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Altered Protein Expression Patterns of *Mycobacterium tuberculosis* Induced by ATB107

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ATB107 is a potent inhibitor of indole-3-glycerol phosphate synthase (IGPS). It can effectively inhibit the growth of clinical isolates of drug-resistant *Mycobacterium tuberculosis* strains as well as *M. tuberculosis* H37Rv. To investigate the mechanism of ATB107 action in *M. tuberculosis*, two-dimensional gel electrophoresis coupled with MALDI-TOF-MS analysis (2-DE-MS) was performed to illustrate alterations in the protein expression profile in response to ATB107. Results show that ATB107 affected tryptophan biosynthesis by decreasing the expression of protein encoded by Rv3246c, the transcriptional regulatory protein of MtrA belonging to the MtrA-MtrB two-component regulatory system, in both drug-sensitive and drug-resistant virulent strains. ATB107 might present a stress condition similar to isoniazid (INH) or ethionamide for *M. tuberculosis* since the altered expression in response to ATB107 of some genes, such as Rv3140, Rv2243, and Rv2428, is consistent with INH or ethionamide treatment. After incubation with ATB107, the expression of 2 proteins encoded by Rv0685 and Rv2624c was down-regulated while that of protein encoded by Rv3140 was up-regulated in all *M. tuberculosis* strains used in this study. This may be the common response to tryptophan absence; however, relations to ATB107 are unknown and further evaluation is warranted.

***Keywords*:** *M. tuberculosis*, ATB107, indole-3-glycerol phosphate synthase, inhibitor, tryptophan biosynthesis

Despite the adjustment of DOTS (directly observed treatment, short course) framework for tuberculosis (TB) control, TB continues to be a major infectious disease with 9.27 million new cases and 1.3 million deaths in 2007 (WHO, 2009). In recent years, the increasing emergence of multidrug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) strains (Gandhi *et al.*, 2006) present a formidable challenge to TB control. The growing burden of drug resistance combined with TB-HIV co-infection indicates an urgent need for novel anti-TB drugs based on new targets.

Indole-3-glycerol phosphate synthase (IGPS) catalyzes the fourth step in the tryptophan biosynthetic pathway, which plays an important role in the growth of *Mycobacterium tuberculosis.* Its absence in mammals makes enzymes in this biosynthetic pathway valuable targets for new anti-TB drug discovery (Lee *et al.*, 2006). The compound ATB107 (Fig. 1), a potent inhibitor of IGPS, can effectively inhibit the growth of not only drug-sensitive strains but also clinical isolated drug-resistant strains (Shen *et al.*, 2009). However, it is not clear how ATB107 affects the metabolism of *M. tuberculosis*. This information will provide valuable clues to elucidate the molecular mechanism of mycobacterial killing.

The genome sequencing of *M. tuberculosis* strains (Cole *et al.*, 1998; Zheng *et al.*, 2008) facilitates functional genomics studies illuminating whole-genome biology. DNA microarray technology has been widely applied to monitor gene expression profiles at a genome-wide scale (Wilson *et al.*, 1999; Betts *et al.*, 2003; Waddell *et al.*, 2004; Denkin *et al.*, 2005; Fu, 2006; Fu and Shinnick, 2007; Karakousis *et al.*, 2008). However, DNA microarray provides only a view of the dynamics associated with cellular responses at a certain time point (Hughes *et al.*, 2006). There is not a direct correlation between mRNA expression and changes in the protein population in response to a stimulus, due in part to post-translational control mechanisms (Mehra *et al.*, 2003). As proteins are the targets for most drugs, understanding of drug-related responses at the level of the proteome will unravel the important dynamics of a drug's mechanism of action and define new pathways for drug

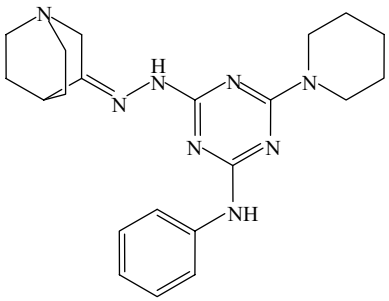

Fig. 1. ATB107 is a nitrogen heterocyclic ligand fused with polycyclic rings.

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discovery (Hughes *et al.*, 2006).

To explore the mechanism of ATB107 action, two-dimensional gel electrophoresis (2-DE) coupled with MALDI-TOF-MS analysis between drug-treated and untreated *M. tuberculosis* H37Rv strains was performed. We also investigated alterations in protein expression profiles of 2 drug-resistant clinical strains (1620 and 1499) and avirulent *M. tuberculosis* H37Ra to provide further insight into the mechanism of ATB107 action.

Materials and Methods

M. tuberculosis strains growth conditions and drug treatment

M. tuberculosis H37Rv and clinical isolated *M. tuberculosis* 1620 (isoniazid-resistant) and 1499 (isoniazid- and rifampin-resistant) were provided by Dr. Jun Yue of Shanghai Pulmonary Hospital of China. *M. tuberculosis* H37Ra (Steenken, 1935), the avirulent counterpart of virulent strain H37Rv, was donated by professor Ying Zhang of the John Hopkins University, USA.

