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Identification of Missing Carbon Fixation Enzymes as Potential Drug Targets in *Mycobacterium Tuberculosis*

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Abstract:

Metabolic adaptation to the host environment has been recognized as an essential mechanism of pathogenicity and the growth of *Mycobacterium tuberculosis* (*Mtb*) in the lungs for decades. The *Mtb* uses CO_2 as a source of carbon during the dormant or non-replicative state. However, there is a lack of biochemical knowledge of its metabolic networks. In this study, we investigated the CO_2 fixation pathways (such as ko00710 and ko00720) most likely involved in the energy production and conversion of CO_2 in *Mtb*. Extensive pathway evaluation of 23 completely sequenced strains of *Mtb* confirmed the existence of a complete list of genes encoding the relevant enzymes of the reductive tricarboxylic acid (rTCA) cycle. This provides the evidence that an rTCA cycle may function to fix CO_2 in this bacterium. We also proposed that as CO_2 is plentiful in the lungs, inhibition of CO_2 fixation pathways (by targeting the relevant CO_2 fixation enzymes) could be used in the expansion of new drugs against the dormant *Mtb*. In support of the suggested hypothesis, the CO_2 fixation enzymes were confirmed as a potential drug target by analyzing a number of attributes necessary to be a good bacterial target.

Keywords: Tuberculosis, CO_2 fixation enzyme, rTCA cycle, dormant phase, drug discovery

1 Introduction

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) is one of the most feared diseases of present times, and is one of the top 10 causes of death worldwide. As per World Health Organization (WHO) records in 2015, the estimated number of TB cases was 10.4 million, followed by 1.8 million TB related deaths annually (http://www.who.int/gho/tb/en/). Though the rate of new TB cases has shrunk, the synergistic relationship between drug resistance TB [multi drug-resistant (MDR); extensively drug-resistant (XDR)] and HIV (TB/HIV coinfection) has transformed TB into a serious public health threat [1], [2], [3]. As an intracellular pathogen, *Mtb* is exposed to a strong host response, including low pH (H+), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and toxic gasses such as nitric oxide (NO), carbon monoxide (CO), and superoxide during the infection [4]. In response to host attack, *Mtb* has evolved a variety of defense mechanisms to counterattack the toxic environment. For example, *Mtb* could utilize superoxide dismutase (SOD, EC 1.15.1.1) and catalase (hydroxyperoxidases, EC 1.11.1.6) enzymes to transform the toxic reactive oxygen intermediates (such as superoxide and H_2O_2) into water and oxygen [5], [6], [7], [8], [9]. Dormancy of *Mtb* is an alternative counter mechanism which allows *Mtb* to survive in the host as well as in the unhostile environment for decades [10], [11], [12], [13], [14], [15]. To adjust to the low oxygen in the dormancy phase, *Mtb* triggers a metabolic switch from the aerobic to anaerobic respiration [16], [17] allowing the survival, growth and persistence of the pathogen. *Mtb* has evolved genes for NO and CO resistance to counterattack NO [5], [6], [7], [8], [9] and CO toxicity [4], [18], [19], vital for long-term survival of TB in the host. On the contrary, harmful effects of CO on *E. coli*, *P. aeruginosa*, and *S. aureus* have been recognized, in which exposures to CO inhibit key enzymes of the electron

transport chain required for bacterial respiration, resulting in microbial death [20], [21]. As *Mtb* resides in the CO_2 rich atmosphere of the lung, it can utilize CO_2 as a source of carbon and energy [22], [23], [24], [25], [26]. *Mtb* retains all the genes requisite to catabolize cholesterol to CO_2 through the tricarboxylic acid cycle (TCA) [27]. Six autotrophic CO_2 fixation pathways are known to date. Three of them (rTCA cycle, the reductive acetyl-CoA pathway and the 3-hydroxypropionate bicycle) were found in bacteria [28]. The existence of a key enzyme of the rTCA cycle, including fumarate reductase, ATP-citrate lyase β-chain, and α-ketoglutarate: ferredoxin oxidoreductase in *Mtb* suggests that a reductive TAC may operate to fix CO_2 in TB [29], [30], [31]. However, a complete list of key enzymes of the rTCA cycle that most likely function in carbon fixation is unknown for *Mtb*. In the present study, two carbon fixation pathways [denoted as "carbon fixation in photosynthetic organisms" and "carbon fixation pathways in prokaryotes" in Kyoto Encyclopedia of Genes and Genomes (KEGG)] most likely involved in energy production and conversion of CO_2 were discovered by mapping the protein sequences of all 23 strains of *Mtb* in KEGG database. Further, as CO_2 is plentiful in the human lungs and the genes involved in the CO_2 fixation pathways are primarily expressed during the dormant phase, inhibition of CO_2 fixation could be used in the development of novel drug treatments against TB.

2 Materials and Methods

2.1 Data

The protein sequences of 23 completely sequenced strains of *M. tuberculosis* were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/genome/genomes/166). List of the studied strains were listed in Supplementary data: Table S1.

2.2 Identification of Putative Orthologous

The protein sequences of *Mtb* were separated according to their type of strain. An all-against-all protein sequence comparison was executed by using Basic Local Alignment Search Tool (BLAST) [32]. The best hit from every other genome was compared to proteins from the given genome. The pair of sequences that were the best match when either sequence was used as a query was defined as Best Bidirectional Hits (BBH), and the sequences in the pair were considered related. The relationship between the sequence pair was not estimated, if the best hit depended on only one direction. In addition to the above criterion, a sequence pair was considered to be related by BBH if the e-value from each BLAST comparison was less than 0.001 in both directions. Since in this methodology only comparisons between protein sequences from two separate genomes were executed, obvious paralogous genes were avoided. Furthermore, the introduction of the stringent e-value cutoff eliminated false matches. The output of this step was a table of putative orthologous genes.

