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Deletion of a unique loop in the mycobacterial F-ATP synthase c subunit sheds light on its inhibitory role in ATP hydrolysis-driven H+ pumping

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Running Title: Inhibitory element of mycobacterial FATP synthase
Abstract

The F$_1$F$_0$ATP synthase is one of the essential enzymes in supplying the energy requirement of both the proliferating aerobic and hypoxic dormant stage of the life cycle of mycobacteria. Most F$_1$ATP synthases consume ATP in the $\alpha_3\beta_3$ headpiece to drive subunit $\gamma$, which couples ATP cleavage with proton pumping in the cring of $F_0$ via the bottom of $\gamma$. Importantly, ATPase driven H$^+$ pumping is latent in mycobacteria. Previously, the presence of a unique 14 amino acid residues loop of the mycobacterial subunit $\gamma$ has been described and aligned in close vicinity to the cring loop (Priya R, Biuković G, Manimekalai MSS, Lim J, Rao SPS, and Grüber G (2013) J. Bioenerg. Biomembr. 45, 121129). Here, we used inverted membrane vesicles (IMVs) of the fast growing Mycobacterium, M. smegmatis, and characterized by a variety of covalent and noncovalent inhibitors the defined ATP hydrolysis activity of the F$_1$F$_0$ATP synthase inside IMVs. This formed a platform to investigate the function of the unique mycobacterial $\gamma$-loop by deleting the respective DNA loop encoding sequence ($\Delta\gamma_{166179}$) in the genome of M. smegmatis. ATP hydrolysis driven H$^+$ pumping was observed in IMVs containing the F$_1$F$_0$ synthase $\Delta\gamma_{166179}$ mutant but not for the wild type. In addition, when compared to the wildtype enzyme, IMVs including the F$_1$F$_0$ synthase $\Delta\gamma_{166179}$ mutant revealed an increase in ATP cleavage and lower levels of ATP synthesis, demonstrating that the loop affects ATPase activity, ATPase driven H$^+$ pumping and ATP synthesis. It further indicates that the loop may affect coupling of ATP hydrolysis and synthesis in a different mode.
**Keywords:** Bioenergetics, FATP synthase, Mycobacterium, tuberculosis, protonmotive force, membrane enzyme

**Abbreviations:** ACMA, 9amino6chloro2methoxyacridine; BDQ, bedaquiline; DCCD, N,N'-Dicyclohexylcarbodiimide; IMV, inverted membrane vesicle; NBDCI, 4chloro7nitrobenzofurazan; NDH2, type II NADH dehydrogenase;
Introduction

The respiratory chain enzyme complexes of mycobacteria, generating a proton motive force (PMF) across the bacterial cytoplasmatic membrane, as well as the F\textsubscript{ATP} synthase, utilizing this force for ATP synthesis, have been identified as potential drug targets against tuberculosis (TB), which is mainly caused by the bacterium Mycobacterium tuberculosis [14]. Mycobacterial F\textsubscript{ATP} synthase has been validated as target of bedaquiline (BDQ), which selectively inhibits this enzyme in a variety of mycobacterial strains [57]. The mycobacterial F\textsubscript{1}F\textsubscript{o} ATP synthase is composed of nine subunits in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon\alpha:\beta:\beta':\varepsilon:9$, and organized in a membrane embedded F\textsubscript{o} domain (a:b:b':c) and a water soluble F\textsubscript{i} part (a:b:b':a:b'b':c) ([1, 8]; Fig. 1). The catalytic F\textsubscript{i} domain contains the $\alpha_3\beta_3$ hexamer, in which ATP synthesis takes place. This catalytic $\alpha_3\beta_3$ headpiece is linked via the two rotating, central stalk subunits $\gamma$, $\varepsilon$ and the peripheral stalk subunits $b$, $b'$ and $\delta$ with the H$^+$ pumping F\textsubscript{o} part. The proton pumping F\textsubscript{o} domain consists of subunit a, and a ring structure of 9 c subunits [8]. The latter is proposed to form a helixloopelix structure, where the loop faces the bottom of the rotary $\gamma$ subunit (Fig. 1a) and enables the coupling of torque energy, derived by ion transport, to the catalytic $\alpha_3\beta_3$ headpiece [9]. The rotational movement of the cring triggers the central subunits $\gamma$ and $\varepsilon$ to rotate. This generated torque energy causes sequential conformational changes in the catalytic $\beta$ and noncatalytic $\alpha$ subunits, resulting in ATP synthesis [10]. In the case of ATP hydrolysis, the energy release of ATP cleavage drives rotation of the $\gamma$ subunit inside the $\alpha_3\beta_3$ hexamer [10]. In this coupling process the coiledcoileddomain of $\gamma$ inside the $\alpha_3\beta_3$ hexamer and the $\gamma$ foot domain connect ATP cleavage with proton pumping in the cring and subunit a [11].

