 2** describes the quinone substrate specificity of both *E. coli* and *M. tuberculosis* cytochrome *bd*. In addition, we show that *M. tuberculosis* contains a redox regulatory disulfide bond in the Q-loop, which allows activation upon disulfide oxidation. It is proposed that this disulfide bond allows enzyme-level regulation of cytochrome *bd* activity, providing a molecular basis for the quick respiratory rerouting found in *M. tuberculosis*. **Chapter 3** describes the molecular basis of quinone and inhibitor binding in *E. coli* cytochrome *bd*-I. We show that *E. coli* cytochrome *bd*-I exists in a dimeric form, stabilizing the Q-loop and allowing structural probing of the active site. This resulted in the first quinone bound structure of cytochrome *bd*, and showed that Aurachin D binding triggers structural rearrangement of the active site, closing the quinone binding pocket. **Chapter 4** describes a high throughput screening method to find cytochrome *bd* inhibitors. Using this method, over 10.000 compounds were screened resulting in a new fragment with a sub µM IC50 value. This fragment showed to bind at the active site, thereby inhibiting cytochrome *bd* by competitively displacing the quinone. **Chapter 5** discusses the alternative hypothesis that heme transporter CydDC, essential for cytochrome *bd* maturation, incorporates heme by direct binding to the CydA subunit during cytochrome *bd* biogenesis. Lastly, **Chapter 6** provides the overall discussion about cytochrome *bd* and the contents of this thesis.