AMERICAN SOCIETY FOR MICROBIOLOGY

Antimicrobial Agents and Chemotherapy®

Deletion of *sigB* Causes Increased Sensitivity to *para*-Aminosalicylic Acid and Sulfamethoxazole in *Mycobacterium tuberculosis*

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ABSTRACT Although the *de novo* folate biosynthesis pathway has been well studied in bacteria, little is known about its regulation. In the present study, the *sigB* gene in *Mycobacterium tuberculosis* was deleted. Subsequent drug susceptibility tests revealed that the *M. tuberculosis* Δ*sigB* strain was more sensitive to *para*-aminosalicylic acid (PAS) and sulfamethoxazole. Comparative transcriptional analysis was performed, and downregulation of *pabB* was observed in the Δ*sigB* strain, which was further verified by a quantitative reverse transcription-PCR and Western blot assay. Then, the production levels of *para*-aminobenzoic acid (*p*ABA) were compared between the *sigB* deletion mutant and wild-type strain, and the results showed that *sigB* deletion resulted in decreased production of *p*ABA. In addition, SigB was able to recognize the promoter of *pabB in vitro*. Furthermore, we found that deleting *pabC* also caused increased susceptibility to PAS. Taken together, our data revealed that, in *M. tuberculosis*, *sigB* affects susceptibility to antifolates through multiple ways, primarily by regulating the expression of *pabB*. To our knowledge, this is the first report showing that SigB modulates *p*ABA biosynthesis and thus affecting susceptibility to antifolates, which broadens our understanding of the regulation of bacterial folate metabolism and mechanisms of susceptibility to antifolates.

KEYWORDS *Mycobacterium tuberculosis*, *pabB*, *para*-aminobenzoic acid, *para*-aminosalicylic acid, *sigB*

Tuberculosis (TB), caused by one of the toughest human pathogens, *Mycobacterium tuberculosis*, remains a serious public health concern in the world. The World Health Organization (WHO) estimated that in 2015 there were 10.4 million new TB cases worldwide, of which 580,000 cases were multidrug-resistant TB (MDR-TB) (1). Traditional effective anti-tubercular drugs, such as rifampin (RFP) and isoniazid (INH), were challenged by the emergence of MDR-TB and extensively drug-resistant TB, which have been major threats to global public health security due to the crisis of their detection and treatment (2, 3). Worst of all, the design of new and repurposed antimicrobial drugs has been slow (4). Thus, a better understanding of the molecular mechanisms medi-

ating resistance or susceptibility in *M. tuberculosis* to preexisting antitubercular drugs is urgently required (5).

Folate is essential for all sorts of life, but mammals are unable to synthesize it, which makes the bacterial *de novo* folate biosynthesis pathway an ideal target for new antimicrobial drug design (6). As is well known, the bacterial *de novo* folate biosynthetic pathway has two branches: one for *para*-aminobenzoic acid (*p*ABA) and the other for pterins. The *p*ABA branch has two enzymes: the aminodeoxychorismate synthase composed of PabA and PabB and the aminodeoxychorismatelysase (PabC) (7–9). Meanwhile, the pterin branch starts with GTP and finally yields 7,8-dihydropterin pyrophosphate (DHPPP). These two branches are joined together by dihydropteroate synthase (DHPS), which synthesizes dihydropteroate (DHP) by using *p*ABA and DHPPP as substrates. The DHP is then converted into dihydrofolate (DHF) through dihydrofolate synthase (DHFS), and is further reduced into tetrahydrofolate (THF) by dihydrofolate reductase (DHFR) (10–12). Thousands of antifolates have been designed targeting bacterial DHPS and DHFR (13). *Para*-aminosalicylic acid (PAS), a second-line drug used in clinical treatment of TB, was also classified as an antifolate that targets the *de novo* folate synthesis of *M. tuberculosis* As an analog of *p*ABA, PAS acts as a prodrug activated by DHPS and DHFS and finally targets DHFR, thus leading to bactericidal effects (14, 15). As a result, *folC* mutation, which encodes the DHFS in *M. tuberculosis*, causes PAS resistance in *M. tuberculosis* clinical isolates by blocking drug activation (16, 17). In addition to *folC* mutations, mutations of the thymidylate synthase coding gene *thyA* or the upstream region of the *ribD* gene also result in PAS resistance in *M. tuberculosis* clinical isolates (18–20). ThyA is involved in folate transformation, which converts methylene THF into DHF (21). So far, mutations in these three genes could be identified in about two-thirds of PAS-resistant clinical isolates, but resistance mechanisms in the remaining third still need to be uncovered.

As essential components of the RNA polymerase (RNAP) holoenzyme, sigma factors provide specific recognition of promoters in bacteria. There are 13 sigma factors in *M. tuberculosis*, making the tough bacteria able to adapt to various environments (22). In *M. tuberculosis*, σ^B, encoded by the well-conserved gene *sigB*, is very close to the major sigma factor σ^A and identified to be positively regulated by the other three extracytoplasmic sigma factors: σ^E (23), σ^H (24), and σ^L (25). σ^B was also found to play a central role in response to various stress conditions, including heat shock in *M. tuberculosis*, sodium dodecyl sulfate (SDS), mild cold shock, cell envelope stress, and hypoxia *in vitro* (26, 27).

Although *de novo* folate biosynthesis has been well studied in bacteria, little is known regarding its regulation, especially in *M. tuberculosis*. Previously, deletion of *sigB* in *Corynebacterium glutamicum* resulted in a significant decrease of expression level of *thyX* and hypersensitivity to the DHFR inhibitor WR99210 (28). Very recently, *sigB* was also found to be an antifolate resistance determinant in *Mycobacterium smegmatis* through chemogenomic screening (29). These observations indicate that SigB might play a role on folate metabolism, thus affecting susceptibility to antifolates in bacteria. In this study, the *sigB* gene of *M. tuberculosis* H37Ra was deleted, and the effects on folate metabolism and susceptibility to different types of antifolates were probed to clarify the role of SigB on regulating folate metabolism in *M. tuberculosis*.

RESULTS

Effects of *sigB* deletion on bacterial growth. Although σ^B is highly homologous to the primary sigma factor σ^A in mycobacteria, *sigB* is a nonessential gene in *M. tuberculosis* (30, 31). To investigate the role of σ^B on susceptibility to antitubercular drugs, we constructed a Δ*sigB* mutant by replacing the *sigB* gene with a hygromycin cassette on the H37Ra genome. A complemented strain, H37RaΔ*sigB*(pMV261::*sigB*), was also constructed by reintroducing the episomal vector pMV261::*sigB* into the H37Ra Δ*sigB* mutant (H37RaΔ*sigB*).