A striking physiological difference of mycobacterial F\textsubscript{ATP} synthases is that they are incapable of ATP driven proton translocation [12]. In parallel, a comparison of the amino acid sequence of subunit $\gamma$ of mycobacterial F\textsubscript{1}F\textsubscript{o} ATP synthase from the $\gamma$ subunit of other pro and eukaryotic F\textsubscript{ATP} synthases showed lower homologies at the bottom region of subunit $\gamma$. This is in particular reflected by the unique insertion of 14 amino acid residues (165\text{TDNGEDQRSDSGEG} \text{178} according to M. tuberculosis numbering), which is not present in any other prokaryotic or eukaryotic $\gamma$ subunit (Fig. 1) [13]. NMR studies showed that peptide M\text{t}\gamma_{165\text{178}} forms a loop of polar residues. This loop has been proposed to be in the vicinity of the polar residues R41, Q42, E44 and Q46 (M. tuberculosis nomenclature) of the cring [13].
Understanding how the unique loop may be related to possible mechanistic processes like the suppression of proton pumping, which may affect the PMF, is essential, since dissipating the PMF is lethal to mycobacteria [14]. Activation of the latent ATP hydrolysis driven $H^+$ pumping would reduce ATP reserves, altering the PMF and decreasing the bacteria’s viability. Using a combination of recombineering and enzymatic assays we report for the first time that the unique $\gamma$ loop is at least one of the regulatory elements inside mycobacteria FATP synthases effecting ATPase activity, preventing ATP driven $H^+$ translocation and is important in coupling proton transport with ATP synthesis. In addition, the effect of covalent and noncovalent inhibitors on the ATP hydrolysis activity of mycobacteria FATP synthase inside inverted membrane vesicles has been studied.

**Results and Discussion Generation of the M. smegmatis FATP synthase $\Delta\gamma 166\text{-}179$ mutant**

A recombineering method with dsDNA in M. smegmatis mc $^\text{\textregistered}155$ as described in Material and Methods has been used to generate the M. smegmatis FATP synthase $\Delta\text{atpG}$ mutant with a deletion of DNA sequence coding for amino acids 166 to 179 of the respective loop of subunit $\gamma$ ($\Delta\gamma 166\text{-}179$). The targeted deletion of the DNA sequence in gene atpG of M. smegmatis (coding for $\gamma$ loop $\gamma 166\text{-}179$), was confirmed by sequencing of the DNA region flanking the deletion site (100 bp DNA sequence upstream and downstream of the loop encoding DNA sequence). The integrity of the ATP synthase operon was confirmed by PCR mapping using sets of primer pairs spanning the whole operon (Table 1; Fig. 2AB). In order to exclude the possibility that a copy of the wild type $\gamma$ gene occurred somewhere else in the genome of M. smegmatis, Southern hybridization was performed (Fig. 2C), and finally the parental and mutant strains were subjected to whole genome sequencing.
ATP hydrolysis assay on M. smegmatis wild type and the Δγ166179 mutant protein

In order to investigate the effect of the loop deletion of the M. smegmatis Δγ166179 mutant protein on ATP hydrolysis, a regeneration assay including the type II NADH dehydrogenase (NDH2) inhibitor thioridazine (80 µM [14]), has been used to measure the ATP hydrolytic activity of M. smegmatis FATP synthase inside inverted membrane vesicles. In M. smegmatis NDH2 represents 95% of total NADH oxidation [15]. As demonstrated in Figures 3AB, IMVs of wild type M. smegmatis revealed an ATP hydrolysis activity of about 36 nmol/min·mg total protein. In order to confirm, that hydrolysis is indeed catalyzed by the M. smegmatis FATP synthase, the effect of various F-type ATP synthase inhibitors like resveratrol, 4chloro7-nitrobenzofurazan (NBDCl), and BDQ, which affect M. smegmatis mc2 155 growth (Supplementary Figure S1), as well as quercetin and efrapeptin C were tested. Resveratrol, which inhibits the rotation of F1Fo ATP synthase by binding to the interface of the α, β subunits and the Cterminus of subunit γ [16], reduced the ATP hydrolytic activity of the M. smegmatis IMV’s to 22% (100 µM of resveratol). A concentration dependent measurement with resveratrol resulted in an IC50 value of about 50 µM (Fig. 4A), which is comparable with those reported for the purified bovine mitochondrial F1ATPase (6.4 µM; [17]). Quercetin, a close analog of resveratrol with the same binding site inside the FATP synthase [17], reduced ATP hydrolysis to 44% (100 µM of quercetin; Fig. 4B). In addition, an IC50 of 75 µM for quercetin could be determined. By comparison, BDQ, a diarylquinoline drug, showed inhibition of ATPase activity with an IC50 comparable to quercetin (75 µM, Fig. 4C). BDQ has been described to inhibit ATP synthesis by binding with its dimethylamino moiety to residue E65 and sitting with its quinoline moiety on the F69 platform of the cring [8]. In addition, as revealed by IMV and whole cell analyses of M. smegmatis mc2 155, bedaquiline has no effect on the membrane potential (Δψ), which may suggest that the charge across the membrane is maintained through as yet undetermined processes during bedaquiline challenge [7]. The authors proposed a mechanism of uncoupling, which involves the binding of bedaquiline to the c subunit and perturbing the ac subunit interface, allowing an uncontrolled proton leak uncoupled from ATP synthesis. Like the inhibitor DCCD, which binds to the catalytic β subunits of F1 [18] and the c subunit of Fo [19], a second possible binding site for BDQ has been proposed to be in the interface of the ε subunit and the cring, where the drug would hinder rotation of the cring relative to the γεpowerstroke in a wedgelike fashion [20]. As ATP hydrolysis is catalysed in the F1 domain the present data imply that BDQ inhibits not only ATP synthesis (IC50 = 4.5 nM, see below) but also ATP cleavage (IC50 around 90 µM) and that this effect may occur via binding to a second binding site inside the FATP synthase complex.
Another potent inhibitor, 4Chloro7nitrobenzofurazan (NBDCI), binds covalently to tyrosine residue 331 of the so called nucleotide empty β subunit (βe) [20], and is also allocated in the proximity of the central stalk. Like resveratol, NBDCI inhibited effective ATP hydrolysis with an IC₅₀ value of around 100 µM (Fig. 4D). While most of the inhibitors showed strong inhibition of ATP hydrolysis, efrapeptin C with a binding site in FATP synthase similar to those for resveratrol and quercetin [22] (Fig. 4E) exhibited a reduction of 20% ATPase activity (100 µM efrapeptin C).
After characterization of the wild type IMV’s, we investigated the effect of the loop deletion of the \( M. \text{sme}gmat\)is \( \Delta\gamma166179 \) mutant protein on ATP hydrolysis. As revealed in Figures 3AB, the hydrolysis rate of the \( \Delta\gamma166179 \) mutant including IMVs increased by about 34\% (55 nmol/min·mg total protein) compared to the vesicles of the wild type complex, which shows that the mutation did alter ATPase activity.