2.3 Pathway Analysis

A complete set of protein sequences from the table of putative orthologous genes was annotated using KEGG Automatic Annotation Server (KAAS; http://www.genome.jp/kegg/kaas/) [33]. KAAS was completed using the BBH method. The identified genes involved in the relevant pathways were compared to reference pathways of Mycobacterium species to identify complete versus incomplete or orphan pathways. A pathway was labeled complete if all the genes in the reference pathway were found in the putative orthologs table, whereas a pathway was considered as an orphan if it was not found in the reference pathways of Mycobacterium species in KEGG database. In this study, two orphan pathways, i.e. "carbon fixation in photosynthetic organisms (ko00710)" and "carbon fixation pathways in prokaryotes (ko00720)" were discovered.

2.4 Enzyme Annotation

Protein sequences from both pathways were annotated for domain (http://pfam.sanger.ac.uk/) [34], molecular function (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) [35], and metabolic pathway (http://www.genome.jp/kegg/). The relevant information was also retrieved from TDR target database V5 (http://tdrtargets.org) [36] and EMBL-EBI InterProScan-5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) [37].

2.5 Potential Targets

All proteins from the pathway were compared with the human proteome by using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The proteins with no human orthologous genes, at query coverage ≥50 % and sequence identity ≥40 % were considered as probable candidates. The relevant information of known targets and related drugs were retrieved from the TDR target database (http://tdrtargets.org) [36] and the DrugBank database (http://www.drugbank.ca/) [38], [39], [40], [41], respectively. The essentiality of genes was calculated by BLAST against the Database of Essential Genes (http://www.essentialgene.org). The antigenic region (epitope) of proteins was predicted by an EMBOSS explorer (http://emboss.bioinformatics.nl/cgi-bin/emboss/antigenic). The transmembrane region and protein mass was predicted by TMHMM-V.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and molecular weight calculator tool (http://www.sciencegateway.org/tools/proteinmw.htm), respectively.

3 Results and Discussion

3.1 Identification of Missing Genes and Enzymes for Carbon Fixation

The present investigation identified and characterized common and orphan pathways using the complete set of protein sequences from the 23 strains of *Mtb*. A total of 90,737 unique protein entries were extracted using Perl script developed in-house (https://gist.github.com/amitkatiyar/). All-against-all comparison showed 7421 unique putative orthologs (bidirectional-best BLAST hits) containing approximately 2558 (35 %) proteins in >90 % of the tested strains. These 7421 unique putative orthologs were mapped to 136 annotated metabolic pathways comprising of 1769 KEGG orthologs (Supplementary data: Table S2). We found one or more proteins from 33 pathways that were formerly not reported in the KEGG for Mycobacteria. Among these pathways, two interesting pathways (i.e. ko00710: carbon fixation in photosynthetic organisms and ko00720: carbon fixation pathways in prokaryotes) belonging to class "energy metabolism" were selected. The extensive pathway analysis revealed a total of 222 and 983 proteins encoding for 10 (ko00710) and 32 (ko00720) enzymes, respectively, which were formerly not annotated for *Mtb* in KEGG (Table 1; Supplementary data: Table S3). These protein enzymes were re-annotated using Pfam domain and KEGG pathways. The results show that protein enzymes were correctly annotated and are involved in the rTCA cycle for CO_2 fixation (Supplementary data: Table S4). These rTCA cycle-specific enzymes were found to be shared conserved among all the strains of TB (Table 2). However, the missing enzymes in a few of the observed strains may be due to issues with computational annotation. The enzyme acetyl-CoA C-acetyltransferase (EC2.3.1.9; K00626) contains the maximum number of orthologous genes (214) and more than seven paralogous genes in each tested strain. The CO_2 fixation pathway enzymes of TB (data compiled from 23 strains) and cyanobacteria (data compiled from 75 strains) were compared that suggesting a close relationship between them (Supplementary data: Table S5). The result shows an identical pathway (ko0710) with eight shared enzymes between Cyanobacteria and TB (Figure 1). The remaining two key enzymes, namely pyruvate, orthophosphate dikinase (K01006; ppdK; EC 2.7.9.1) and fructose-1, 6- bisphosphatase II (K02446; glpX; EC 3.1.3.11) were only present in *Mtb*. Notably, we did not find pathways-00720 in Cyanobacteria as found in TB.

Table 1: Carbon fixation enzymes of the reductive citric acid cycle (rTCA) in *Mycobacterium tuberculosis.*

*KO	Enzyme description	*EC number
	(A) ko00710: carbon fixation in photosynthetic organisms	
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37
K00134	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	EC:1.2.1.12
K00615	Transketolase (TK)	EC:2.2.1.1
K00927	Phosphoglycerate kinase (PGK)	EC:2.7.2.3
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1
K01624	Fructose-bisphosphate aldolase, class II (FBA)	EC:4.1.2.13
K01783	Ribulose-phosphate 3-epimerase (rpe)	EC:5.1.3.1
K01803	Triosephosphate isomerase (TIM)	EC:5.3.1.1
K01808	Ribose 5-phosphate isomerase B (rpiB)	EC:5.3.1.6
K02446	Fructose-1,6-bisphosphatase II (glpX)	EC:3.1.3.11
	(B) ko00720: carbon fixation pathways in prokaryotes	
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37
K00031	Isocitrate dehydrogenase (IDH1)	EC:1.1.1.42