A previous study showed that inactivation of *sigB* in H37Rv did not affect the survival of H37Rv in macrophages but could make bacteria more sensitive to SDS stress and
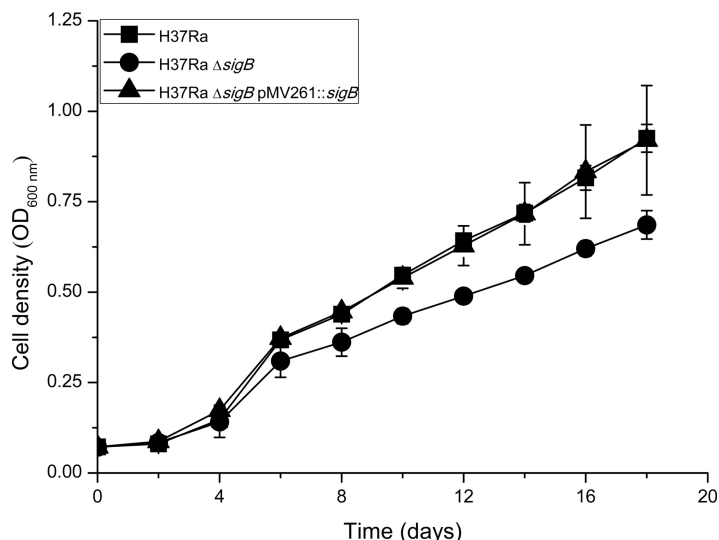
FIG 1 Growth curves of H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) in liquid culture at 37°C. The OD$_{600}$ was measured by using a SynergyH1 Hybrid reader (BioTek, USA) every 48 h. Data represent the means of three biological replicates, and error bars denote the standard deviations.

hypoxia *in vitro* (27). To probe the effect of *sigB* deletion on the bacterial growth of H37Ra, growth curves of H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) were compared. As shown in Fig. 1, the Δ*sigB* mutant did show an obvious lag of growth compared to the wild-type and complemented strains.

**Deletion of *sigB* led to increased susceptibility to various antitubercular drugs.** The susceptibility of H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) to various antitubercular drugs was tested. The results showed that *sigB* deletion led to increased sensitivities to most of the antitubercular drugs tested, as determined by MIC tests (Table 1). As shown in Table 1, the MICs of four drugs (RFP, streptomycin [SM], ethambutol [EMB], and norfloxacin) of the Δ*sigB* mutant were about 2- to 4-fold lower than for the wild-type strain, whereas MICs of two drugs (INH and ofloxacin) did not show any difference. Interestingly, we observed a remarkable decrease of MICs for the two antifolates (PAS and sulfamethoxazole [SMX]) in the Δ*sigB* mutant (16-fold lower than that of the wild-type strain). Moreover, changes in susceptibility to those drugs for the Δ*sigB* mutant could be completely reversed in the complemented strain.

Effect of *sigB* deletion on the bactericidal effect of PAS against *M. tuberculosis*. To further investigate the effect of *sigB* deletion on the bactericidal effect of PAS against *M. tuberculosis*, PAS kill kinetics were compared between the *sigB* knockout mutant and the wild-type strain. As shown in Fig. 2, we found that a dramatic decrease of viable bacterial cell number (from 7 to 5.5 log$_{10}$ CFU/ml) was observed for the Δ*sigB* mutant after 24 h of PAS treatment but that only a slight decrease (~0.5 log$_{10}$ CFU/ml) was

TABLE 1 MICs of various antitubercular drugs against H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) strains

Drug	MIC (μg ml^{-1})			Fold change
	H37Ra	H37RaΔ*sigB*	H37RaΔ*sigB*(pMV261::*sigB*)	
para-Aminosalicylic acid	0.04	0.0025	0.04	16
Sulfamethoxazole	25	1.5625	25	16
Rifampin	0.04	0.01	0.04	4
Isoniazid	0.1	0.1	0.1	1
Streptomycin	1.0	0.5	1.0	2
Ofloxacin	0.4	0.4	0.4	1
Norfloxacin	3.2	1.6	3.2	2
Ethambutol	1.25	0.625	1.25	2
WR99210	25.62	6.405	25.62	4
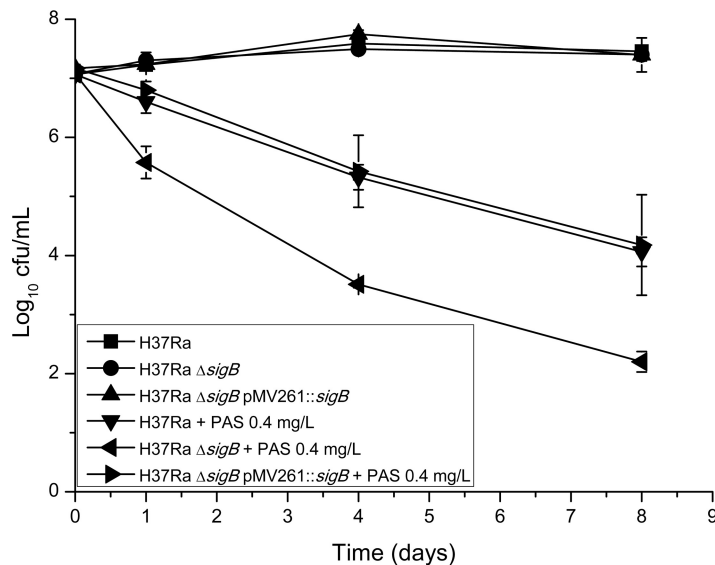
FIG 2 Killing curves of different *M. tuberculosis* strains after exposure to PAS at 0.4 μg ml^{-1} in liquid medium (7H9 plus OADC). Experiments were performed in three biological replicates. Standard deviations are indicated by error bars.

observed for the wild-type strain. After treatment for 4 days, the viable bacterial cell number of the $\Delta sigB$ mutant decreased to 3.5 $\log_{10}$ CFU/ml, while that of the wild-type strain fell to 5.5 $\log_{10}$ CFU/ml. The viable cell number of the $\Delta sigB$ mutant continued decreasing sharply from 8 days and onward, and no viable bacterial cells could be detected in 10-μl cultures dropped on the 7H10 medium after 16 days of treatment (data not shown). However, viable cell numbers of the wild-type strain could still be detected after 16 days of PAS treatment (data not shown). During the course of PAS treatment, survival of the complemented strain was very similar to that of the wild-type strain.

Transcriptome analysis of the $\Delta sigB$ mutant. To obtain further insights on how *sigB* deletion led to increased susceptibility to multiple antitubercular drugs, we compared the global transcription profile between the $\Delta sigB$ mutant and wild-type strain using transcriptome sequencing (RNA-seq) technology. Consequently, 175 genes were identified to be significantly regulated, including 33 upregulated genes and 142 downregulated genes (see Table S1 in the supplemental material). Based on gene ontology (GO) pathway enrichment analysis, changes in the expression level of many genes involved in stress responses and pathogenesis could be observed (see Table S2 in the supplemental material), which was in consistent with the fact that σ^B plays a central role in stress responses (27). We also found that 37 genes involved in bacterial cell wall biosynthesis and 26 genes involved in bacterial plasma membrane biosynthesis were downregulated, which might affect the permeability of the bacteria. In addition, several transcriptional regulators were found to be up- or downregulated in the $\Delta sigB$ mutant (Table 2). For example, the expression of *whiB6*, which plays a role on regulating the ESX-1 secretion system and the Dos dormancy regulon in *Mycobacterium marinum* (32), was downregulated in H37Ra$\Delta sigB$.

Very importantly, we found that *pabB*, an essential component of the *p*ABA synthesis machinery in *M. tuberculosis*, was also downregulated in the $\Delta sigB$ mutant (Table 2), and this was further proved by the subsequent quantitative real-time PCR (qRT-PCR) analysis (Fig. 3A).