To confirm that the observed difference in ATP hydrolysis of the wild type and \( \Delta\gamma166179 \) mutant IMVs is indeed caused by the loop deletion, we complemented the \( \Delta\text{atpG} \) mutant strain with the wild type copy of the \text{atpG} \text{gene}. This was done by overexpression of \text{atpG} under the strong constitutive promoter hsp60 on plasmid pMV262 [23] transformed into \( M. \text{sme}gmat\)is \( \Delta\text{atpG}(166179) \), harbouring the gene \( \Delta\text{atpG} \) coding for \( \Delta\gamma \) (see Material and Methods). As revealed in Figure 3C, the hydrolysis rate of IMVs derived from the constitutive expression system described above decreased (42.2 nmol/min·mg total protein) when compared to the one of the \( \Delta\gamma166179 \) mutant, reflecting that the complementation was successful and that the loop deletion caused the observed increase of ATPase activity of the \( \Delta\gamma166179 \) mutant strain.

**Protontranslocation of WT and the \( \Delta\gamma166179 \) mutant protein**

In order to determine, whether ATP hydrolysis is coupled with protonpumping, IMVs of \( M. \text{sme}gmat\)is WT were measured in the presence of the fluorescent dye 9amino6chloro2methoxyacridine (ACMA). As shown in Figure 5A, adding the substrate ATP to the IMV containing assay resulted besides the typical slight ATPcaused quenching signal in no further drop of fluorescence. This indicated that the F\text{ATP} synthe\text{ase} of \( M. \text{sme}gmat\)is does not show an ATP hydrolysis driven proton transport, which is in line with previous findings of Haagsma et al. [12]. These studies demonstrated that irrespective of the Mg:ATP ratio or the pH value (pH 5.5–8.0) nor ATPasedriven proton pumping could be detected in slow or fast growing mycobacteria [12]. In order to exclude the leakiness of the IMV’s, NADH has been added to the assay, which caused a significant quenching of ACMA, generated by the respiratory chain complexes (Fig. 5A). Addition of the uncoupler SF6847 revealed the associated increase in fluorescence, confirming that the IMV’s are intact. In comparison, when ATP was added to IMV’s including the \( M. \text{sme}gmat\)is \( \Delta\gamma166179 \) mutant protein protonconduction occurred, resulting in a quenching of about 61\%. This result confirms also that the ATPase activity detected above is of intact IMV’s with complete F\text{i}F\text{o} ATP synthe\text{ase} complexes. Afterwards, SF6847 resolved the generated proton pumping shown by an increase in fluorescence (Fig. 5B). Furthermore, NADH driven proton pumping of the IMV’s of the \( M. \text{sme}gmat\)is \( \Delta\gamma166179 \)
mutant protein revealed a quenching result similar to that for the wildtype IMV’s, indicating that the observed change in ATP-driven proton pumping of the IMV’s of the M. smegmatis Δγ166179 mutant is caused by the loop deletion in the FATP synthase mutant form. Furthermore, the data show for the first time that the additional 14 amino acid loop at the bottom of the globular domain of the mycobacteria γ subunit, proposed to be in vicinity to the polar loop residues of the cring (Fig. 1), is at least one critical structural element preventing ATP-driven H⁺ pumping of mycobacterial FATP synthases.
The data above and revealed recently [12] demonstrate that although at a relatively low level, ATP becomes hydrolyzed by the fast growing M. smegmatis FATP synthase without coupled protonpumping. Furthermore, slow growing mycobacteria like M. bovis BCG show neither detectable ATPase activity [13, 24] nor H⁺ pumping translocation of their FATP synthase [13]. The question arises, why does the fast growing M. smegmatis FATP synthase hydrolyse ATP to ADP although it does not pump protons? As previously discussed by Zharova and Vinogradov [25] in studies on FATP synthases of tightly coupled vesicles of Paracoccus denitrificans, two alternative mechanisms of respiratory control have been described so far. A first mechanism invokes the so-called “thermodynamic” control [26, 27].
In this model, an increase in the respiratory activity by ADP is considered as a change in the ATP/ADP ratio that results in activation of redox reactions to restore the disturbed equilibrium. A second model suggests that respiration is kinetically controlled by ADP via either the adenine nucleotide translocase (in case of mitochondria) or some intrinsic mechanisms of the F ATP synthase [28, 29]. Since the essence of the respiratory control is that ADP accelerates respiration, ADP generated by the M. smegmatis F ATP synthase may actually stimulate the respiratory chain, leading to the formation of ATP. This may be one aspect enabling this mycobacterium to grow faster compared to its counterpart M. bovis BCG, whose F ATP synthase does not show detectable ATP hydrolysis.