M. tuberculosis H37Rv strains were cultured in fresh Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.05% Tween-80, and 10% oleic acid-albumin-dextrose-catalase-enriched Middlebrook (OADC) at 37°C without shaking. After 2 weeks, ATB107 was added into bacteria cultures to achieve a final concentration of 25 ng/ml (1/4 MIC), and the cultures were incubated at 37°C for another week before collection.

Sample preparation

Bacteria cells were harvested by centrifugation at 3,000×g for 30 min, washed with phosphate buffered saline (PBS, pH 7.4), and inactivated via γ-irradiation. Cell pellets were suspended in 120 μl double-distilled water supplemented with a protease inhibitor mixture (1 mM phenylmethanesulfonyl fluoride, 10 mM EDTA). Lysis of tubercle bacilli was obtained by sonication until a uniform suspension was achieved. To bring proteins into solution, 150 μl of the suspension was combined with 0.141 g urea, 0.0386 g thiourea, 16 μl dithiothreitol (2.8 M), 9.6 μl SDS (10%), 0.8 μl Biolyte (pH 3-5), and 0.8 μl Biolyte (pH 5-8) (Bio-Rad, USA).

2D-PAGE

An aliquot of the lysate containing 100 μg of proteins was added to 350 μl rehydration buffer (7.77 M urea, 2.4 M thiourea, 4% CHAPS, 2.8% dithiothreitol, 1.05 μl Biolyte; pH 3-5, 0.7 μl Biolyte; pH 5-8) and used for IPG rehydration at 50 V for 13 h. Isoelectric focusing was performed at 17°C on 17 cm Immobiline pH 4-7 strips using a Protean IEF cell focusing system (Bio-Rad, USA). The isoelectric focusing was initiated at a voltage of 250 V for 2 h, stepped to 1,000 V and held for 2.75 h, after which a linear gradient voltage increase from 1,000 V to 8,000 V for 5 h was applied to the strips. The final voltage was 8,000 V to achieve 88,000 V/h. Prior to the second dimension the strips were incubated for 15 min in 4 ml equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris; pH 8.8, 20% glycerol) first with 130 mM dithiothreitol, and then with 135 nM iodoacetamide. Each equilibrated strip was put onto a 0.75 cm thick, 18 cm wide, 24 cm long, 12% polyacrylamide gel, and the 2D was run at 60 mA for 5 h in the Protean@ iiXi Cell 2D gel system (Bio-Rad). The gels were stained with silver nitrate (Scheler *et al.*, 1998; Gharahdaghi *et al.*, 1999), and scanned using a GS-800 calibrated densitometer (Bio-Rad).

Gel images were analyzed using PDQuest 7.1 software (Bio-Rad). Though different parameters were applied to gel images obtained from different *M. tuberculosis* strains, images of drug-treated and untreated bacteria from the same *M. tuberculosis* strain shared the same parameters. For gel images gained from H37Rv, the spot detection parameter wizard was set to a sensitivity of 0.39 combined with a scale size of 3 and minimum peak value of 4506. Streaks were removed by parameters of a vertical radius of 25 and a horizontal radius of 29. Background was subtracted by the floating ball method with a radius of 25. Smoothing was done by a kernel size of 3×3.

In gel images obtained from drug-treated bacteria, one spot was regarded as down-regulated if its density was lower than 80% of the density of the corresponding spot in the other gel images of untreated bacteria of the same *M. tuberculosis* strain. Similarly, one spot was regarded as up-regulated when its density was more than 120% of that of the corresponding spot. Other spots were regarded as having no significant change.

In-gel tryptic digest

The protein spots of interest were manually excised from the gels and then destained in a mixture of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) for 20 min at room temperature. The gel dots were then washed twice with deionized water and dehydrated in acetonitrile (ACN). Dried gel particles were rehydrated at 4°C for 45 min with 2 μl per well trypsin (Promega, USA) and then incubated in 25 mM ammonium bicarbonate at 37°C for 12 h, after which the peptide mixtures were extracted using 8 μl extraction solution (50% ACN, 0.5% TFA) per well at 37°C for 1 h. The extracts were dried under the protection of N_2.

MALDI-TOF/TOF MS and MS/MS analysis

The peptides were eluted with 0.8 μl matrix solution containing α-cyano-4-hydroxy-cinnamic acid (CHCA, Sigma, USA) in 0.1% TFA and 50% ACN before being spotted on the target plate and air-dried. Analysis was accomplished with a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, UK) equipped with a 355 nm Nd:YAG laser. Proteins were identified by peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) using the program MASCOT V2.1 (Matrix Science, UK) against the NCBInr database with GPS explorer software (Applied Biosystems) which restricted the search to *Mycobacterium* species. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 53 combined with at least 2 identified peptides were considered statistically significant ($P < 0.05$).