Deletion of *sigB* led to decreased expression level of PabB. A Western blot assay was also performed to compare the expression level of PabB between different strains, and the results showed that expression was significantly decreased in H37Ra$\Delta sigB$ compared to the wild-type strain, which could be restored in the complemented strain (Fig. 3B).

TABLE 2 Genes differentially regulated in the H37RaΔ*sigB* strain versus the wild-type H37Ra strain

| Category and gene	Description[a]	Fold change	Tendency
Growth			
yrb1B	Membrane protein	1.54	Up
yrbE4B	ABC transporter permease	1.50	Up
pabB	Aminodeoxychorismate synthase component I	0.54	Down
MRA_0320	Hypothetical protein	0.54	Down
MRA_0637	Hypothetical protein	0.57	Down
MRA_0881	Hypothetical protein	0.58	Down
mprB	Two-component sensor histidine kinase	0.65	Down
cysD	Sulfate adenylyltransferase subunit 2	0.60	Down
cysN	Adenylyl-sulfate kinase	0.65	Down
MRA_1350	Membrane protein	0.67	Down
MRA_2389	Hypothetical protein	0.61	Down
MRA_2684	Antitoxin	0.21	Down
MRA_2056	Sugar ABC transporter substrate-binding lipoprotein	0.63	Down
MRA_0067	RNase VapC1	0.59	Down
MRA_3842	Membrane protein	0.60	Down
sigB	RNA polymerase sigma factor SigB	0.00	Down
Metabolic process			
MRA_1007	Acetyltransferase	1.64	Up
msrA	Peptide-methionine (*S*)-S-oxide reductase	0.56	Down
fabD2	Malonyl CoA-ACP transacylase	0.49	Down
pncA	Bifunctional pyrazinamidase nicotinamidase	0.59	Down
Transcriptional regulator			
MRA_0471	Transcriptional regulator	0.53	Down
MRA_1735	Transcriptional regulator	0.46	Down
MRA_1828	Transcriptional regulator	1.76	Up
MRA_2649	Transcriptional regulator	0.54	Down
MRA_2671	Transcriptional regulator	0.62	Down
MRA_2909	Transcriptional regulator	1.67	Up
whiB6	Transcriptional regulator	0.43	Down

[a]CoA, coenzyme A.

Overexpression of *pabB* in *M. tuberculosis* led to PAS resistance. To further verify that decreased expression of *pabB* was responsible for increased susceptibility to both PAS and SMX in the Δ*sigB* strain, *pabB* was overexpressed in both H37RaΔ*sigB* and the wild-type strain. The results showed that the overexpression of *pabB* resulted in PAS resistance (Table 3).

σ^B specifically recognized the promoter of *pabB* in vitro. To further investigate whether σ^B could recognize the promoter of *pabB* directly, we performed an *in vitro* transcriptional assay (Fig. 3C). We found that σ^B in combination with mycobacterial RNAP core enzymes successfully initiated transcription from the promoter of the *pabB* gene.

Deletion of *sigB* caused decreased the production of *p*ABA. The *pabB* (*MRA_1014*) gene product was characterized to be the aminodeoxychorismate synthase, an indispensable enzyme for the biosynthesis of *p*ABA from chorismate in folate *de novo* synthesis in bacteria. Consequently, decreased expression of PabB may lead to decreased production of *p*ABA. To determine production levels of *p*ABA in H37Ra and H37RaΔ*sigB* mutant strains, an *Escherichia coli* W3110 Δ*pabB* mutant strain (W3110Δ*pabB*) was constructed, which needed exogenous *p*ABA to support its growth both in E minimal medium and 7H9 plus oleic acid-albumin-dextrose-catalase (OADC) medium (see Fig. S1 in the supplemental material). We also found that the *in vitro* growth of *E. coli* W3110Δ*pabB* was *p*ABA dose dependent (see Fig. S1A in the supplemental material). When *E. coli* W3110Δ*pabB* was cultured in E minimal medium plus culture filtrates from different H37Ra strains, there was obviously less growth when
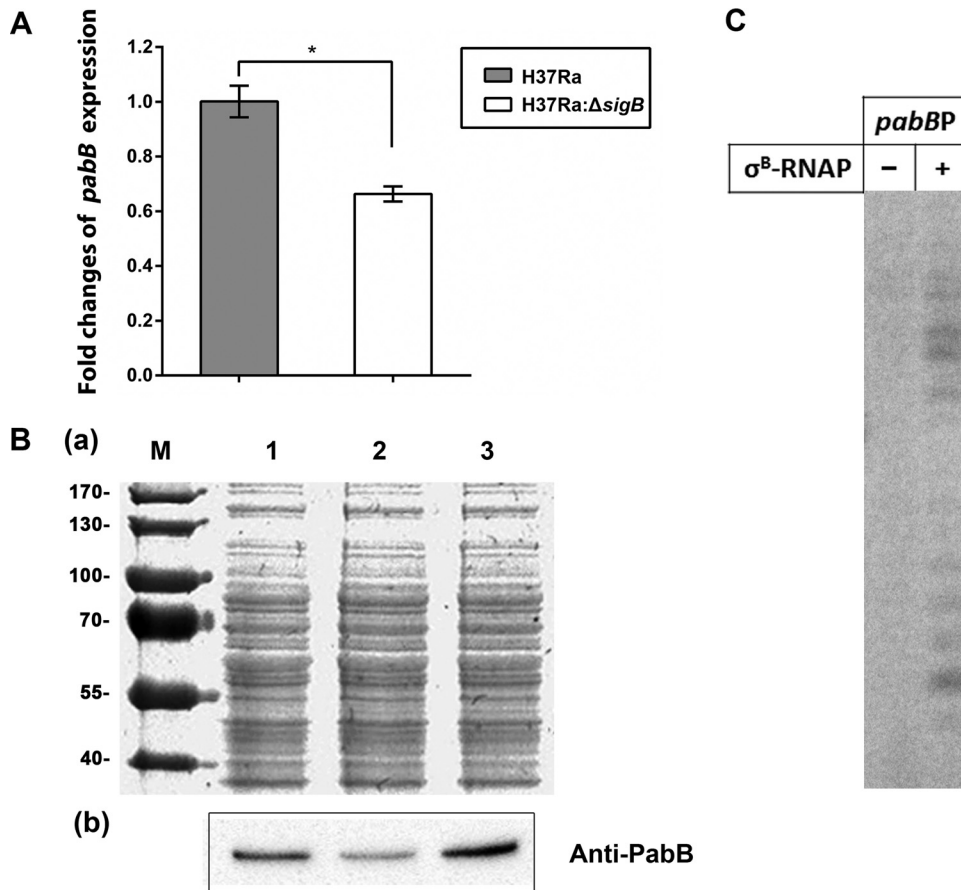
FIG 3 PabB was regulated directly by σ^B in *M. tuberculosis* H37Ra. (A) Comparison of the transcriptional level of the gene *pabB* during the exponential phase in H37Ra (WT) and H37RaΔ*sigB* (KO) strains by qRT-PCR. The expression levels of GAPDH mRNA were normalized as an endogenous control. Data are representative of three experiments, and the statistical significance is indicated by an asterisk: *, $P < 0.01$. (B) Comparison of the expressional level of PabB during the exponential phase in H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) as determined by a Western blot assay. Experiments were repeated at least three times, and representative results are shown. (a) Total protein was normalized to 20 μg of each strain and then electrophoresed by SDS-PAGE and stained by Coomassie brilliant blue. Lane M, the prestained protein marker; lane 1, total protein of H37Ra; lane 2, total protein of H37RaΔ*sigB*; lane 3, total protein of H37RaΔ*sigB*(pMV261::*sigB*). (b) Western blot analysis of total protein immunoblotted with mice antisera of anti-PabB. Lane 1, anti-PabB immunoblotted to the total protein of H37RaΔ*sigB*; lane 2, anti-PabB immunoblotted to the total protein of H37RaΔ*sigB*; lane 2, anti-PabB immunoblotted to the total protein of H37RaΔ*sigB*(pMV261::*sigB*). (C) [^{32}P]RNA products synthesized in the *in vitro* transcription assay from the indicated promoter of *pabB*. Transcription was performed by RNAP holoenzyme containing σ^B.