K00031	Isocitrate dehydrogenase (IDH2)	EC:1.1.1.42
K00174	2-oxoglutarate ferredoxin oxidoreductase subunit alpha (korA)	EC:1.2.7.3
K00175	2-oxoglutarate ferredoxin oxidoreductase subunit beta (korB)	EC:1.2.7.3
K00239	Succinate dehydrogenase flavoprotein subunit (sdhA)	EC:1.3.99.1
K00240	Succinate dehydrogenase iron-sulfur subunit (sdhB)	EC:1.3.99.1
K00241	Succinate dehydrogenase cytochrome b556 subunit (sdhC)	EC:1.3.99.1
K00242	Succinate dehydrogenase membrane anchor subunit (sdhD)	EC:1.3.99.1
K00244	Fumarate reductase flavoprotein subunit (frdA)	EC:1.3.99.1
K00245	Fumarate reductase flavoprotein subunit (frdB)	EC:1.3.99.1
K00246	Fumarate reductase subunit C (frdC)	EC:1.3.99.1
K00247	Fumarate reductase subunit D (frdD)	EC:1.3.99.1
K00626	Acetyl-CoA C-acetyltransferase (2.AA440)	EC:2.3.1.9
K00925	Acetate kinase (ackA)	EC:2.7.2.1
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1
K01491	Methylenetetrahydrofolate dehydrogenase (NADP+)//Methenyltetrahydrofolate cyclohydrolase (fold)	EC:1.5.1.5 EC:3.5.4.9
K01679	Fumarate hydratase, class II	EC:4.2.1.2
K01681	Aaconitate hydratase (ACO)	EC:4.2.1.3
K01782	3-hydroxyacyl-CoA dehydrogenase//enoyl-CoA hydratase//3-hydroxybutyryl-CoA epimerase (fadJ)	EC:1.1.1.35// EC:4.2.1.17// EC:5.1.2.3
K01847	Methylmalonyl-CoA mutase (MUTB) AA750	EC:5.4.99.2
K01895	Acetyl-CoA synthetase (ACSS)	EC:6.2.1.1
K01902	Succinyl-CoA synthetase alpha subunit (sucD)	EC:6.2.1.5
K01903	Succinyl-CoA synthetase beta subunit (sucC)	EC:6.2.1.5
K01958	Pyruvate carboxylase (PC)	EC:6.4.1.1
K01963	Acetyl-CoA carboxylase carboxyl transferase subunit beta (accD)	EC:6.4.1.2
K01965	Propionyl-CoA carboxylase alpha chain (PCCA)	EC:6.4.1.3
K01966	Propionyl-CoA carboxylase beta chain (PCCB)	EC:6.4.1.3
K03518	Carbon-monoxide dehydrogenase small subunit	EC:1.2.99.2
K03519	Carbon-monoxide dehydrogenase medium subunit	EC:1.2.99.2
K03520	Carbon-monoxide dehydrogenase large subunit	EC:1.2.99.2
K05606	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase (MCEE)	EC:5.1.99.1
K13788	Phosphate acetyltransferase (PTA)	EC:2.3.1.8

These key enzymes were found in two pathways, namely ko00710[#] and ko00720[#], containing 10 and 32 unique enzymes, respectively. The pathway analysis revealed the role of identifying enzymes in CO_2 fixation. [#]ko00710: Carbon fixation in photosynthetic organisms; [#]ko00720: Carbon fixation pathways in prokaryotes; *KO: KEGG orthologs; *EC number: Enzyme commission number.

Table 2: Comparative assessment of existing carbon fixation enzymes of rTCA cycle in 23 strains of *Mycobacterium tuberculosis*.

*KO	Enzyme description	*EC	No of strains	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
(A) ko00710: carbon fixation in photosynthetic organisms																										
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00134	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	EC:1.2.1.12	22	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00615	Transketolase (TK)	EC:2.2.1.1	22	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
K00927	Phosphoglycerate kinase (PGK)	EC:2.7.2.3	22	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1	24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
K01624	Fructose-bisphosphate aldolase, class II (FBA)	EC:4.1.2.13	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K01783	Ribulose-phosphate 3-epimerase (rpe)	EC:5.1.3.1	22	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K01803	Triosephosphate isomerase (TIM)	EC:5.3.1.1	22	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
K01808	Ribose 5-phosphate isomerase B (rpiB)	EC:5.3.1.6	22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
K02446	Fructose-1,6-bisphosphatase II (glpX)	EC:3.1.3.11	20	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1
(B) ko00720: carbon fixation pathways in prokaryotes																										
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00031	Isocitrate dehydrogenase (IDH)	EC:1.1.1.42	23	2	3	2	2	2	1	2	2	2	2	2	1	1	2	2	2	2	2	1	2	2	2	2
K00174	2-oxoglutarate ferredoxin oxidoreductase subunit alpha (korA)	EC:1.2.7.3	22	1	1	2	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00175	2-oxoglutarate ferredoxin oxidoreductase subunit beta (korB)	EC:1.2.7.3	22	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00239	Succinate dehydrogenase flavoprotein subunit (sdhA)	EC:1.3.99.1	23	2	2	1	2	2	2	2	2	2	2	2	1	1	2	1	2	2	2	2	2	2	2	2
K00240	Succinate dehydrogenase iron-sulfur subunit (sdhB)	EC:1.3.99.1	23	2	2	2	2	2	1	2	2	2	2	2	2	3	2	2	2	2	2	2	2	2	2	2
K00241	Succinate dehydrogenase cytochrome b556 subunit (sdhC)	EC:1.3.99.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

KO	Description	EC	N																						
K00242	Succinate dehydrogenase membrane anchor subunit (sdhD)	EC:1.3.99.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00244	Fumarate reductase flavoprotein subunit (frdA)	EC:1.3.99.1	21	1	0	1	0	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1
K00245	Fumarate reductase flavoprotein subunit (frdB)	EC:1.3.99.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00246	Fumarate reductase subunit C (frdC)	EC:1.3.99.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00247	Fumarate reductase subunit D (frdD)	EC:1.3.99.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K00626	Acetyl-CoA C-acetyltransferase	EC:2.3.1.9	23	9	10	7	9	9	8	10	10	9	10	7	11	8	10	10	10	10	8	10	11	8	11
K00925	Acetate kinase (ackA)	EC:2.7.2.1	22	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
K01491	Methylenetetrahydrofolate dehydrogenase (NADP+) / Methenyltetrahydrofolate cyclohydrolase (fold)	EC:1.5.1.5 EC:3.5.4.9	22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
K01679	Fumarate hydratase, class II	EC:4.2.1.2	20	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1
K01681	Aaconitate hydratase (ACO)	EC:4.2.1.3	19	1	1	0	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1
K01782	3-hydroxyacyl-CoA dehydrogenase / enoyl-CoA hydratase / 3-hydroxybutyryl-CoA epimerase (fadJ)	EC:1.1.1.35 EC:4.2.1.17 EC:5.1.2.3	23	1	1	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1	2	1	1	1	1
K01847	Methylmalonyl-CoA mutase (MUTB)	EC:5.4.99.2	20	2	2	0	2	2	3	2	1	2	2	0	2	2	2	2	2	2	0	2	2	2	2
K01895	Acetyl-CoA synthetase (ACSS)	EC:6.2.1.1	22	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1
K01902	Succinyl-CoA synthetase alpha subunit (sucD)	EC:6.2.1.5	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K01903	Succinyl-CoA synthetase beta subunit (sucC)	EC:6.2.1.5	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K01958	Pyruvate carboxylase (PC)	EC:6.4.1.1	21	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1
K01963	Acetyl-CoA carboxylase carboxyl transferase subunit beta (accD)	EC:6.4.1.2	21	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1
K01965	Propionyl-CoA carboxylase alpha chain (PCCA)	EC:6.4.1.3	20	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1
K01966	Propionyl-CoA carboxylase beta chain (PCCB)	EC:6.4.1.3	22	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K03518	Carbon-monoxide dehydrogenase small subunit	EC:1.2.99.2	20	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	0	1	0	1