**ATP synthesis activity of wild type and the Δγ166179 vesicle mutant protein**

To demonstrate an effect of the γ loop deletion in ATP synthesis, the ATP production of the wild type and the Δγ166179 mutant containing vesicles were measured. The wildtype F ATP synthase of the M. smegmatis IMVs showed an ATP synthesis formation of 11.3 nmol. In comparison, 5.8 nmol have been synthesized by IMV’s of the Δγ166179 mutant protein and therefore 54% of the wildtype protein (Fig. 6A). Like for the ATP hydrolysis studies, IMVs isolated from the complementation strain with the constitutive expression system described above have been used to demonstrate that the difference in ATP synthesis is caused by the alteration of γ inside the Δγ166179 mutant. Figure 3B shows that out of the 54% difference synthesis formation seen above, 30% ATP synthesis is recovered by the IMVs of the γ loop deletion strain M. smegmatis ΔatpG(166179). This confirms the effect of the γ166179 loop in ATP synthesis as well as ATP hydrolysis (see above).

As revealed in Figure 6C, the addition of inhibitor BDQ reduced ATP synthesis. To determine the halfmaximal inhibitory concentration, we varied the BDQ concentration from 0.04 nM to 5 µM. The IC50 value of about 4.5 nM determined is in line with those reported
recently for M. smegmatis (2.5 – 12.9 nM [30, 31] and M. phlei (20 – 25 nM, [8]). In comparison, although the Δγ166179 mutant membrane vesicles showed a lower overall ATP synthesis formation, the determined IC50 value of BDQ is about 0.4 nM (Fig. 6D) and therefore lower than for the wildtype protein (Fig. 6C). This reflects that the deletion of the Δγ166179 in M. smegmatis F ATP synthase increases the accessibility of BDQ, and that this loop may be in proximity to the additional binding site of γε and cring interface as mentioned recently [13].

The increase in ATP hydrolysis driven H+ pumping and reduced ATP formation by the IMVs of the Δγ166179 mutant protein presented, may imply that ATP synthesis may not simply be the reversal of ATP hydrolysis or at least may differ in minor point(s), as described for FATP synthases of mitochondrial particles [25, 32] or liposomes of co-reconstituted bacteriorhodopsin and Geobacillus stearothermophilus FATP synthase [33]. It may be speculated that the unique 14-residue loop of mycobacterial FATP synthases may block the relative rotation of the γε rotor relative to the cring by its interaction with the cring loop, and that the energy provided from the low ATP hydrolysis activity of the fast growing M. smegmatis presented and described recently [12] is not sufficient to unlock the inhibitory loop-loop interaction.

In the case of ATP synthesis direction the PMF may be sufficient to alter the looploop interaction of subunit γ and c. As shown in Figure 7, subunit γ is asymmetric at its globular foot domain and tilted relative to the symmetric cring [34, 35]. At any given time during rotation of γ as well as the cring, one of the c subunit loops is in or without contact of a particular region of the central γ subunit. In the case of the mycobacterial γ and its unique polar loop, the latter would interact with the cring and would affect the speed of rotation. As proposed recently, the mycobacterial γ loop is linked to α4 in subunit γ, which forms a structural path via β3 to α3, which directly interacts with the C terminal domain of the catalytic β subunit, causing the change of an open to a closed (ATPbound) form (Fig. 7; [13]). In this context we hypothesize that the mycobacterial γ loop would not only effect rotation but also the velocity of nucleotide binding and/or release in the catalytic sites via the α4β3α3 structural path. Since the γ166179 loop is deleted in the M. smegmatis Δγ166179 mutant protein, the γ166179 loop effect on nucleotide binding and/or release is reduced, resulting in decreased ATP formation as shown in Figure 6A.
Interestingly, the low resolution structure of the M. tuberculosis ε subunit as well as the NMR solution structure of the Cterminal region of M. tuberculosis subunit ε (ε103120), has been determined [20]. A comparison of these data with the εinhibited state in the Escherichia coli F1 ATPase, which blocks both hydrolysis and synthesis of ATP [34], showed that the mycobacterial ε subunit has a shorter Cterminal domain, which will not reach the upper region of the αβ interface close to the adeninebinding pocket and the Nterminal and rotating part of γ [36]. The surface potential of Mtε103120 reveals highly positive surface charge due to the presence of arginine residues, giving the helix a very positive surface, and enabling this region to interact strongly with the Cterminus of one of the catalytic subunits and thereby regulate catalytic processes. Therefore, the inhibiting mode in ATP hydrolysis subunit ε in most prokaryotic F1ATP synthases was proposed to be different in mycobacterial F1ATP synthases, because of their shorter Cterminus [20]. In combination with the findings of the mycobacterial γloop presented, the unique features of the mycobacterial ε and γ seem to cause concerted actions, in which subunit ε regulates the low or nondetectable ATPase activity in fast- and slow growing mycobacterial strains [12], respectively, whereby the extra loop of mycobacterial subunit γ inhibits ATPase driven H+ pumping and affects ATP synthesis.
**Conclusion**