culture filtrates from H37RaΔ*sigB* were used (Fig. 4), suggesting a decreased production of *p*ABA in the H37RaΔ*sigB* mutant.

Disruption of *p*ABA synthesis caused increased susceptibility to PAS. In *M. tuberculosis*, *pabA*, *pabB*, and *pabC* are all essential genes for bacterial *in vitro* growth. To confirm that decreased production of *p*ABA finally caused increased susceptibility to SMX and PAS, the *p*ABA biosynthesis pathway was disrupted by deleting *pabC* in H37Ra. We found that although *pabC* was predicted to be essential for *in vitro* growth of *M. tuberculosis*, the first generation of the H37RaΔ*pabC* mutant did grow on 7H10 solid plates without exogenous *p*ABA and continued growing when inoculating into

TABLE 3 PAS MICs against different mycobacterial strains

Strain	Description	MIC (μg ml^{-1})
H37Ra(pMV261)	H37Ra transformed with pMV261	0.04
H37Ra(pMV261::*pabB*)	H37Ra transformed with pMV261::*pabB*	0.64
H37RaΔ*sigB*(pMV261::*pabB*)	H37RaΔ*sigB* transformed with pMV261::*pabB*	0.16
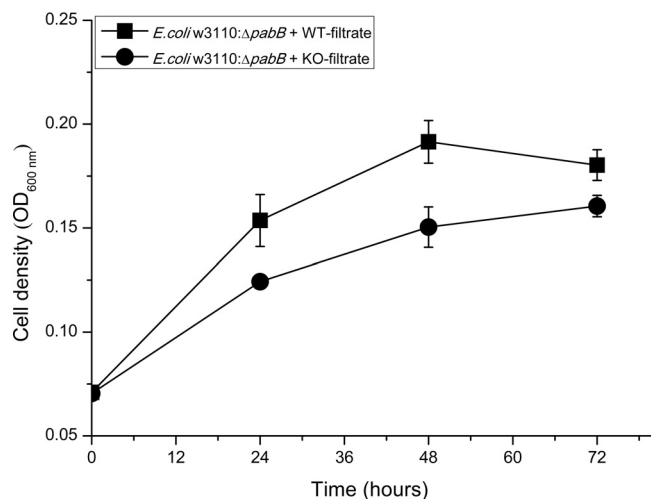
FIG 4 The synthesis of *p*ABA was impaired in H37Ra∆*sigB*. The growth curves of *E. coli* W3110∆*pabB* were determined in the presence of filtrates from culture of H37Ra (WT-filtrate) and H37Ra ∆*sigB* (KO-filtrate) strains separately. The means of three biological replicates are shown, and error bars denoted the standard deviations.

liquid medium in the absence of *p*ABA but stopped growing after it was subcultured into liquid medium. Although the H37Ra∆*pabC* mutant did not grow as well as the wild-type strain in the absence of *p*ABA on solid medium (see Fig. S2 in the supplemental material), it could be restored by the addition of a rather low concentration of *p*ABA (0.01 μg ml^{-1}). Then, the susceptibility to PAS of the H37Ra∆*pabC* mutant were determined by MIC tests. The results showed that deletion of *pabC* led to increased susceptibility to PAS in the presence of a limited amount of exogenous *p*ABA (0.01 and 0.05 μg ml^{-1}) (Table 4).

DISCUSSION

Previously, Thiede et al. reported that disruption of *p*ABA biosynthesis potentiates the antitubercular effect of anti-folates by up to 1,000-fold (33). Here, we showed that SigB regulates the expression of *pabB* gene in *M. tuberculosis* and, as a result, deletion of *sigB* results in decreased expression of *pabB*, impaired *p*ABA production, and increase susceptibility to antifolates.

Although the folate *de novo* biosynthesis pathway has been well studied in bacteria, very little is known about its regulation. In 2010, a eubacterial riboswitch class that selectively binds derivatives of folate was discovered, indicating a role of riboswitches for the regulation of folate biosynthesis (34). However, further evidence is required to prove this. Very recently, a vitamin B$_{12}$-binding light sensing transcriptional regulator (PhrR) was found in *Halomonas*, which modulates the expression of three genes related to folate biosynthesis (*folE*, *folK*, and *folM*) and shows the existence of transcriptional regulation of folate biosynthesis in bacteria (35). Further studies are necessary to see whether there would be any homologue of PhrR in other bacteria beyond gammaproteobacteria.

Except for PhrR, *sigB*, a well-known transcriptional regulator in bacteria, has also been shown to be related to folate metabolism. Previously, the alternative thymidylate synthase ThyX, which is involved in folate transformation, was found to be regulated by SigB (28). Since *M. tuberculosis* also has an alternative thymidylate synthase gene *thyX*

TABLE 4 PAS MICs against H37Ra and H37Ra∆*pabC* strains supplemented with *p*ABA

*p*ABA concn (μg ml^{-1})	MIC (μg ml^{-1})	
	H37Ra	H37Ra∆*pabC*
0.01	0.32	0.02
0.05	>0.32	0.08

(36), we wondered whether SigB could also regulate the expression of *thyX* in *M. tuberculosis*. Therefore, we deleted the *sigB* gene in H37Ra and tested its effect on *thyX* expression through Western blot analysis. Meanwhile, the susceptibility of the H37RaΔ*sigB* mutant to different types of antitubercular drugs, including antifolates, was also tested. To our surprise, although deletion of *sigB* in *M. tuberculosis* caused increased susceptibility to PAS and SMX, it did not affect the expression of *thyX* (see Fig. S3 in the supplemental material). These data suggested that in *M. tuberculosis* SigB does not regulate the expression of *thyX* and that the increased susceptibility to PAS and SMX caused by *sigB* deletion was not related to *thyX*.

