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Central metabolism in *Mycobacterium smegmatis* during the transition from O₂-rich to O₂-poor conditions as studied by isotopomer-assisted metabolite analysis

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Abstract Isotopomer-assisted metabolite analysis was used to investigate the central metabolism of *Mycobacterium smegmatis* and its transition from normal growth to a non-replicating state under a hypoxic environment. Tween 80 significantly promoted aerobic growth by improving O₂ transfer, while only small amount was degraded and metabolized via the TCA cycle for biomass synthesis. As the bacillus encountered hypoxic stress, isotopomer analysis suggested: (1) isocitrate lyase activity increased, which further induced glyoxylate pathway and glycine dehydrogenase for replenishing NAD⁺; (2) the relative amount of acetyl-CoA entering the TCA cycle was doubled, whereas little entered the glycolytic and pentose phosphate pathways.

Keywords Glycine · Glyoxylate · NAD⁺ · Non-replicating · Tween 80

Introduction

Mycobacterium tuberculosis is a sophisticated pathogen that is able to persist in the human host for decades. It is estimated that up to one-third of the world's population is latently infected with this bacillus (Boshoff and Barry 2005), which enters a so-called non-replicating persistence (NRP) state. The NRP state is characterized by a lack of bacillus division and basic changes in metabolic activity. These non-replicating bacilli are difficult to eradicate because they are not reliant on the biosynthetic machinery targeted by conventional antibiotics (Höner zu Bentrup and Russell 2001). Thus, a deeper understanding of the characteristics of central metabolism and its changes during shifts from growth to the NRP state might open new avenues for pathogen clearance.

One of the key factors in the host environment responsible for inducing the NRP state is O₂ limitation in infected tissues and necrotic lesions (Wayne and Sohaskey 2001). Microarray and proteomic studies have shown many changes in gene expression as the bacillus undergoes hypoxic stress prior to metabolic dormancy (Boshoff and Barry 2005). In this study, isotopomer labeling was applied to

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investigate mycobacterial central metabolism along with its change in response to hypoxia. Isotopomer-assisted metabolite analysis can quantitatively track metabolic pathways and identify enzyme activities in cells (Tang et al. 2009). When the carbon substrate (e.g. glycerol) is labeled in specific positions with a non-radioactive isotope (^{13}C), the resulting isotopic label pattern of cellular metabolites reveals the active metabolic pathways. GC-MS is a sensitive method to measure mass isotopomer distributions in metabolites. The most widely used GC-MS protocol is based on derivatizing amino acids from proteins with tert-butyldimethylsilyl (TBDMS) moieties (Wahl et al. 2004). Proteinogenic amino acids are predominantly synthesized during the growth phase; thus, the labeling pattern reflects cellular metabolism in the active growth phase. More reflective of the metabolism during hypoxic response would be the label pattern in free metabolites that are rapidly turned over (Toya et al. 2007). BSTFA [*N,O*-bis-(trimethylsilyl)trifluoroacetamide] is an attractive approach for isotopomer analysis of free metabolites in cancerous cells (Meadows et al. 2008). The label information in free metabolites can be used to capture the dynamic changes in metabolism when mycobacterium growth is under environmental perturbations.

Materials and methods

Growth and hypoxic stress of bacteria

Mycobacterium smegmatis was grown in Middlebrook 7H9 medium to middle growth-phase (OD about 1); then cells were transferred to a modified Sauton liquid medium (Dubos and Davis 1946) (inoculation volume 1:1,000) consisting of 99% labeled [$2\text{-}^{13}\text{C}$]glycerol (Cambridge Isotope, USA), 2.5 g/l; KH_2PO_4 , 0.5 g/l; MgSO_4 , 0.5 g/l; NH_4Cl , 3.24 g/l; 1% ZnSO_4 , 0.1 ml/l and 20 mM PIPES at a final pH of 7.0. Two types of detergents, 0.05% Tween 80 or 0.025% non-hydrolyzable Tyloxapol (Sigma-Aldrich, neither labeled with ^{13}C), can be added into the initial medium to promote cell growth (Vandal et al. 2008). Cell density was monitored at 600 nm. Aerobic cultures were maintained at 37°C with shaking at 200 rpm in 250 ml flasks. To initiate a hypoxic stress, a 100 ml culture was grown under aerobic conditions to middle growth phase ($\text{OD}_{600} \sim 1$) and then transferred to a sealed

glass bottle with a septum cap. The ratio of head-space volume to liquid volume was approximately 1:2. The hypoxic culture was grown at 37°C. The hypoxic culture was harvested 3.5 h after initiation of O_2 depletion. As a control, the aerobic culture of the same volume was collected from the shaking flask at the same time.