The studies here provide a first insight into the unique γ loop in the foot domain of mycobacterial FATP synthases. Generating the FATP synthase Δγ166179 mutant form sheds light on the influence of this loop on ATP hydrolysis, proton pumping and ATP synthesis. Considering the available Mtγ165178 loop structure, its proximity to the cring as well as its link to helix α4 in subunit γ, which forms a structural path via β3 and α3 to the C terminal domain of the catalytic β subunit, implications and hypotheses concerning functional conformations were discussed. Furthermore, the data demonstrate that ATP becomes hydrolyzed by the fast growing M. smegmatis FATP synthase without coupled proton pumping. Therefore, unraveling how the energy release of ATP cleavage becomes transferred is an ongoing goal.

**Materials and Methods Biochemicals**

Phusion and Taq DNA polymerases were obtained from Thermo Fischer Scientific (USA); restriction enzymes were purchased from NEB (USA). Chemicals and antibiotics: kanamycin, hygromycin, rifampicin, streptomycin and dequalinium chloride, were purchased from SigmaAldrich (USA), antibiotic bedaquiline was purchased from Genegobio (USA). Media and their supplements for strain cultivation: Middlebrook 7H9, Middlebrook 7H10, Luria-Bertrand (LB), Dubos oleic agar, ADC and OADC, were purchased and prepared according to the manufacturer’s instructions from Difco Laboratories (USA). CellTiterGlo 2.0 was purchased from Life Technologies (USA), Random prime labeling kit (Roche USA). All other chemicals were of analytical grade and were obtained from Biomol (Hamburg, Germany), Merck (USA), SigmaAldrich (USA), Roche (USA) or Serva (USA).
**Genetic manipulation**

DNA manipulations were done according to standard protocols [37]. Plasmid and DNA isolations were done using Qiagen kits and their protocols. Mycobacterial DNA manipulations were done with some minor modifications according to the published protocols [38]. Primers and dsDNA oligonucleotides were synthesized by IDT (Integrated DNA Technologies, USA) and sequencing of DNA was done by AIT Biotech, Singapore. Cultivation and preparation of electrocompetent cells of *M. smegmatis* mc² 155 was done according to the protocol described by Goude and Parish [39]. For recombineering, *M. smegmatis* cells containing the episomal plasmid pJV53, with the inducible genes che60 and che61 under control of the acetamide promoter, were used, and electrocompetent cells were prepared as described [40]. In this particular case, cells after overnight growth were washed and expanded in 7H9 medium supplemented with succinate (0.2% v/v) to an OD₆₀₀ value of 0.6. Prior to cooling the cells on ice, recombineering genes were induced with acetamide (0.2% v/v) for 3h at 37 °C. To create the complementation construct for *M. smegmatis ΔatpG(166179)*, an open reading frame atpG from *M. smegmatis* wild type was amplified by PCR using oligonucleotides carrying BamHI(Forward) and PstI (Reverse) restriction enzyme cutting sites (see Table 1). The internal BamHI restriction site in gene atpG was deleted by silenced PCRmutagenesis using oligonucleotides For_Inter_atpG/Rev_Inter_atpG. The 1.05 kb PCR product was cloned in the BamHI/PstI cloning site of the *E. coli / Mycobacterium* shuttle plasmid pMV262 [23], creating complementation vector pMV262atpG. The construct was verified by sequencing, and introduced by electroporation into *M. smegmatis ΔatpG(166179)*, which was then used for subsequent complementation studies.
Recombineering with dsDNA in M. smegmatis

Recombineering was done according to the published protocols [40, 41]. In order to generate a M. smegmatis mc² 155 FATP synthase mutant form with a deletion of amino acids 166 to 179 of the loop of subunit γ (Δγ166179), a double stranded 100 bp DNA oligonucleotide with homology with DNA upstream and downstream of the gene atpG (coding for the γ loop γ166179) was synthesized by IDT (USA). This oligonucleotide was further extended by 100 bp by PCR using forward and reverse extending primers (Table 1 supplement), resulting in a 200 bp dsDNA oligonucleotide (Table 1).

Electrocompetent cells of M. smegmatis containing plasmid pJV53 (Kan<sup>k</sup>) were mixed with 200 ng double stranded DNA (dsDNA) oligonucleotide and 100 ng cotransforming plasmid pSJ25. Transformed cells were recovered by shaking for 4 h at 37 °C in 7H9 supplemented with 10% ADC and 0.05% Tween80, and then plated on 7H10 agar supplemented with 10% OADC, kanamycin and 50 µg/ml hygromycin. 300 hygromycin resistant transformants were streaked on 7H10 master agar and tested by colony PCR. ColonyPCR was done as follows: cells were resuspended in 200 µl H₂O and boiled 95 °C for 20 min, immediately cooled on ice and an aliquot of 1/10 of volume of the mix was used as a template in PCR detection using Forward/ Reverseflanking primers (Table 1). PCR parameters used in detecting recombinants were as follows: TaqDNA polymerase (Thermo Fischer), initial denaturation (95 °C for 3 min), cycle denaturation (95 °C/30 sec), annealing (55 °C/30 sec), extension (72 °C for 30 sec), final extension (72 °C for 5 min), number of cycles 25. Detected recombinants were restreaked several times on nonselective 7H10 agar plates in order to remove recombineering plasmid pJV53 (Kan<sup>k</sup>).