KO	Enzyme	EC number	No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23
K03519	Carbon-monoxide dehydrogenase medium subunit	EC:1.2.99.2	19	1	0	2	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
K03520	Carbon-monoxide dehydrogenase large subunit	EC:1.2.99.2	20	1	1	2	1	0	1	1	1	1	1	1	1	2	1	0	1	1	1	1	1	1	1	1
K05606	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase (MCEE)	EC:5.1.99.1	22	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
K13788	Phosphate acetyltransferase (PTA)	EC:2.3.1.8	23	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Strain-wise comparisons showed that most of the strains (except for a few strains that lack either one or two genes) contain genes encoding for respective enzymes in both pathways. *KO: KEGG orthologs; *EC number: Enzyme commission number; *Mycobacterium tuberculosis (strains): given below. S1: CCDC5180 uid161941; S2: H37Rv uid170532; S3: Haarlem3 NITR202 uid202216; S4: CDC1551 uid57775; S5: CCDC5079 uid161943; S6: RGTB423 uid162179; S7: CTRI 2 uid161997; S8: KZN 4207 uid83619; S9: CCDC5079_uid203790; S10: Beijing NITR203 uid197218; S11: EAI5 NITR206 uid202218; S12: F11 uid58417; S13: RGTB327 uid157907; S14: Erdman ATCC 35801 uid193763; S15: CAS NITR204 uid202217; S16: EAI5 uid212307; S17: KZN 605 uid54947; S18: UT205 uid162183; S19: H37Rv uid57777; S20: H37Ra uid58853; S21: Haarlem uid54453; S22: KZN 1435 uid59069; S23: uid185758.
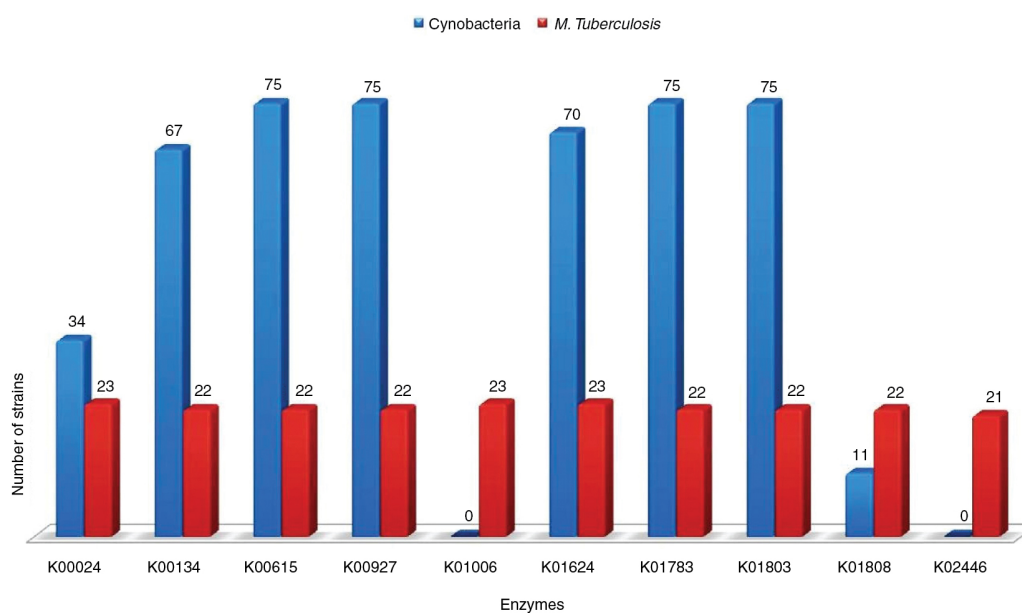
Figure 1: The comparison of CO_2 fixation pathway enzymes (ko00710) between *M. tuberculosis* and Cyanobacteria. The comparison showed that Cyanobacteria shared eight enzymes with *M. tuberculosis*.