Extraction of metabolites

We modified a procedure for extracting free metabolites using methanol/chloroform (Winder et al. 2008) as follows. Cell suspensions (50 ml) from either hypoxic or aerobic cultures were plunged rapidly into an equal volume of 60% (v/v) methanol–water (pre-chilled on dry ice, pH = 7.5, 60 mM HEPES buffer), then centrifuged at $10,000\times g$ (-10°C) for 5 min. The supernatant was quickly removed, and 5 ml cold 100% methanol (pre-chilled on dry ice) was added to the cell pellets. The cell/methanol mixture (setting on dry ice) was sonicated for 1.5 min with a 2 s on/3 s off cycle. The resulting cell lysate was added to a mixture of HPLC-grade chloroform (5 ml) and HPLC-grade water (2 ml), vortexed, and centrifuged for 2 min at $10,000\times g$ at 4°C . The aqueous layer (top) was collected (~ 6 ml) and mixed with 7.5 ml HPLC-grade water. The solution was frozen with liquid N_2 and lyophilized overnight. We also prepared proteogenic amino acids from biomass grown under aerobic and hypoxic conditions. In brief, 10 ml cell culture was centrifuged, and the bacterial pellet was hydrolyzed (in 6 M HCl) at 100°C for 24 h before it was completely dried under air flow.

Metabolite derivatization and isotopomer analysis

To prepare bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) (BSTFA)-derivatized samples for GC-MS analysis, we pre-derivatized the dried samples with a solution (0.3 ml) of 2% (w/v) $\text{NH}_2\text{OH} \cdot \text{HCl}$ in pyridine (Sigma-Aldrich) (MOX solution) for 90 min at 40°C while shaking at 200 rpm. The MOX solution was then centrifuged for 2 min at $17,600\times g$, and 0.15 ml of the supernatant was derivatized by reaction with 0.25 ml BSTFA for 25 min at 40°C (shaking at 200 rpm). This derivatization step added trimethylsilyl groups to carboxyls and converted oxoacids (e.g. pyruvate) to oximes. One microliter

of the derivatized sample was injected into a gas chromatograph. The column was held at 60°C for 1 min after injection and then heated at 20°C/min to 130°C, 4°C/min to 150°C, and finally 40°C/min to 260°C, where it was held for 3 min (Meadows et al. 2008). The *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (TBDMS) was used to derivatize proteinogenic amino acids. The sample preparation for GC-MS analysis was performed as previously reported (Tang et al. 2007). All GC-MS measurements were carried out using a gas chromatograph (HP6890 series, Agilent Inc.) equipped with a DB5 column (J&W Scientific Inc.) and a mass spectrometer (5973 Network, Agilent Inc.). Because the natural abundance of isotopes alters the mass isotopomer spectrum in derivatized molecules, we corrected these changes using a published algorithm (Wahl et al. 2004).

Results and discussion

BSTFA metabolite derivatization

TBDMS-derivatized proteinogenic amino acids and fragment patterns have been studied extensively (Antoniewicz et al. 2007). On the other hand, BSTFA is a more sensitive method, which can also derivatize the functional groups –COO, –OH, –SH, and –NH₂.