The targeted deletion of the DNA sequence in atpG of M. smegmatis (coding for γ loop γ166179), was confirmed by sequencing. The integrity of the ATP synthase operon was analyzed by PCR mapping using Taq DNA polymerase (Thermo Fischer) and sets of primer pairs spanning the whole operon (Table 1, Fig. 2AB).
Furthermore, Southern blotting was performed using (i) a synthesized 42 bp DNA (γ166179) probe with DIG labelling at its 3’ends, and (ii) the 200 bp DNA recombineering oligonucleotide, to confirm the deletion of the loop sequence. The latter was labelled using the random primed DNA labelling kit from Roche. Approximately 6 µg of genomic DNAs were double digested overnight with BamHI/StuI, and DNA fragments were separated by 1% agarose gel electrophoresis. The gel was treated with depurination, denaturation and neutralization solution (according to Roche protocol) and then transferred overnight by capillary action to a nylon membrane. DNA fixation was done using UV crosslinking. The membrane was prehybridized (3 hrs) and then hybridized (overnight) in DIG easy Hyb solution at 44 °C (42 bp probe) and 52 °C (200 bp probe). The next day the detection was done using the DIG Nucleic acid Detection kit (Roche). Finally the mutant and parental strains were subjected to whole genome sequencing (AIT Singapore) to confirm that the only difference between the two strains consists of the loop encoding sequence.

Preparation of inverted membrane vesicles from M. smegmatis

In order to purify membrane fractions (inverted vesicles) of M. smegmatis for ATP synthesis, hydrolysis and proton pumping assays, cells were grown overnight at 37 °C in 7H9 supplemented with 10% ADC, 0.5% glycerol and 0.05% Tween80 until it reached OD₆₀₀ 0.6. The culture was expanded in 200 ml supplemented 7H9 and grown in 1 litre shake flasks (180 rpm) until OD₆₀₀ 0.6. This culture was used to inoculate a 500 ml culture that was grown overnight in 2 litre shake flasks (180 rpm) until an OD₆₀₀ of 0.6. About 5 g (wet weight) of wild type M. smegmatis, the Δγ166179 mutant and γ conform were resuspended in 20 ml membrane preparation buffer (50 mM MOPS, 2 mM MgCl₂, pH 7.5) containing EDTAfree protease inhibitor cocktail (1 tablet in 20 ml buffer, RocheUSA) and 1.2 mg/ml lysozyme. The suspension was stirred at room temperature for 45 min and additionally supplemented with 300 µl 1 M MgCl₂ and 50 µl DNase I (Thermo Fischer, USA), and continued stirring for another 15 min at room temperature. All subsequent steps were performed on ice. Cells were broken by 3 passages through an ice precooled Model M110L Microfluidizer processor (M110L) at 18000 psi. The suspension containing lysed cells was centrifuged at 4200 x g at 4 °C for 20 min. The supernatant containing membrane fraction was further subjected to ultracentrifugation 45000 x g at 4 °C for 1 h. The supernatant was discarded and the precipitated membrane fraction was resuspended in membrane preparation
buffer containing 15% glycerol, aliquoted, snap frozen and stored at 80 °C. The concentrations of the proteins in the vesicles were determined by the BCA method. Inverted membrane vesicles were stored at 80 °C.

**ATP hydrolysis activity measurements**

A continuous ATP hydrolysis assay was applied to measure the ATPase activity of wild type membrane vesicles from M. smegmatis, the Δγ166179 mutant and γ_con form. In this assay ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was detected at a wavelength of 340 nm as described [42, 43]. Consumption of NADH by the type II NADH dehydrogenase (NDH2) was inhibited by thioridazine (80 µM). In addition, IMVs were also measured in the absence of ATP and presence of thioridazine to identify any slight background oxidation of NADH, and to determine the amount of MgATP hydrolyzed per minute and total protein by the wildtype M. smegmatis ATP synthase and the Δγ166179 mutant. ATP synthase inhibitors were added to the solution before measurement and the vesicles were added at the beginning of the measurement. Recombinant αβγ complex of Geobacillus stearothermophilus (formerly Bacillus PS3) ATP synthase was used as a control.
Assay for ATP-driven proton translocation

ATP-driven proton translocation into IMV’s of M. smegmatis was measured by a decrease of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence using a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Palo Alto) according to Haagsma et al. [12]. IMV’s (0.18 mg per ml) were preincubated at 37 °C in 10 mM HEPES KOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂ containing 2 µM ACMA and a baseline was monitored for 5 min. The reaction was started by adding 2 mM ATP or 2 mM NADH. After about 8 min, any proton gradient was collapsed by the addition of 2 µM of the uncoupler SF6847. The excitation and emission wavelengths were 410 nm and 480 nm, respectively.