3.2   Evidence of CO_2 Fixation by Reductive TCA Cycle

The citrate cycle (TCA cycle, Krebs cycle) is used by all aerobic organisms to produce energy by the oxidation of pyruvate. The TCA cycle is characterized as oxidative or reductive by the presence of three enzymes known as ATP-citrate lyase, 2-oxoglutarate synthase, and fumarate reductase [42], [43], [44]. The rTCA cycle reverses the reactions of the oxidative citric acid cycle by the replacement of three enzymes: the succinate dehydrogenase has to be replaced by the fumarate reductase, the NAD+-dependent 2-oxoglutarate dehydrogenase has to be replaced by the ferredoxin-dependent 2-oxoglutarat synthase and the citrate synthase has to be replaced by the ATP citrate lyase. The *Mtb* genome of 23 strains was annotated to encode a complete TCA cycle in this study. The results disclosed the presence of citrate synthase (EC 2.3.3.1), 2-oxoglutarate dehydrogenase (EC 1.2.4.2) and succinate dehydrogenase (EC 1.3.5.1) enzymes in *Mtb* (Figure 2). The findings indicate evidence of the existence of an oxidative TCA cycle in *Mtb*. Furthermore, the findings of fumarate reductase (EC 1.3.99.1), 2-oxoglutarate synthase (EC 1.2.7.3) and a homologue for citrate-oxaloacetate lyase (EC 4.1.3.6) enzymes showed evidence of an reduvtive TCA cycle for CO_2 fixation in *Mtb* (Figure 2). Most of the enzymes (such as cis-aconitase, EC 4.2.1.3; isocitrate dehydrogenase, EC 1.1.1.42; succinyl-CoA ligase, EC 6.2.1.5; fumarate hydratase, EC 4.2.1.2 and malate dehydrogenase, EC 1.1.1.37) of the oxidative or reductive TCA cycle were common (Figure 2). These results are consistent with the previous hypothesis that Mycobacterium can activate the metabolic switch to anaerobic respiration where a complete or partial TCA cycle may operate in the reductive mode. This switch permits both carbon fixation and restoration to the balance of oxidative and reductive reactions in environmental changes, thus allowing the pathogen to survive, grow, and persist [31]. The first key enzyme of the rTCA pathway is fumarate reductase (Figure 2, reaction 3), which catalyzes the reduction of fumarate to succinate using Ubiquinol as an electron donor. We observed that the genome of *Mtb* encodes four distinct fumarate reductase (EC 1.3.99.1) namely, flavoprotein subunit A (frdA; K00244), subunit B (frdB; K00245), subunit C (frdC; K00246) and subunit D (frdD; K00247) (Table 1; Supplementary data: Table S4). The study also confirmed that the *Mtb* grnome encodes two distinct 2-oxoglutarate ferredoxin:oxidoreductase (OGOR; EC 1.2.7.3) enzymes, namely, subunit alpha (korA; K00174) and subunit beta (korB; K00175) (Table 1; Supplementary data: Table S4). The enzyme OGOR is the key enzyme of the rTCA cycle that fixes carbon dioxide [45], [46], [47]. The enzyme OGOR (Figure 2, reaction 5) catalyzes the reductive carboxylation of succinyl-CoA to 2-oxoglutarate. An anaerobic enzyme "ATP-citrate lyase" catalyzes the division of citrate to oxaloacetate and acetyl-coA. A homologue for citrate lyase subunit beta (citE; K01644; EC 4.1.3.6), synonym of citrate-oxaloacetate was observed in the genome of *Mtb*. Remarkably, we did not find the key activity of the pyruvate synthase (EC 1.2.7.1) in an rTCA cycle of *Mtb*. This may be due to a single or a set of alternative enzymes that can fill the missing reactions. Another important enzyme of the rTCA cycle is "isocitrate dehydrogenase or IDH" (Figure 2, reaction 6) that catalyzes the reversible conversion of isocitrate to 2-oxoglutarate in prokaryotes. Here, we observed that the genome of *Mtb* encodes two types of isocitrate dehydrogenase (EC 1.1.1.42), i.e. IDH1 (dimeric) and IDH2 (monomeric) as reported previously in *Mtb* [48]. Notably, all 23 strains of *Mtb* having IDH enzymes indicated their importance

for the organism. Multiple sequence alignment revealed a closer similarity of isocitrate dehydrogenase (ICD)-1 from *Mtb* to eukaryotic NADP$^+$-dependent ICDs, whereas ICD-2 from *Mtb* groups with bacterial ICDs (data not shown). We also explored the TDR database and observed the expression of many rTCA cycle-specific enzymes in dormant phase of TB (Table 3). The findings demonstrated that *Mtb* can activate reductive rTCA (during low oxygen tension or anaerobic environment) for the fixation of CO_2 in TB. In support of this theory, several studies have been presented that *Mtb* could operate a reductive TCA half cycle under anaerobic situations, permitting it to metabolize glucose by generating succinate as an obligatorily secreted, fermentation product of fumarate, following reductive carboxylation of pyruvate or phosphoenolpyruvate to malate and/or oxaloacetate [49]. In addition, several mycobacterial strains including *Mtb* can grow on CO as the sole source of carbon and energy. A remarkable attribute of *Mtb* is its capability to survive with minimal growth inhibition [50] during the elevated CO concentration [30] by expressing CO resistance genes. Carbon monoxide dehydrogenase (CO-DH) is an enzyme catalyzing the oxidation of CO to carbon dioxide in Mycobacterium [4], [30]. Here, we observed that the genome of *Mtb* encodes three copies of the CO-DH (EC 1.2.99.2) enzymes, namely CO dehydrogenase-small subunit (K03518), medium subunit (K03519) and large subunit (K03520). However, the genome of three strains (RGTB423_uid162179; RGTB327_uid157907; UT205_uid162183) of *Mtb* did not encode for CO-DH enzyme. This might be due to lack of annotation or absence of CO resistance genes.
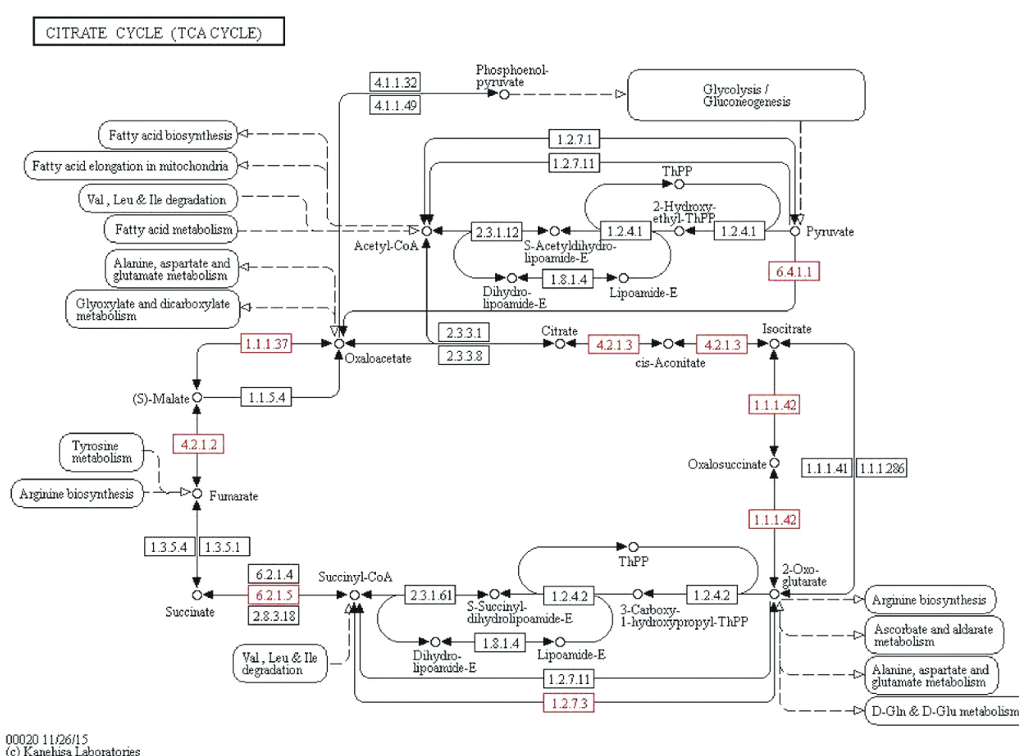
Figure 2: Pictorial representation of reductive citric acid cycle for autotrophic CO_2 fixation. The enzyme catalyzed reactions are shown by arrows. Enzyme activities: 1, malate dehydrogenase (EC 1.1.1.37); 2, fumarate hydratase (fumarase) (EC 4.2.1.2); 3, fumarate reductase; 4, succinyl-CoA synthetase (EC 6.2.1.5); 5, 2-oxoglutarate:ferredoxin oxidoreductase (EC 1.2.7.3); 6, isocitrate dehydrogenase (EC 1.1.1.42); 7, aconitate hydratase (aconitase) (EC 4.2.1.3); 8, ATP citrate lyase (EC 2.3.3.8); and 9, pyruvate: ferredoxin oxidoreductase (EC 1.2.7.1). Fd$_{red}$, reduced ferredoxin. The enzymes observed in the genome of tuberculosis strains were highlighted with red color.