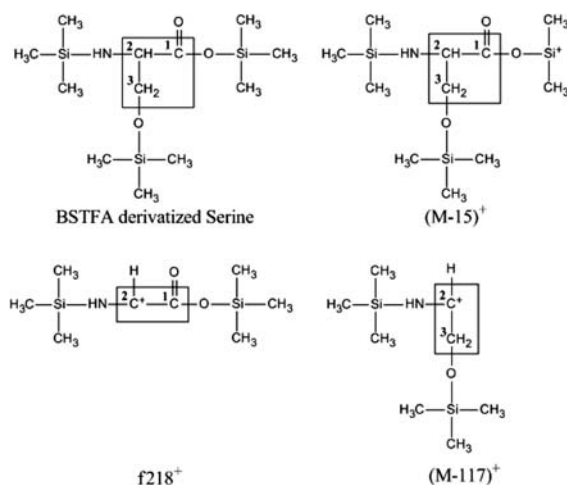


Fig. 1 BSTFA derivatized serine (serine C-skeleton was boxed) and its common ion fragments: fragments (M – 15)⁺ (–CH₃ loss of TMS), f218⁺ (loss of –R group from serine backbone) and (M – 117)⁺ (loss of –COO[–] and –TMS)

Thus, it can be used to determine intra-cellular free amino acids and organic acids as well as their labeling pattern (Meadows et al. 2008). Figure 1 shows an example of the structures of BSTFA-derivatized serine fragments in mass spectrum. Loss of a methyl group from the trimethylsilyl (TMS) group yields the fragment (M – 15)⁺, which still contains the complete amino acid. The (M – 117)⁺ ion originates from the loss of C(O)–O–TMS. The f218⁺ results from cleavage of the bond between the R group and α-carbon atom of the amino acid. Using this BSTFA method, we identified 10 key metabolites in *M. smegmatis* extracts, including amino acids and several organic acids (Table 1).

Growth and metabolism of *M. smegmatis* under aerobic conditions

M. smegmatis is a fast-growing, non-virulent mycobacterium. When growing *M. smegmatis* in [¹³C]-labeled glycerol minimal medium without any detergent (Tween 80 or Tyloxapol), cells formed large clumps. The doubling time was over 7 h, and the final OD₆₀₀ was only 0.35 (Fig. 2). When the medium was supplemented with Tween 80 (or Tyloxapol), we observed its doubling time to be much faster (~3.5 h) and its final OD₆₀₀ to be over 2 (for both Tween-grown or Tyloxapol-grown cultures). During aerobic growth, glycerol was completely utilized to produce the biomass, and no extra-cellular acetate or lactate was detected.

These findings indicate that *M. smegmatis* completely utilizes the majority of the carbon source (glycerol) for aerobic growth. It was noticed decades ago that *Mycobacterium* spp. all require the detergent supplemented in the medium for optimal growth (Mizuno and Tsukamura 1978). One reason for the faster growth is that oleic acid, the hydrolytic product of Tween 80 metabolism, can be converted into triacylglycerols as carbon sources for biosynthesis of lipid and other biomass building blocks (Saito et al. 1983). However, non-hydrolyzable Tyloxapol (Vandal et al. 2008) significantly enhanced growth under aerobic conditions with similar growth rate and final cell density compared to that of Tween 80 culture. Another possibility is that Tween 80 stimulates the growth by improving O₂ transfer to the cells rather than by providing extra nutrients or energy. When *M. smegmatis* grows in a detergent-free medium, the bacilli form large aggregates due to the extremely

Table 1 Labeling of free metabolites, derived with BSTFA, showing changes of labeling pattern under hypoxic condition ($n = 2$)