Although the microarray data of H37Rv Δ*sigB* under normal growth and stress conditions had been reported (27), we failed to find any reasonable clue as to why the *sigB* knockout mutant was more sensitive to antifolates. From the known action mechanisms of PAS and SMX, we can see that *p*ABA is able to antagonize the antitubercular effects of both drugs, which means disruption of the *p*ABA biosynthesis pathway may result in increased sensitivity to both drugs. Thus, we speculated that the deletion of *sigB* might affect the expression of genes involved in *p*ABA biosynthesis. The data from our comparative RNA-seq analysis between H37Ra and its *sigB* deletion mutant strain showed that in *M. tuberculosis* the expression of *pabB* was regulated by SigB, which was confirmed by subsequent qRT-PCR and Western blot analysis.

In addition, our data of *in vitro* transcriptional analysis further confirmed that *sigB* is indeed able to recognize the promoter of *pabB* and initiate gene transcription. Although the transcription of *pabB* could be initiated by σ^B *in vitro*, the expression of *pabB* was not completely eliminated in the Δ*sigB* mutant (Fig. 3), suggesting the existence of other transcriptional regulators, such as SigA, for *pabB* in *M. tuberculosis*. The alternative sigma factor SigB of *M. tuberculosis* shows 62.9% identity to its principal sigma factor SigA in amino acid sequences (37, 38). Furthermore, a previous study showed that these two sigma factors recognize similar promoters with a conserved −10 element in TANNNT (39). In accordance, the *pabB* promoter contains a 5′-TAAGAT-3′ as the −10 element upstream of the transcriptional start site identified by Shell et al. (40). Thus, SigA and SigB may both be in charge of the initiation of *pabB* transcription in *M. tuberculosis*.

Since *pabB* is essential for *p*ABA biosynthesis and thus essential for *in vitro* bacterial growth, we speculated that a decrease in *pabB* expression might result in decreased production of *p*ABA in the Δ*sigB* mutant. Thus, we used the *E. coli* W3110 Δ*pabB* mutant to compare the *p*ABA production level between different H37Ra strains, and the results showed that the deletion of *sigB* led to decreased *p*ABA production. Considering the impairment to *p*ABA synthesis caused by *sigB* deletion, SigB should not be only regarded as an environmental stress responder in *M. tuberculosis*. In fact, it has been reported that in *C. glutamicum*, SigB was involved in the positive regulation of glucose metabolism even during the aerobic exponential phase (41). In addition, the homologue of SigB in *E. coli*, RpoS, was also shown to play an important role in iron acquisition and other metabolism gene regulation during exponential growth (42). Our data (in combination with those previous observations) suggested that SigB also played important roles under standard physiological growth conditions, and as a result, inactivation of *sigB* led to a slower growth under normal *in vitro* growth conditions (Fig. 1).

It has been previously reported that in *Pseudomonas aeruginosa*, RpoS (the homologue of SigB) was involved in tolerance to multiple antibiotics (43, 44). Therefore, except for antifolates, susceptibility to other antitubercular drugs was also tested for the H37RaΔ*sigB* mutant (Table 1). The results showed that the deletion of *sigB* in *M. tuberculosis* also led to increased susceptibility to many other antitubercular drugs. To obtain further insights into how *sigB* deletion affects susceptibility to multiple antitubercular drugs, we returned to our comparative RNA-seq data and found that deletion resulted in the downregulation of 37 genes involved in biosynthesis of the bacterial cell wall and 26 genes involved in biosynthesis of bacterial the plasma membrane, which might change bacteria permeability. However, further studies are required to probe the effect of *sigB* deletion on *M. tuberculosis* cell permeability.

Taken together, we found that deletion of *sigB* affected the *in vitro* growth of H37Ra and susceptibility to various antitubercular drugs, including PAS and SMX. It seemed that, in *M. tuberculosis* H37Ra, *sigB* could affect susceptibility to PAS and SMX through at least two different ways: first, by regulating the expression of *pabB*, which has been verified in this study, and second, by modulating the permeability of the bacterial cell, which requires further verification. So far, this is the first report showing that SigB modulates the biosynthesis of *p*ABA by regulating the expression of *pabB*, which broadens our understanding of both the physiological function of SigB and the regulation mechanisms of folate biosynthesis in mycobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *M. tuberculosis* strains were cultured at 37°C in 7H9 medium consisting of Middlebrook 7H9 broth (Difco), 10% (vol/vol) OADC (Difco), 0.5% (vol/vol) glycerol, and 0.05% (vol/vol) Tween 80 (Sigma-Aldrich) or on 7H10 agar medium (Difco) supplemented with 10% (vol/vol) OADC (Difco) and 0.5% (vol/vol) glycerol. *Mycobacterium smegmatis* mc²155 was grown in Middlebrook 7H9 medium or on 7H10 agar medium, both without adding OADC. *E. coli* trains HB101 and BL21(DE3) were cultured in Luria-Bertani (LB) medium (Difco) at 37°C or in E minimal medium (citric·H_2O [2 g/liter], $MgSO_4·7H_2O$ [0.2 g/liter], $K_2HPO_4·3H_2O$ [13.09 g/liter], $NaNH_4HPO_4·4H_2O$ [3.5 g/liter]) supplemented with 0.5% D-(+)-glucose. Plasmid pET-21a (Novagen) and pMV261 were used for the construction of expression plasmids. The gene-specific primers used for the construction of recombinant plasmids are listed in Table S3 in the supplemental material. Where appropriate, the culture medium was supplemented with hygromycin at 75 μg ml^{-1} for mycobacteria and 150 μg ml^{-1} for *E. coli*, kanamycin at 25 μg ml^{-1} for mycobacteria and 50 μg ml^{-1} for *E. coli*, and ampicillin at 100 μg ml^{-1} for *E. coli*.

Construction of mycobacterial mutant and complemented strains. A modified strategy for specialized transduction was used to construct the *M. tuberculosis* H37RaΔ*sigB* mutant. Genomic regions flanking *sigB*, 705 bp upstream (region containing *MRA_2737* and *MRA_2736*) and 838 bp downstream (region containing *ideR*), were amplified by PCR. The primers used for amplification of the upstream of *sigB* were *sigBko*LFP and *sigBko*LRP, and those for the region downstream were *sigBko*RFP and *sigBko*RRP. The recombinant plasmid p0004s-L+R was constructed by inserting the Van91I-digested PCR products into the plasmid p0004s digested with Van91I. Then, the p0004s-L+R was digested with PacI and ligated to the PacI-digested shuttle phasmid vector phAE159. After ligation, the recombinant cosmid phAE159-p0004s-L+R was transduced into *E. coli* HB101 in an *in vitro* λ-packaging reaction (Epicentre Biotechnologies, MaxPlax Lambda packaging extracts). Phasmid DNA prepared from confirmed selected hygromycin-resistant transductants was electroporated into *M. smegmatis* mc²155 to generate the specialized transducing phage. As described in a previous study (45), the transducing phage at the most efficient titer was used to infect H37Ra at a multiplicity of infection of 10. Successful specialized transduction of H37Ra was confirmed by comparing the size of the PCR-amplified product of hygromycin-resistant colonies with wild-type H37Ra using primers *sigB*LYZ and *sigB*RYZ (see Table S3 in the supplemental material). The gene *sigB* was amplified from *M. tuberculosis* H37Ra genomic DNA using the specific primers *sigB*-L and *sigB*-R (see Table S3) and then cloned into pMV261 to yield pMV261::*sigB*. The complemented strain was constructed by electrotransforming the recombinant plasmid pMV261::*sigB* into the competent cell of H37RaΔ*sigB* and plated on 7H10 medium supplemented with hygromycin at 75 μg ml^{-1} and kanamycin at 25 μg ml^{-1}.