Table 3: Carbon fixation enzymes accepted as a potential drug target in *Mycobacterium tuberculosis*.

*KO	Enzyme description	$EC number	Human homologues (Query coverage/Sequence identity)	Gene expression (dormant phase)			Epitopes/Antigenicity	&Number of homologues in DEG	Trans domains	Associated compounds/-Druggability	Low mass (<100 kDa)
				Lower (%)	Mid (%)	Upper (%)					
(A) ko00710: carbon fixation in photosynthetic organisms											
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37	Human positive: 98/52 %	00–20	NA	NA	13; 33.0 (67.0%)	3	Absent	Nitrofurazone (E. coli)	34.33
K00134	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	EC:1.2.1.12	Human positive: 97/51 %	20–40	NA	NA	16; 72.2 (22.8%)	22	Absent	No drug	35.96
K00615	Transketolase (TK)	EC:2.2.1.1	Human negative: 87/25 %	00–20	NA	NA	30; 51.2 (48.8%)	16	Absent	No drug	79.97
K00927	Phosphoglycerate kinase (PGK)	EC:2.7.2.3	Human negative: 98/38 %	00–20	NA	NA	20; 82.5 (17.5%)	21	Absent	No drug	42.52
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1	Human negative: 07/41 %	00–20	NA	40–60	21; 53.1 (46.9%)	1	Absent	No drug	54.7
K01624	Fructose-bisphosphate aldolase, class II (FBA)	EC:4.1.2.13	Human negative: 27/29 %	00–20	NA	60–80	12; 16.6 (17.5%)	17	Absent	No drug	36.55
K01783	Ribulose-phosphate 3-epimerase (rpe)	EC:5.1.3.1	Human positive: 95/44 %	00–20	40–60	NA	11; 75.7 (24.3%)	14	Absent	No drug	24.24
K01803	Triosephosphate isomerase (TIM)	EC:5.3.1.1	Human negative: 93/39 %	20–40	NA	NA	11; 51.5 (48.5%)	18	Absent	No drug	27.41
K01808	Ribose 5-phosphate isomerase B (rpiB)	EC:5.3.1.6	Human negative: 50/24 %	60–80	NA	80–100	08; 82.5 (17.5%)	7	Absent	No drug	17.66
K02446	Fructose-1,6-bisphosphatase II (glpX)	EC:3.1.3.11	Human negative: 35/26 %	20–40	40–60	NA	13; 33.9 (66.1%)	3	Absent	No drug	39.74
(B) ko00720: Carbon fixation pathways in prokaryotes											
K01782	3-hydroxyacyl-CoA dehydrogenase / Enoyl-CoA hydratase / 3-hydroxybutyryl-CoA epimerase (fadJ)	EC:1.1.1.35 EC:4.2.1.17 EC:5.1.2.3	Human negative: 97/32 %	Unknown	Unknown	Unknown	33;NA	2	Absent	No drug	76.11
K00024	Malate dehydrogenase (MDH)	EC:1.1.1.37	Human positive: 98/52 %	0–20	NA	NA	13; 33.0 (67.0%)	3	Absent	Nitrofurazone (E. coli)	34.33
K00031	Isocitrate dehydrogenase (IDH2)	EC:1.1.1.42	Human negative: 14/25 %	20–40	NA	80–100	38; 87.2 (12.8%)	3	Absent	No drug	82.56