Synthesis from central pathways	Metabolites	Aerobic		Hypoxia for 3.5 h	
		(M – 15) ⁺ or *f(218) ⁺	(M – 117) ⁺	(M – 15) ⁺ or f(218) ⁺	(M – 117) ⁺
Glycolysis pathway	Ala ^a				
	M0	Overlapping peak	0.08 ± 0.02	Overlapping Peak	0.10 ± 0.01
	M1		0.89 ± 0.03		0.88 ± 0.01
	M2		0.02 ± 0.01		0.02 ± 0.01
	Pyruvate ^b				
	M0	0.02 ± 0.02	Overlapping peak	0.12 ± 0.02	Overlapping Peak
	M1	0.94 ± 0.03		0.82 ± 0.02	
	M2	0.04 ± 0.02		0.05 ± 0.01	
	M3	0.02 ± 0.01		0.01 ± 0.01	
	Gly				
	M0	0.10 ± 0.02	0.27 ± 0.01	0.26 ± 0.01	0.37 ± 0.01
	M1	0.86 ± 0.02	0.73 ± 0.01	0.71 ± 0.01	0.63 ± 0.01
	M2	0.03 ± 0.01	0	0.03 ± 0	0
	Ser ^c				
	M0	0.09 ± 0.02*	0.09 ± 0.04	0.15 ± 0.01*	0.17 ± 0.01
	M1	0.88 ± 0.01*	0.83 ± 0.07	0.81 ± 0.04*	0.69 ± 0.04
	M2	0.03 ± 0.02*	0.08 ± 0.03	0.04 ± 0.03*	0.13 ± 0.04
Pentose phosphate pathway	Phe ^d				
	M0	0.10 ± 0.03*	0	0.12 ± 0.03*	0
	M1	0.85 ± 0.03*	0	0.86 ± 0.04*	0
	M2	0.04 ± 0.01*	0.06 ± 0.02	0.01 ± 0.01*	0.08 ± 0.02
	M3	0	0.66 ± 0.04	0	0.64 ± 0.04
TCA cycle	M4	0	0.22 ± 0.03	0	0.21 ± 0.03
	Asp ^e				
	M0	Insufficient ions	0.11 ± 0.01	Insufficient ions	0.21 ± 0.02
	M1		0.63 ± 0.02		0.59 ± 0.01
	M2		0.25 ± 0.03		0.19 ± 0.02
	M3		0.01 ± 0.01		0.01 ± 0.01
	Glu				
	M0	0.01 ± 0	0.03 ± 0.01	0.03 ± 0.01	0.07 ± 0.02
	M1	0.15 ± 0.03	0.24 ± 0.04	0.23 ± 0.03	0.39 ± 0.04
	M2	0.63 ± 0.04	0.69 ± 0.05	0.52 ± 0.04	0.49 ± 0.04
	M3	0.21 ± 0.03	0.05 ± 0.02	0.18 ± 0.03	0.04 ± 0.02
	Citrate ^f				
	M0	Insufficient ions	0.38 ± 0.05	Insufficient ions	0.58 ± 0.07
	M1		0.21 ± 0.04		0.23 ± 0.06
	M2		0.29 ± 0.04		0.14 ± 0.02
	M3		0.11 ± 0.02		0.04 ± 0.01
	M4		0.01 ± 0		0.01 ± 0

Table 1 continued

Synthesis from central pathways	Metabolites	Aerobic		Hypoxia for 3.5 h	
		(M – 15) ⁺ or *f(218) ⁺	(M – 117) ⁺	(M – 15) ⁺ or f(218) ⁺	(M – 117) ⁺
TCA cycle	Succinate ^g				
	M0	0.05 ± 0.01	Not fragmented	0.12 ± 0.02	Not fragmented
	M1	0.22 ± 0.03		0.40 ± 0.04	
	M2	0.66 ± 0.04		0.41 ± 0.03	
	M3	0.05 ± 0.01		0.06 ± 0.03	

Note: The final isotopomer data were reported as M0 unlabeled fraction, M1 singly labeled fraction, M2 fraction with two labeled carbons, M3 fraction with labeled three carbons, etc. *f(218)⁺ peak is used instead of (M – 15)⁺. (1) Alanine's (M – 15)⁺ peak overlaps the f(218)⁺ peak. (2) Pyruvate's (M – 117)⁺ peak overlaps other anomalous ion peaks. (3) Serine's f(218)⁺ fragment is better for quantitative purposes than (M – 15)⁺ due to high signal to noise ratio in the latter. (4) Phenylalanine's f(218)⁺ fragment is better for qualitative purposes used for these amino acids than (M – 15)⁺ due to high signal to noise ratio in the latter. (5) Aspartic acid's (M – 15)⁺ and f(218)⁺ peak have high background noise. Not recommended to be used. (6) Citrate (M – 117)⁺ fragment is better for quantitative purposes than (M – 15)⁺ due to high signal to noise ratio in the latter. (7) Succinate's (M – 117)⁺ ion is not present, i.e., MS cannot fragment succinate