Construction of the H37RaΔ*pabC* mutant was performed by the same strategies described above except for the last screening step in which, screening was performed on 7H10 medium supplemented with hygromycin at 75 μg ml^{-1} and an additional 10 μg ml^{-1} *p*ABA.

Drug susceptibility tests. Mycobacterial cells were cultured to optical density at 600 nm (OD$_{600}$) of 0.5 to 1.0 and diluted to about 10^5 CFU ml^{-1} by 10-fold serial dilutions in fresh 7H9 medium with or without 10% OADC. Bacteria were plated onto 7H10 agar solid plates containing various concentrations of different drugs: PAS (0, 0.00125, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, and 0.32 μg ml^{-1}), SMX (0, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg ml^{-1}), WR99210 (0, 1, 2, 4, 8, 16, 32, 64, and 128 μM), INH (0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 μg ml^{-1}), RIF (0, 0.000625, 0.00125, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 μg ml^{-1}), SM (0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, and 8 μg ml^{-1}), EMB (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, and 10 μg ml^{-1}), ofloxacin (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 μg ml^{-1}), and norfloxacin (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 μg ml^{-1}). All antibiotics were purchased from Sigma-Aldrich and solubilized according to the manufacturer's recommendations. Cultures were incubated at 37°C for 21 days. The MIC was defined as the lowest required concentration of antibiotics to inhibit the growth of 99% bacterial CFU. MIC tests for the H37RaΔ*pabC* mutant were performed on the 7H10 medium in the presence of different concentrations of *p*ABA (0.01, and 0.05 μg ml^{-1}) with various concentrations of PAS as described above.

Kill kinetics of PAS against *M. tuberculosis*. Bacteria were grown to OD$_{600}$ of 0.5 to 1.0 and diluted to about OD$_{600}$~0.1 (10^7 CFU ml^{-1}) in fresh 7H9 medium with OADC, and 0.4 μg ml^{-1} (10×MIC of WT) of PAS was used in PAS treatment. Cultures were incubated at 37°C, and aliquots of samples were taken and plated on 7H10 medium after serial dilutions at days 0, 1, 4, and 8, separately.

RNA-seq. Total RNA was isolated using an RNeasy minikit (Qiagen, Germany). Library constructions were prepared using TruSeq stranded total RNA sample preparation kit (Illumina, USA), and RNA sequencing was conducted using an Illumina HiSeq 2500 at Shanghai Biotechnology Corporation. The insert size conformation of purified libraries was validated by an Agilent 2100 bioanalyzer (Agilent

Technologies, USA). Bowtie2 v2-2.0.5 was used to map the cleaned reads to the *M. tuberculosis* H37Ra genome acquired from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/148659757?report=GenBank). Then, HTSeq v2.1.1 was run with a reference annotation to generate fragments per kilobase of exon model per million mapped reads values for estimation of fold changes. Three biological replicates were used in RNA-seq and the *P* and *q* values were calculated. The differentially expressed genes were selected using the following filter criteria: a false discovery rate of ≤0.05 and a fold change of ≥1.5.

Quantitative real-time PCR assays. Total RNA was extracted as described previously, and cDNA was synthesized with a ReverTra Ace qPCR kit (Toyobo) according to the manufacturer's instructions. Quantification of the gene expression levels was performed by real-time qPCR analysis on a 7900 HT sequence detection system (ABI, USA) with ABI Power SYBR green PCR master mix. Primers specific to *pabB* were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Expression levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA were normalized as an endogenous control. The gene-specific primers *pabB*-qRT-L and *pabB*-qRT-R used are listed in Table S3 in the supplemental material.

In vitro transcription assay. The recombinant *M. tuberculosis* RNAP core enzyme containing 6×His tag at the C terminus of the β' subunit was purified from *E. coli* BL21(DE3) carrying pMR4 plasmid as described previously (39). Mycobacterial SigB was expressed in *Escherichia coli* BL21(DE3) using pET28a plasmid and purified as previously described (39). *In vitro* transcription was performed in transcription buffer (20 mM Tris-HCl [pH 7.9], 50 mM NaCl, 5 mM MgSO$_4$, 1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol) in a total volume of 5 μl. RNAP holoenzyme was assembled by mixing the 600 nM SigB with 200 nM core RNAP, followed by incubation for 5 min. A 15 nM promoter DNA fragment was added, followed by incubation at 37°C for 10 min. Transcription was initiated by the addition of 50 μM ATP, GTP, and CTP and 3 μCi of [α-³²P]UTP and then carried out for 10 min at 37°C. Reactions were stopped by adding 8 M urea, and the synthesized RNA products were analyzed on denaturing (7 M urea) 18% polyacrylamide gel electrophoresis (PAGE). The primers *pabB*-p-L and *pabB*-p-R listed in Table S3 in the supplemental material were designed according to the reported transcription start site of *pabB* analyzed by Shell et al. for *in vitro* transcriptional assay (40).

Purification of recombinant histidine-tagged PabB. PabB was amplified from *M. tuberculosis* H37Ra genomic DNA using the specific primers M*pabB*-L and M*pabB*-R (see Table S3) and cloned into pET21a to yield pET21a::*pabB*. After sequence verification, these recombinant plasmids were transformed into *E. coli* BL21(DE3). The transformed *E. coli* BL21(pET21a::*pabB*) cells were grown at 37°C in LB broth to an OD$_{600}$ of ~0.6. Then, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, followed by incubation at 16°C for 20 h. The cells were harvested by centrifugation after incubation and resuspended in 50 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole (pH 8.0). Suspensions were disrupted by sonication and clarified by high-speed centrifugation. After centrifugation, the supernatants were mixed with prewashed nickel-nitrilotriacetic acid His-Trap HP affinity resin (GE Healthcare) at 4°C overnight, and nonspecifically bound protein was removed by washing the resin with 50 mM Tris-HCl, 0.5 M NaCl, and 60 mM imidazole (pH 8.0). Meanwhile, recombinant PabB was eluted with 50 mM Tris-HCl, 0.5 M NaCl, and 200 mM imidazole (pH 8.0) separately. SDS-PAGE was used to analyze the acquired elution.

Antibody preparation of PabB. Five mice as a group were immunized with purified recombinant PabB. They were injected intramuscularly with 0.5 mg of recombinant proteins in a ratio of 1:1 (vol/vol) with Freund complete adjuvant for the first immunization. Then, every 2 weeks a similar injection was subcutaneously administered, except that Freund incomplete adjuvant was used. Immune sera were collected after four injections, and mice were bled a week after the fourth injection. The specificity of antisera against recombinant proteins was tested by Western blotting assay.

Western blot analysis. The H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*) strains were cultured in 50 ml of 7H9 medium at 37°C and harvested at log phase by centrifugation. For Western blot analysis, pellets were resuspended in 50 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole (pH 8.0) and then lysed by using zirconium beads. Protein samples acquired from the supernatant after centrifugation were separated by SDS–10% PAGE and immediately transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) by a Bio-Rad SD device (Bio-Rad Laboratories, Hercules, CA) at 15 V for 25 min. Finally, the proteins were probed with mice antisera against PabB.