ATP synthesis assay

ATP synthesis was measured in flat bottom white microtiter 96 well plates (Corning USA). The reaction mix (50 µl), made in assay buffer (50 mM MOPS, pH 7.5, 10 mM MgCl₂) containing 10 µM ADP, 250 µM Pᵢ and 1 mM NADH. Concentration of Pᵢ was adjusted by addition of 100 mM KH₂PO₄ salt dissolved in the assay buffer. ATP synthesis was started by adding inverted vesicles of the M. smegmatis wild type, the Δγ₁₆₆₁₇₉ mutant protein or γ_con protein to a final concentration of 5 µg/ml. The reaction mix was incubated at room temperature for 30 min before 50 µl of the CellTiterGlow reagent was added and the mix was incubated for another 10 min in dark at room temperature. Produced luminescence, which is correlated to the synthesized ATP, was measured by a Tecan plate reader Infinite 200 Pro (Tecan USA), using the following parameter: luminescence, integration time 500 ms, attenuation none.
Acknowledgments

We thank Dr. H. Sielaff (School of Biological Sciences, NTU) for his help on data analysis. We are grateful to Dr S. S. M. Malathy (School of Biological Sciences, NTU) for the art work of the Figures 1 and 7. We are grateful to Prof. Dr A. D. Vinogradov, Moscow State University, Russia and Dr S. P. Rao for helpful discussions as well as the Professors. R. W. Bates and H. Hirao, NTU, for critical reading the manuscript. This research was supported by the Ministry of Health (MOH), Singapore (NMRC, CBRG12nov049; G. Grüber and T. Dick).

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Author contributions


and G.G. wrote the paper.
References


**Figure legends**

**Fig. 1.** Structural model of the mycobacterial F1F0 ATP synthase and amino acid sequence alignment of γ subunits. Subunits α (orange) and β (green) form the static hexameric catalytic part. One α and β subunit were removed to reveal subunit γ (yellow), which acts as the central stalk rotor. The regulatory ε subunit in blue is in the extended form reaching in to the catalytic subunit. The unique mycobacterial γ loop is shown in red in the vicinity to the cring loop residues Q46 to E48 of M. phlei (wheat; [8]). The subunits a, b, b’ and δ are shown as cartoon in the red, light blue and light green, respectively. (B) Amino acid sequence alignment of subunit γ of the E. coli, human mitochondrial, of the nonpathogenic M. smegmatis and the pathogenic M. tuberculosis HR37v as well M. africanum F1F0 ATP synthase. The secondary structure of E. coli γ is based on the crystal structure PDB ID 3OAA. The residues of the unique γ loop are highlighted in red frame, whereby identical amino acids are marked in red and identical residues only in the two pathogenic forms are marked in green. The Q residue is proposed to interact with the loop of subunit c [13]. Helix α3, β3 and α4 (salmon) are described to form a structural path to interact with the C-terminal domain of the catalytic β subunit.

**Fig. 2.** Confirmation of the loop deletion in the generated M. smegmatis Δγ166179 mutant.

(A) Recombineering scheme: 200 bp dsDNA allelic exchange substrate was constructed by PCR extension of a synthesized 100 bp dsDNA spanning the intended loop deletion site of the γ subunit gene. dsDNA homologous recombination after transforming M. smegmatis mc² 155 was facilitated by recombineering proteins Gp60 and Gp61. (B) PCR mapping scheme of the F1F0 synthase operon of M. smegmatis and sizes of obtained PCR products. (C) Southern blot hybridization of M. smegmatis wild type and ΔatpG166179 mutant showing the deletion of the 42 bp γ loop encoding sequence. Genomic DNA from M. smegmatis mc² 155 (lane 1 and 3) and M. smegmatis ΔatpG166179 (lane 2 and 4) were double digested with BamHIStuI. The blot labelled lane 1 and 2 were probed with a probe representing the 42 bp γ loop DNA. As shown in lane 1, wild type bacteria showed a 700 bp BamHIStuI fragment, whereas the deletion mutant had lost this sequence from its genome (lane 2). The blot labelled lane 3 and 4 were probed with a 200 bp probe flanking the deletion site. In this cases, wild type bacteria show again the 700 bp BamHIStuI fragment (lane 3). In comparison, the deletion mutant revealed the shorter BamHIStuI fragment (lane 4), indicating the sucessful deletion of the loop encoding sequence. M, marker lane.
**Fig. 3.** ATP hydrolysis of wild type and Δγ166179 mutant M. smegmatis IMV’s. (A) Continuous ATPase activity of WT and the M. smegmatis Δγ166179 mutant using IMVs measured at 2 mM MgATP. Line 1 (grey), WTIMV’s in the presence of the NDH2 inhibitor thioridazine (80 µM) without MgATP, showing the inhibitory effect of thioridazine. Line 2 (blue) and 3 (red), WTand Δγ166179 mutant IMV’s in the presence of thioridazine and MgATP. (B) Specific ATPase activity of WT and Δγ166179 mutant FATP synthase. (C) Specific ATPase activity of WT, γcon (complementation construct M. smegmatis ΔatpGx pMV262atpG) and Δγ166179 mutant FATP synthase. Values are mean ± SD based on the three independent determinations.