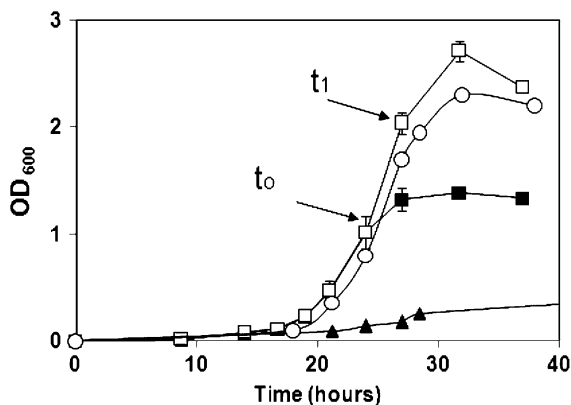


Fig. 2 *M. smegmatis* growth. The symbol (filled triangle): the growth curve without any detergent. The symbols (filled square and empty squares): the growth with Tween 80. The empty squares: growth under aerobic conditions. The filled squares: growth under hypoxic stress. The symbol (empty circle): the growth with detergent Tyloxapol. t_0 : start of hypoxia, the culture was transferred to the serum bottle; t_1 : biomass samples were taken for isotopomer analysis

waxy nature of the cell surface. Therefore, the O₂ availability in the center of such a clump may be low.

We compared the isotopomer labeling patterns of five key proteinogenic amino acids produced from cultures grown in Tween 80-containing and Tween 80-free media (Fig. 3). Specifically, precursors to alanine (pyruvate) and leucine (acetyl-CoA) are key metabolites bridging glycolysis and the TCA cycle, while the precursors to aspartic acid (oxaloacetate),

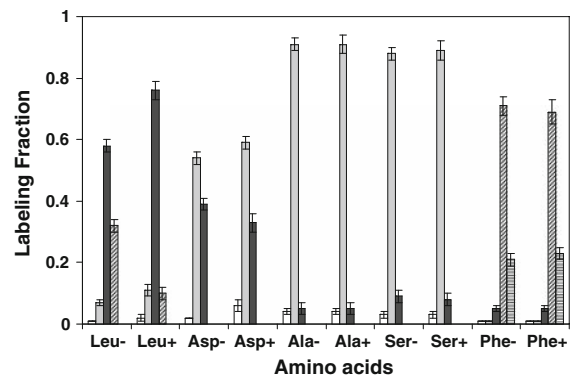


Fig. 3 Effect of Tween 80 on proteinogenic amino acid labeling. The bars from left to right represent isotope peaks of M0 unlabeled fraction, white bar; M1 singly labeled fraction, gray bar; M2 fraction with two labeled carbons, dark bar; M3 fraction with labeled three carbons, diagonal hash; M4 fraction with labeled four carbons, horizontal hash. (–) Indicates the culture without any detergent; (+) indicates the culture with Tween 80. The (M – 57)⁺ fragment was used for Asp, Ala, Ser, and Phe. The (M – 159)⁺ fragment was used for Leu due to its overlapping peak with (M – 57)⁺

serine (3-phosphoglycerate), and phenylalanine (phosphoenolpyruvate and erythrose 4-phosphate) are derived from the TCA cycle, glycolysis, and pentose phosphate pathway, respectively. Therefore, if unlabeled Tween 80 was metabolized via β -oxidation (Muñoz-Elías and McKinney 2006), it would be incorporated into these amino acids and change their labeling patterns. As shown in Fig. 3, addition of Tween 80 caused measurable dilution of the label in

leucine (the fractions of the M3 isotopes in leucine were moderately deceased). Because acetyl-CoA and pyruvate (synthesis from glycerol) are precursors for leucine synthesis, the change in its label pattern confirmed previous report that unlabeled Tween 80 could be oxidized to acetyl-CoA (Höner zu Bentrup and Russell 2001; Schnappinger et al. 2003) and thus decreased the labeling concentrations in leucine. The label in aspartic acid was minimally diluted (up to 5%, just above the measurement noise), which indicated that only a small fraction of unlabeled acetyl-CoA from Tween 80 entered the TCA cycle. Amino acids derived from glycolysis and the pentose phosphate pathway (e.g., alanine, serine, and phenylalanine) did not show any changes in their label patterns. Thus, acetyl-CoA derived from Tween-80 did not enter the aforementioned pathways. These results show that only small amount of Tween 80 is utilized for synthesis of biomass building blocks under aerobic condition. Tween 80 or non-hydrolyzable Tyloxapol enhances mycobacterium aerobic growth mainly because it enhances O₂ transfer.