K number	Enzyme	EC	Human positive/negative							Drug	Value
	Isocitrate dehydrogenase (IDH1)		Human positive: 97/66 %			60–80	19; 68.4 (31.6%)	1			45.52
K00175	2-oxoglutarate ferredoxin oxidoreductase subunit beta (korB)	EC:1.2.7.3	Human negative: 12/32 %	Unknown	Unknown	Unknown	17; NA	4	Absent	Nitrofurantoin (E. coli)	46.76
K00174	2-oxoglutarate ferredoxin oxidoreductase subunit alpha (korA)	EC:1.2.7.3	Human negative: 11/30 %	Unknown	Unknown	Unknown	29; NA	3	Absent	No drug	69.68
K03519	Carbon-monoxide dehydrogenase medium subunit	EC:1.2.99.2	Human negative: 56/24 %	NA	40–60	60–80	15; 92.2 (7.8 %)	2	Absent	No drug	31.56
K03520	Carbon-monoxide dehydrogenase large subunit	EC:1.2.99.2	Human negative: 97/23 %	NA	40–60	60–80	33; 45.2 (54.8 %)	2	Absent	No drug	85.9
K03518	Carbon-monoxide dehydrogenase small subunit	EC:1.2.99.2	Human negative: 86/39 %	20–40	NA	60–80	06; 27.4 (72.6 %)	1	Absent	No drug	17.22
K00241	Succinate dehydrogenase cytochrome b556 subunit (sdhC)	EC:1.3.99.1	Human negative: 24/49 %	20–40	NA	80–100	3; 5.5 (94.5 %)	Unknown 3		No drug	16.3
K00242	Succinate dehydrogenase membrane anchor subunit (sdhD)	EC:1.3.99.1	Human negative: 57/25 %	20–40	NA	60–80	4; 6.1 (93.9 %)	1	2	No drug	17.37
K00246	Fumarate reductase subunit C (frdC)	EC:1.3.99.1	Human negative: 66/26 %	NA	NA	60–80	4; 15.8 (84.2 %)	Unknown 3		No drug	14.82
K00247	Fumarate reductase subunit D (frdD)	EC:1.3.99.1	Human negative: 29/38 %	NA	40–60	60–80	5; 52.5 (47.5 %)	Unknown 3		No drug	13.75
K00239	Succinate dehydrogenase flavoprotein subunit (sdhA)	EC:1.3.99.1	Human negative: 84/32 %	20–40	NA	NA	24; 26.9 (73.1 %)	17	Absent	Thiabendazole (E. coli)	70.69
			Human positive:-94/48 %				22; 27.3 (72.7%)				64.83
K00244	Fumarate reductase flavoprotein subunit (frdA)	EC:1.3.99.1	Human negative: 88/37 %	20–40	NA	NA	22; 27.3 (72.7%)	17	Absent	Thiabendazole (E. coli)	63.77
K00240	Succinate dehydrogenase iron-sulfur subunit (sdhB)	EC:1.3.99.1	Human positive: 85/41 %	20–40	NA	NA	10; 31.6 (68.4 %)	8	Absent	No drug	29.34
			Human negative: 87/25 %				10; 42.1 (57.9 %)				28.63
K00245	Fumarate reductase flavoprotein subunit (frdB)	EC:1.3.99.1	Human negative: 89/38 %	20–40	NA	NA	10; 31.6(68.4 %)	8	Absent	No drug	27.24
K01491	Methylenetetrahydrofolate dehydrogenase (NADP+) / Methenyltetrahydrofolate cyclohydrolase (fold)	EC:1.5.1.5 EC:3.5.4.9	Human positive: 91/40 %	0–20	NA	NA	11; 36.2 (63.8 %)	18	Absent	No drug	32.09

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KO	Enzyme	EC number	Human homolog									
K13788	Phosphate acetyltransferase (PTA)	EC:2.3.1.8	Human negative: 12/30 %	NA	40–60	60–80	31; 66.8 (33.2 %)	8		Absent	No drug	75.1
K00626	Acetyl-CoA C-acetyltransferase	EC:2.3.1.9	Human negative: 04-99/21-44 %	Unknown	Unknown	Unknown	03–38; NA	2+1		Absent	No drug	8.8–53.57
K00925	Acetate kinase (ackA)	EC:2.7.2.1	Human negative: 04-22/27-41 %	Unknown	40–60	Unknown	17–38; 61.4 (38.6 %)-NA	6–7		Absent	No drug	41.32–95.4
K01006	Pyruvate,orthophosphate dikinase (ppdK)	EC:2.7.9.1	Human negative: 07/41 %	0–20	NA	40–60	21; 53.1 (46.9 %)	1		Absent	No drug	54.7
K01679	Fumarate hydratase, class II	EC:4.2.1.2	Human negative: 33/30 %	NA	NA	40–60	20; 48.0 (52.0 %)	5		Absent	No drug	29.36
K01681	Aaconitate hydratase (ACO)	EC:4.2.1.3	Human positive: 98/49 %	20–40	NA	80–100	40; 51.6 (48.4 %)	11		Absent	No drug	102.46
K05606	Methylmalonyl-CoA/ethylmalonyl-CoA epimerase (MCEE)	EC:5.1.99.1	Human negative: 86/32 %	Unknown	Unknown	Unknown	6; NA	1		Absent	No drug	16.63
K01847	Methylmalonyl-CoA mutase (MUTB)	EC:5.4.99.2	Human positive: 97/61 %	NA	NA	80–100	38; 86.0 (14.0 %)	Unknown	Unknown	Absent	No drug	80.64
			Human negative: 73/28 %			60–100	27; 57.7 (42.3 %)					64.75
K01895	Acetyl-CoA synthetase (ACSS)	EC:6.2.1.1	Human positive: 95/46 %	NA	40–60	NA	29; 62.3 (37.7 %)	Unknown	Unknown	Absent	No drug	71.48
K01903	Succinyl-CoA synthetase beta subunit (sucC)	EC:6.2.1.5	Human negative: 96/37 %	0–40	NA	NA	15; 33.4 (66.6 %)	6		Absent	No drug	40.93
K01902	Succinyl-CoA synthetase alpha subunit (sucD)	EC:6.2.1.5	Human positive: 95/48 %	0–20	NA	NA	16; 91.3 (8.7 %)	5		Absent	No drug	31.23
K01958	Pyruvate carboxylase (PC)	EC:6.4.1.1	Human positive: 96/47 %	0–40	NA	NA	54; 79.1 (20.9 %)	24		Absent	No drug	124.34
K01963	Acetyl-CoA carboxylase carboxyl transferase subunit beta (accD)	EC:6.4.1.2	Human negative: 81/25 %	Unknown	Unknown	Unknown	26; NA	36		Absent	No drug	51.78
K01966	Propionyl-CoA carboxylase beta chain (PCCB)	EC:6.4.1.3	Human positive: 92/53 %	0–40	NA	NA	26; 75.0 (25.0 %)	38		Absent	No drug	60.38
K01965	Propionyl-CoA carboxylase alpha chain (PCCA)	EC:6.4.1.3	Human negative: 99/39 %	Unknown	Unknown	Unknown	33; NA	32		Absent	No drug	71.13