**Fig. 4.** ATP hydrolysis and inhibition of wild type M. smegmatis IMV’s. Concentration dependent inhibitory effects on the ATPase activity of IMV’s of wildtype M. smegmatis by resveratrol (A), quercetin (B), BDQ (C), 4Chloro7-nitrobenzofurazan (NBDCI) (D), and efrapeptin c (E). The inhibitors (magenta) and their respective binding sites insight the crystal structure of F₁ or FATP synthase are shown.
**Fig. 5.** Substrate driven proton pumping in IMV’s. (A) M. smegmatis wild type membrane vesicles were diluted to 0.18 mg/ml in buffer containing 10 mM HEPES KOH (pH 7.5), 100 mM KCl, 5 mM MgCl₂ and 2 µM ACMA. Fluorescence quenching of ACMA by wildtype IMV’s was studied after the addition of a substrate (2 mM ATP (profile 2)) or 2 mM NADH (profile 3). The uncoupler (SF6847) was added at the shown time point to collapse the proton gradient. In the control experiment, buffer was added in place for substrate (profile 1). (B) Fluorescence quenching of ACMA by IMV’s of the Δγ166179 mutant after addition of ATP (profile 2) in comparison to the wild-type IMV’s (profile 1). Profile 3 reveals the quenching of wild type IMV’s in the presence of 2 mM NADH as a comparison.

**Fig. 6.** Comparison of ATP synthesis of the wild type and γ mutant containing IMV’s. (A) ATP synthesis measured for WT (blue) and Δγ166179 mutant (red) IMVs of M. smegmatis. (B) Comparison of ATP synthesis of the Δγ166179 mutant and the complemented strain γ_con containing IMVs. Effect of bedaquiline on ATP synthesis using the M. smegmatis WT (C) and Δγ166179 mutant IMVs (D).

**Fig. 7.** Proposed communication of the cring rotation and catalytic events in the α₃:β₃:headpiece via the mycobacterial γ loop, α4, β3 and α3 in subunit γ. Structure of the bovine mitochondrial F₁ATPase (PDB ID: 1E79; [44]) with Mtγ₁₆₅-₁₇₈ (red) and the cring (wheat) of M. phlei (PDB ID: 4V1G; [9]). The mycobacterial γ loop is linked to α4 in subunit γ, which forms a structural path via β3 to α3 (salmon), which directly interacts with the C terminal domain of the catalytic β subunit (inset). The position of the Mtγ₁₆₅₁₇₈ peptide with respect to the βDP and αTP sites are shown. The sites are defined based on [45], where βTP contains an ATP analog (AMPPNP), βDP with ADP and βE site none. Subunit α (green) contains the ATP analog in all the three sites. The vertical line represents the axis of rotation for the γ subunit.
(Inset) Two views of the Mtγ165178 peptide showing the polar conserved residues (green) in slow growing species like M. tuberculosis, that are substituted by nonpolar residues in fast growing species like M. smegmatis. These residues are closer to the loop region (represented by yellow dotted line) that connects the α2 helix with β2 strand and might differentially affect the interaction of α2 helix with the Cterminal domain of the βsubunit in fast and slow growing species.
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Table 1: List of oligonucleotides used in these studies
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Figure 3A-C
Figure 4AE
Figure 5AB
Figure 6A-C

(A) (B)
Double stranded DNA homology exchange by recombineering

Construct 200 bp dsDNA AES by PCR

- 50bp
- 50bp

dsDNA AES

- Upstream homology to atpG
- Downstream homology to atpG
- 100bp
- 100bp

dsDNA homology exchange by proteins Gp60 and Gp61

- Upstream atpG
- 42 bp loop
- Downstream atpG
| atpB | atpE | atpFH | atpA | atpG | atpD | atpC |
Deletion of the specific subunit $\gamma$-loop166-179 of mycobacterial F-ATP synthases sheds light on the inhibition of ATP hydrolysis driven $H^+$-pumping of this class of engines

Adam Hotra$^{1,2,3}$, Manuel Suter$^4$, Goran Biuković$^4$, Ragunathan Priya$^1$, Subhashri Kundu$^4$, Thomas Dick$^4,*$ and Gerhard Grüber$^{1,*}$

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Running Title: Inhibitory element of mycobacterial F-ATP synthase Supplementary Figure Legends Figure S1: As presented resveratrol (A) showed a weak bacterial growth inhibition at concentration of 200 – 400 µM. Interestingly, quercetin (B), with a similar structure to resveratrol and with the identical binding site, didn’t inhibit growth of *M. smegmatis* at all, even at the high concentrations used. Although similar in structure, the inhibitor may not pass the bacterial wall or membrane (the F1-headpiece is oriented inside the bacterial lumen).

BDQ
(C) inhibited *M. smegmatis* growth with an IC$_{50}$ of 62.5 nM. In comparison, NBD-Cl (D) displayed moderate growth inhibition with a half maximal inhibitory concentration (IC$_{50}$) of approximately 12.5 µM. These experiments were carried out in 96 well plate assays with *M. smegmatis mc²* 155 strains. The bacterial growth after 24 hour incubation, measured as bacterial concentration, was determined by absorbance at 600 nm (OD$_{600}$) with a Tecan plate reader (Middlebrook 7H9 growth media with 10% ADC, 0.5% glycerol and 0.05% Tween80; initial OD$_{600}$ 0.05). Ciprofloxacin and drug free were used as positive and negative control, respectively.