Growth curves of the *E. coli* W3110 Δ*pabB* mutant. Filtrates from cultures of the three strains [H37Ra, H37RaΔ*sigB*, and H37RaΔ*sigB*(pMV261::*sigB*)] were collected, respectively, through sterile filters (Millipore). *E. coli* W3110Δ*pabB* was cultured to log phase in LB medium at 37°C and then harvested by centrifugation. After that, the pellet was washed twice and resuspended for inoculation (at ca. 10⁶ to 10⁷ CFU/ml) into fresh E medium supplemented with 0.5% D-(+)-glucose for starvation. After incubation at 37°C to consume the endogenous *p*ABA for 72 h, the *E. coli* W3110Δ*pabB* mutant was inoculated (10⁵ CFU/ml) into 2 ml of ETG plus 8-ml filtrates from the different strains mentioned above. The growth of bacterial cultures was measured by monitoring the OD$_{600}$ every 24 h using a spectrophotometer (Bio-Rad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00551-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

REFERENCES

1. World Health Organization. 2016. Global tuberculosis report 2016. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/global_report/en/.

2. Nguyen L. 2016. Antibiotic resistance mechanisms in *Mycobacterium tuberculosis*: an update. Arch Toxicol 90:1585–1604. https://doi.org/10.1007/s00204-016-1727-6.

3. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients coinfected with tuberculosis and HIV in a rural area of South Africa. Lancet 368:1575–1580. https://doi.org/10.1016/S0140-6736(06)69573-1.

4. Wallis RS, Maeurer M, Mwaba P, Chakaya J, Rustomjee R, Migliori GB, Marais B, Schito M, Churchyard G, Swaminathan S, Hoelscher M, Zumla A. 2016. Tuberculosis-advances in development of new drugs, treatment regimens, host-directed therapies, and biomarkers. Lancet Infect Dis 16:e34–e46. https://doi.org/10.1016/S1473-3099(16)00070-0.

5. Nguyen L, Jacobs MR. 2012. Counterattacking drug-resistant tuberculosis: molecular strategies and future directions. Expert Rev Anti Infect Ther 10:959–961. https://doi.org/10.1586/eri.12.97.

6. Bermingham A, Derrick JP. 2002. The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. Bioessays 24:637–648. https://doi.org/10.1002/bies.10114.

7. Ye QZ, Liu J, Walsh CT. 1990. *p*-Aminobenzoate synthesis in *Escherichia coli*: purification and characterization of PabB as aminodeoxychorismate synthase and enzyme X as aminodeoxychorismate lyase. Proc Natl Acad Sci U S A 87:9391–9395. https://doi.org/10.1073/pnas.87.23.9391.

8. Green JM, Nichols BP. 1991. *p*-Aminobenzoate biosynthesis in *Escherichia coli*: purification of aminodeoxychorismate lyase and cloning of *pabC* J Biol Chem 266:12971–12975.

9. Nichols BP, Seibold AM, Doktor SZ. 1989. *para*-Aminobenzoate synthesis from chorismate occurs in two steps. J Biol Chem 264:8597–8601.

10. Richey DP, Brown GM. 1969. The biosynthesis of folic acid. IX. Purification and properties of the enzymes required for the formation of dihydropteroic acid. J Biol Chem 244:1582–1592.

11. Shiota T, Baugh CM, Jackson R, Dillard R. 1969. The enzymatic synthesis of hydroxymethyldihydropteridine pyrophosphate and dihydrofolate. Biochemistry 8:5022–5028. https://doi.org/10.1021/bi00840a052.

12. Blakley RL, Mcdougall BM. 1961. Dihydrofolic reductase from *Streptococcus faecalis* R. J Biol Chem 236:1163–1167.

13. Estrada A, Wright DL, Anderson AC. 2016. Antibacterial antifolates: from development through resistance to the next generation. CSH Perspect Med 6:a028324. https://doi.org/10.1101/cshperspect.a028324.

14. Chakraborty S, Gruber T, Barry CE, III, Boshoff HI, Rhee KY. 2012. *para*-Aminosalicylic acid acts as an alternative substrate of folate metabolism in *Mycobacterium tuberculosis*. Science 339:88–91. https://doi.org/10.1126/science.1228980.

15. Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, Jang J, Nam J, Dick T, Walker JR, Pethe K, Camacho LR. 2013. *para*-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. J Biol Chem 288:23447–23456. https://doi.org/10.1074/jbc.M113.475798.

16. Zhao F, Wang XD, Erber LN, Luo M, Guo AZ, Yang SS, Gu J, Turman BJ, Gao YR, Li DF, Cui ZQ, Zhang ZP, Bi LJ, Baughn AD, Zhang XE, Deng JY. 2014. Binding pocket alterations in dihydrofolate synthase confer resistance to *para*-aminosalicylic acid in clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 58:1479–1487. https://doi.org/10.1128/AAC.01775-13.

17. Zhang X, Liu L, Zhang Y, Dai G, Huang H, Jin Q. 2015. Genetic determinants involved in *p*-aminosalicylic acid resistance in clinical isolates from tuberculosis patients in northern China from 2006 to 2012. Antimicrob Agents Chemother 59:1320–1324. https://doi.org/10.1128/AAC.03695-14.

18. Rengarajan J, Sassetti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. 2004. The folate pathway is a target for resistance to the drug *para*-aminosalicylic acid (PAS) in mycobacteria. Mol Microbiol 53:275–282. https://doi.org/10.1111/j.1365-2958.2004.04120.x.

19. Moradigaravand D, Grandjean L, Martinez E, Li H, Zheng J, Coronel J, Moore D, Torok ME, Sintchenko V, Huang H, Javid B, Parkhill J, Peacock SJ, Koser CU. 2016. *dfrA thyA* double deletion in *para*-aminosalicylic acid-resistant *Mycobacterium tuberculosis* Beijing strains. Antimicrob Agents Chemother 60:3864–3867. https://doi.org/10.1128/AAC.00253-16.

20. Cheng YS, Sacchettini JC. 2016. Structural insights into *Mycobacterium tuberculosis* Rv2671 protein as a dihydrofolate reductase functional analogue contributing to *para*-aminosalicylic acid resistance. Biochemistry 55:1107–1119. https://doi.org/10.1021/acs.biochem.5b00993.

21. Carreras CW, Santi DV. 1995. The catalytic mechanism and structure of thymidylate synthase. Annu Rev Biochem 64:721–762. https://doi.org/10.1146/annurev.bi.64.070195.003445.

22. Rodrigue S, Provvedi R, Jacques PE, Gaudreau L, Manganelli R. 2006. The sigma factors of *Mycobacterium tuberculosis*. FEMS Microbiol Rev 30:926–941. https://doi.org/10.1111/j.1574-6976.2006.00040.x.

23. Manganelli R, Voskuil MI, Schoolnik GK, Smith I. 2001. The *Mycobacterium tuberculosis* ECF sigma factor σ^E: role in global gene expression and survival in macrophages. Mol Microbiol 41:423–437. https://doi.org/10.1046/j.1365-2958.2001.02525.x.