The selected enzymes were verified by a number of attributes defined as good drug targets (e.g. non-human homologs, unique function, essentiality of genes, gene expression, etc.). Druggability analysis exposed that to this date, no drugs are available against the proposed drug targets. #ko00720: carbon fixation pathways in prokaryotes; *KO: KEGG orthologs; $EC number: Enzyme commission number; &DEG: Database of Essential Genes; NA: Not found.

3.3 Carbon Fixation Enzymes as New Anti-TB Targets

The discovery of new targets that are hard to repress by mutation and essential for the bacterial survival are the primary requirement in the drug discovery process [51]. In the present study, we focused on a number of parameters to confirm "carbon fixation enzymes" as a good antibiotic drug target in TB. The considered parameters included genes homologous to humans, genes essential to the pathogen growth and survival, genes expressed during adaptation to dormancy, chokepoint reaction, low molecular weight of a protein, presence or absence of transmembrane helices, and target publication(s) in PubMed. This study does not include drug findings against carbon fixation enzymes, therefore we neither checked for crystallographic structures nor performed homology modeling. The study is evaluated based on known information. Initially implemented BLAST analysis showed that the proposed targets of TB were non-homologous to human genes (Table 3). This reduces the chances of uninvited host-drug interactions and therefore avoids host toxicity [52]. Next, the proposed targets were examined for the gene essentiality to pathogen growth and survival using the "Database of Essential Genes (DEG)." As a result, we found essential homologues for 35 key enzymes commonly from the pathways ko00710 and ko00720 (Table 3; Supplementary data: Table S6). The three enzymes, namely propionyl-CoA carboxylase beta chain (K01966), acetyl-CoA carboxylase carboxyl transferase subunit beta (K01963) and propionyl-CoA carboxylase alpha chain (K01965) contain the highest number of homologues i.e. 38, 36 and 32, respectively. Targeting for any of these essential genes will prevent or kill bacterial growth. In the analysis, homologues for five enzymes (K00241; K00246; K00247; K01847 and K01895) were not observed, potentially due to lack of essential genes or gene annotation in database of essential genes. Furthermore, the "TDR target database" showed the higher expression (80–100 %) of ribose 5-phosphate isomerase B (rpiB; K01808; EC 5.3.1.6), isocitrate dehydrogenase (IDH2; K00031; EC 1.1.1.42), succinate dehydrogenase cytochrome b556 subunit (sdhC; K00241; EC 1.3.99.1), aconitate hydratase (ACO; K01681; EC 4.2.1.3), and methylmalonyl-CoA mutase (MUTB; K01847; EC 5.4.99.2) enzymes during TB adaptation to dormancy [53] (Table 3). The expression of these and other key enzymes during TB adaptation to dormancy signifies the need to survive in a dormant state. The proposed target genes were also selected by the uniqueness of their essential functions in the metabolome (metabolic chokepoint). We clustered "carbon fixation enzymes" by their unique enzyme commission (EC) number as it might perform unique reactions [54]. As a result, 20 clusters or unique chokepoint reactions were detected from ko00720 pathway of $M.$ $tuberculosis.$ Interestingly, we observed unique chokepoints reactions for all 10 enzymes from ko00710 pathway of $M.$ $tuberculosis$ (Table 1). Enzymes involved in unique essential chokepoint reactions are the good metabolic drug targets as their function cannot be compensated by another enzyme. In addition, the proposed targets also satisfied additional key properties of good drug targets [54] such as low mass [<100 kDa]; no transmembrane domain; and publication in PubMed (Table 3). The selected properties of the proposed targets will be useful due to the following reasons: (1) cloning efficiency is significantly higher at low molecular weight (<100 kDa) as compared with high molecular weight (<100 kDa), (2) protein with no transmembrane domain is easy to crystallize as compared to membrane proteins which are difficult to crystallize due to their heterogeneous nature. In addition, the detergent micelle or liposome used for solubilizing and stabilizing the membrane proteins can interfere with protein crystallization, (3) the publication on the target protein will help to identify unwanted off-target effects, molecular function and structure related information. These results confirmed "carbon fixation enzymes" as a good metabolic drug target of $M.$ $tuberculosis.$ Additionally, several studies have described the essentiality of carbon fixation enzymes for bacterial survival and therefore may be used as a potential target. For instance, six autotrophic pathways and ICD, a key regulatory enzyme (released during late exponential growth phase) in the citric acid cycle are used as autolysis markers [55]. Malate dehydrogenase and the ICD has been reported as a potential antigen for serodiagnosis [56], [57]. Similarly, Mtb requires the enzyme isocitrate lyase (ICL) for survival during nutrient starvation, growth and virulence in $vivo$. The essentiality of icl1 has been reported for slow growth on glycerol [25] and the multiplication of mtb in macrophages and mice [53], [54]. It has been also reported that physiologically significant levels of CO_2 evade the growth arrest of $Mycobacterium$ $bovis$ BCG under persistent hypoxia [30]. Therefore, inhibition of CO_2 fixation enzymes could support the development of novel drug treatments against TB.

4 Conclusions

In this study, we identified and characterized the common and orphan pathways in $M.$ $tuberculosis$. We bioinformatically confirmed the existence of a complete list of genes by encoding the relevant enzymes of the reductive rTCA, most likely involved in energy production and conversion of CO_2. The CO_2 fixation pathway enzymes of TB and cyanobacteria were compared that suggesting a close relationship between them. The CO_2 fixation enzymes were confirmed as a potential drug target by analyzing a number of attributes necessary to be a good bacterial target.

Acknowledgments

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