Growth and metabolism of *M. smegmatis* adapted to hypoxia

Previous studies showed that *M. smegmatis* can adapt to the non-replicating persistence state with growth kinetics and antibiotic resistance similar to *M. tuberculosis* under O₂-limited culture (Dick et al. 1998). In this study, the hypoxic condition was established by gradually depriving an actively Tween 80-growing culture of O₂ in a sealed glass bottle, and thus cell growth eventually stopped within 3 h (Fig. 2). Isotopomer analysis of proteinogenic amino acids derived from biomass harvested after 3 h of hypoxia did not show a significant difference in the labeling patterns of the hypoxic cells compared with those of proteins extracted from cells in the aerobic, active growth phase (data not shown), because proteinogenic amino acids from the O₂-deprived culture were derived primarily from the aerobic growth stage. To capture the transition in metabolism from aerobic to hypoxic conditions, we derivatized the free metabolites with BSTFA and analyzed their isotopic label patterns.

As shown in Table 1, the TCA cycle acids, i.e. citrate and succinate, had the highest label dilution (e.g., the M0 peak of citrate increased from 38% to 58%), indicating that a large fraction of unlabeled carbon entered central metabolism (compared to the

aerobic condition) and caused a significant decrease in the isotope labeling of TCA cycle acids. These unlabeled metabolites in the TCA cycle originated from unlabeled hydrolysis products of Tween 80. Second, the isotopically labeled fractions of aspartate and glutamate from the hypoxic culture were lower compared with those from the aerobic culture (the unlabeled M0 peak of Asp increased from 11% to 21%, and that of Glu from 3% to 7%). Under hypoxic conditions, the two amino acids were either synthesized from oxaloacetate and 2-ketoglutarate, respectively, or from the degradation of protein grown under aerobic conditions, and as such, the change in labeling in amino acids was less significant than fast turnover TCA cycle intermediates (e.g., citrate). Lastly, we did not observe a dramatic change in the label patterns of free alanine and phenylalanine, which indicated that the Tween 80 catabolites were not used to synthesize their precursors (e.g., pyruvate, phosphoenolpyruvate, and erythrose 4-phosphate) under hypoxia. Taken together, the isotopomer data of free metabolites indicate that catabolism of Tween 80 was relatively enhanced compared to that of glycerol so as to serve as a secondary source of carbon assimilated mainly via the TCA cycle under hypoxic stress. This finding confirmed the transcript studies showing that expression of genes encoding enzymes involved in fatty acid oxidation are up-regulated under O₂ limited conditions (Muñoz-Elías and McKinney 2006; Schnappinger et al. 2003).

Glyoxylate and glycine metabolism in *M. smegmatis*

The glyoxylate metabolism plays a major role in enabling mycobacteria to utilize carbon sources when TCA cycle is shut down, particularly in infected tissues where O₂ and nutrients are limited (Boshoff and Barry 2005). The activity of isocitrate lyase, the first enzyme in the glyoxylate pathway, has been shown to increase in hypoxic culture of *M. tuberculosis* (Muñoz-Elías and McKinney 2005; Muttucumaru et al. 2004). Interestingly, the activity of glycine dehydrogenase (GDH), which channels glyoxylate out of the cycle, was detected in the cell extracts of *M. tuberculosis* by biochemical assays (Boshoff and Barry 2005). GDH catalyzes the reductive conversion of glyoxylate to glycine (concomitantly oxidizing NADH to NAD⁺) and was found to be

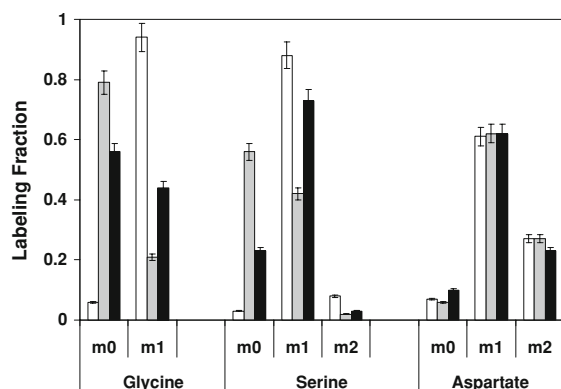


Fig. 4 Effect of addition of glycine and glyoxylate (100 mg/l) on the isotopomer distribution in proteinogenic amino acids. The $(M - 57)^+$ fragment was used. White bar (control), gray bar (addition of glycine), black bar (addition of glyoxylate)

strongly activated in microaerophilic cultures of *M. tuberculosis*.