24. Manganelli R, Voskuil MI, Schoolnik GK, Dubnau E, Gomez M, Smith I. 2002. Role of the extracytoplasmic-function sigma factor σ^H in *Mycobacterium tuberculosis* global gene expression. Mol Microbiol 45:365–374. https://doi.org/10.1046/j.1365-2958.2002.03005.x.

25. Dainese E, Rodrigue S, Delogu G, Provvedi R, Laflamme L, Brzezinski R, Fadda G, Smith I, Gaudreau L, Palu G, Manganelli R. 2006. Posttranslational regulation of *Mycobacterium tuberculosis* extracytoplasmic-function sigma factor sigma L and roles in virulence and in global regulation of gene expression. Infect Immun 74:2457–2461. https://doi.org/10.1128/IAI.74.4.2457-2461.2006.

26. Manganelli R, Dubnau E, Tyagi S, Kramer FR, Smith I. 1999. Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. Mol Microbiol 31:715–724. https://doi.org/10.1046/j.1365-2958.1999.01212.x.

27. Fontan PA, Voskuil MI, Gomez M, Tan D, Pardini M, Manganelli R, Fattorini L, Schoolnik GK, Smith I. 2009. The *Mycobacterium tuberculosis* sigma factor σ^B is required for full response to cell envelope stress and hypoxia *in vitro*, but it is dispensable for *in vivo* growth. J Bacteriol 191:5628–5633. https://doi.org/10.1128/JB.00510-09.

28. Cho S, Yang S, Rhie H. 2012. The gene encoding the alternative thymidylate synthase ThyX is regulated by sigma factor SigB in *Corynebacterium glutamicum* ATCC 13032. FEMS Microbiol Lett 328:157–165. https://doi.org/10.1111/j.1574-6968.2011.02494.x.

29. Guzzo MB, Nguyen HT, Pham TH, Wyszczelska-Rokiel M, Jakubowski H, Wolff KA, Ogwang S, Timpona JL, Gogula S, Jacobs MR, Ruetz M, Krautler B, Jacobsen DW, Zhang GF, Nguyen L. 2016. Methylfolate trap promotes bacterial thymineless death by sulfa drugs. PLoS Pathog 12:e1005949. https://doi.org/10.1371/journal.ppat.1005949.

30. Gomez M, Doukhan L, Nair G, Smith I. 1998. *sigA* is an essential gene in *Mycobacterium smegmatis*. Mol Microbiol 29:617–628. https://doi.org/10.1046/j.1365-2958.1998.00960.x.

31. Lee JH, Karakousis PC, Bishai WR. 2008. Roles of SigB and SigF in the *Mycobacterium tuberculosis* sigma factor network. J Bacteriol 190:699–707. https://doi.org/10.1128/JB.01273-07.

32. Chen ZK, Hu YB, Cumming BM, Lu P, Feng LP, Deng JY, Steyn AJC, Chen SY. 2016. Mycobacterial WhiB6 differentially regulates ESX-1 and the Dos regulon to modulate granuloma formation and virulence in zebrafish. Cell Rep 16:2512–2524. https://doi.org/10.1016/j.celrep.2016.07.080.

33. Thiede JM, Kordus SL, Turman BJ, Buonomo JA, Aldrich CC, Minato Y, Baughn AD. 2016. Targeting intracellular *p*-aminobenzoic acid production potentiates the anti-tubercular action of antifolates. Sci Rep 6:38083. https://doi.org/10.1038/srep38083.

34. Ames TD, Rodionov DA, Weinberg Z, Breaker RR. 2010. A eubacterial

riboswitch class that senses the coenzyme tetrahydrofolate. Chem Bio 17:681–685. https://doi.org/10.1016/j.chembiol.2010.05.020.

35. Romine MF, Rodionov DA, Maezato Y, Anderson LN, Nandhikonda P, Rodionova IA, Carre A, Li X, Xu C, Clauss TR, Kim YM, Metz TO, Wright AT. 2017. Elucidation of roles for vitamin B$_{12}$ in regulation of folate, ubiquinone, and methionine metabolism. Proc Natl Acad Sci U S A 114: E1205–E1214. https://doi.org/10.1073/pnas.1612360114.

36. Fivian-Hughes AS, Houghton J, Davis EO. 2012. *Mycobacterium tuberculosis* thymidylate synthase gene *thyX* is essential and potentially bifunctional, while *thyA* deletion confers resistance to *p*-aminosalicylic acid. Microbiology 158:308–318. https://doi.org/10.1099/mic.0.053983-0.

37. Hu YM, Coates ARM. 1999. Transcription of two sigma 70 homologue genes, *sigA* and *sigB*, in stationary-phase *Mycobacterium tuberculosis*. J Bacteriol 181:8.

38. Manganelli R, Proveddi R, Rodrigue S, Beaucher J, Gaudreau L, Smith I. 2004. Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. J Bacteriol 186:895–902. https://doi.org/10.1128/JB.186.4.895 -902.2004.

39. Hu YB, Morichaud Z, Perumal AS, Roquet-Baneres F, Brodolin K. 2014. *Mycobacterium* RbpA cooperates with the stress-response σ^{B} subunit of RNA polymerase in promoter DNA unwinding. Nucleic Acids Res 42: 10399–10408. https://doi.org/10.1093/nar/gku742.

40. Shell SS, Wang J, Lapierre P, Mir M, Chase MR, Pyle MM, Gawande R, Ahmad R, Sarracino DA, Ioerger TR, Fortune SM, Derbyshire KM, Wade JT,

Gray TA. 2015. Leaderless transcripts and small proteins are common features of the mycobacterial translational landscape. PLoS Genet 11: e1005641. https://doi.org/10.1371/journal.pgen.1005641.

41. Ehira S, Shirai T, Teramoto H, Inui M, Yukawa H. 2008. Group 2 sigma factor SigB of *Corynebacterium glutamicum* positively regulates glucose metabolism under conditions of oxygen deprivation. Appl Environ Microbiol 74:5146–5152. https://doi.org/10.1128/AEM.00944-08.

42. Dong T, Kirchhof MG, Schellhorn HE. 2008. RpoS regulation of gene expression during exponential growth of *Escherichia coli* K-12. Mol Genet Genomics 279:267–277. https://doi.org/10.1007/s00438-007-0311-4.

43. Murakami K, Ono T, Viducic D, Kayama S, Mori M, Hirota K, Nemoto K, Miyake Y. 2005. Role for *rpoS* gene of *Pseudomonas aeruginosa* in antibiotic tolerance. FEMS Microbiol Lett 242:161–167. https://doi.org/ 10.1016/j.femsle.2004.11.005.

44. Kayama S, Murakami K, Ono T, Ushimaru M, Yamamoto A, Hirota K, Miyake Y. 2009. The role of *rpoS* gene and quorum-sensing system in ofloxacin tolerance in *Pseudomonas aeruginosa*. FEMS Microbiol Lett 298:184–192. https://doi.org/10.1111/j.1574-6968.2009.01717.x.

45. Bardarov S, Bardarov S, Jr, Pavelka MS, Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, and Jacobs WR, Jr. 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG, and *M. smegmatis*. Microbiology 148:3007–3017. https://doi.org/10 .1099/00221287-148-10-3007.