Here we revisited the *in vivo* activity of GDH by using ^{13}C isotopomer labeling. In order to test whether GDH is active in aerobically grown bacilli, we supplemented the labeled culture medium with unlabeled glyoxylate or glycine (100 mg/l) and analyzed the label patterns of proteinogenic amino acids. A marked change in the labeling of glycine and serine was observed but not in any other amino acids measured (Fig. 4). After addition of unlabeled glyoxylate, the unlabeled fraction (M0) of proteinogenic glycine increased from 5% to 57%, which indicates that over 50% of glycine in the biomass was derived from the unlabeled glyoxylate. A dilution of the label in serine, which was less significant than that in glycine, suggested that glycine could be a precursor for serine synthesis. Therefore, the reactions glyoxylate \rightarrow glycine \rightarrow serine is an alternate pathway for glycine and serine biosynthesis in *M. smegmatis* regardless of the O_2 concentration. Under hypoxic conditions, glyoxylate synthesis is induced because isocitrate lyase activity (isocitrate \rightarrow glyoxylate + succinate) is up-regulated (Muñoz-Elías and McKinney 2005). Thus, the flux through the pathway from glyoxylate to glycine and serine would be expected to have a concurrent increase. Indeed, the label in free glycine and serine were dramatically diluted ($>10\%$, Table 1) under hypoxic conditions even without addition of unlabeled glyoxylate to the medium. Such dilution resulted primarily from a large fraction of acetyl-CoA (derived from unlabeled Tween 80)

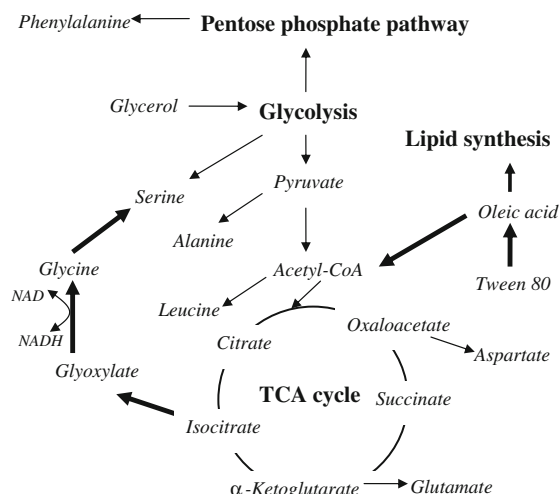


Fig. 5 Central metabolic pathways of *M. smegmatis* revealed by isotopomer analysis. The thick arrows indicate the pathways up-regulated under hypoxia. The metabolites are in *italics*

entering the TCA cycle. Since the alternate pathway for glycine and serine synthesis was up-regulated (i.e., isocitrate \rightarrow glyoxylate \rightarrow glycine \rightarrow serine), the unlabeled carbon was thus introduced into glycine and serine from the TCA cycle. Figure 5 summarizes the central metabolic pathways of *M. smegmatis* and highlights all the up-regulated pathways (thick arrows) under hypoxic stress. It is noteworthy that the glyoxylate \rightarrow glycine \rightarrow serine pathway, with concomitant oxidation of NADH to NAD^+ , may be of particular importance for bacilli responding to O_2 shortage in the non-replicating persistence state by facilitating lipid utilization and maintaining the redox balance.

Conclusion

Isotopomer-assisted metabolite analysis revealed change of Tween 80 metabolism under O_2 rich and O_2 poor conditions. It also demonstrated an increase in the flux through the pathway that transforms glyoxylate to serine (glyoxylate \rightarrow glycine \rightarrow serine) under hypoxic conditions, which might contribute to the bacillus' survival and persistence in the O_2 -limited host tissue. The impact of hypoxia on mycobacterial metabolism and growth has been studied using other molecular biology tools before. The isotopomer-assisted metabolite analysis described herein is an

alternate and complementary tool to these previously-used technologies, and thus it may have broad application in other complicated biological systems.

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