Results and Discussion

Proteins regulated by ATB107 treatment in *M. tuberculosis* H37Rv

To monitor the protein expression profiles in response to ATB107 treatment in *M. tuberculosis* H37Rv strain, two-dimensional gel electrophoresis (2-DE) was employed to separate proteins according to their isoelectric point (pI) and molecular mass. The silver-stained 2-DE protein patterns contain more than 600 spots in each gel (Fig. 2). Protein expression patterns between drug-treated and untreated strains were compared 3 times and differences confirmed in all comparisons were accepted. Using mass spectrometry, 33 protein spots with obvious expression alterations were successfully identified. The MS spectrum of spot No. 25 with a match score of 165 was identified as MtrA (Rv3246c) (Fig. 3).

There were 30 spots identified as single gene products. Among them, proteins of spots No. 1 and 2 were both identified as products of Rv3140, proteins of spot No. 12, 13

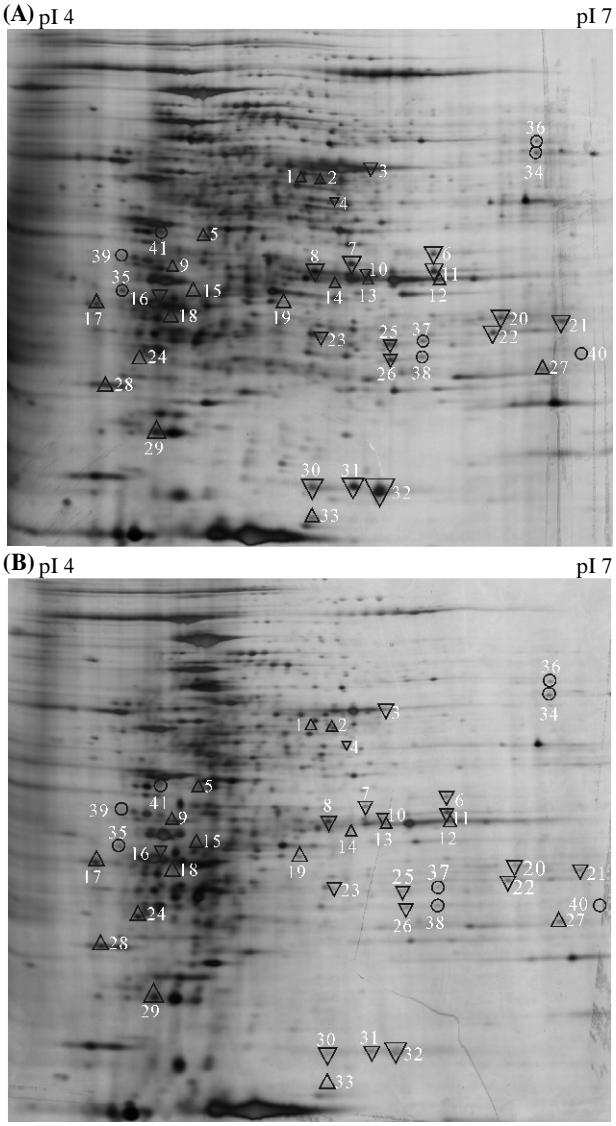

Fig. 2. Two-dimensional electrophoresis patterns of cell total proteins of *M. tuberculosis* H37Rv strains without ATB107 treatment (A) and those incubated with ATB107 (B). 100 μg proteins were separated first by isoelectric focusing in the pH range between 4.0 and 7.0 and then by 12% SDS-PAGE in 2D. The 2D gels were stained with silver nitrate and the spot intensity was quantified by the PDQuest-7.1.0 software (Bio-Rad). (△) increased spot intensity after ATB107 treatment; (▽) decreased spot intensity after ATB107 treatment; (○) no significant change in spot intensity.

and 14 were products of Rv2623, and proteins of spot No. 32 and 30 were products of Rv1284. Moreover, there were 3 protein spots identified as product mixtures of several genes. Spot No. 24 was identified as the mixture product of Rv0560c and Rv3676 genes, spot No. 23 was mixture product of Rv0554 and Rv3400, and spot No. 22 was mixture product of Rv1484 and Rv1109c. In all, 32 genes encoding the identified proteins were recognized (Table 1).

Among the differentially expressed protein spots in response to ATB107 treatment, 16 spots were up-regulated

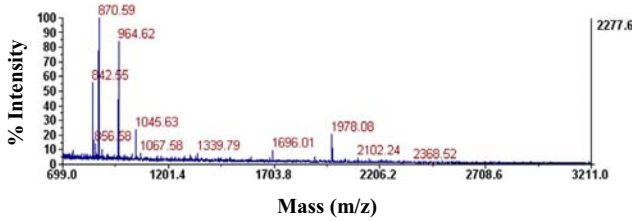

Fig. 3. MALDI-TOF spectra obtained for Rv3246c (spot 25). The spectrum of peptide at 964.62 Da was matched to the peptide sequence LVNVHVQR, the peptide at 1339.79 Da was matched to the peptide sequence DPENPTVVLTVR, the peptide at 1696.01 Da was matched to the peptide sequence VEKDPENPTVVLTVR, the peptide at 1978.08 Da was matched to the peptide sequence GVREGFDTAVIGDGT QALTAVR, and the peptide at 2102.24 Da was matched to the peptide sequence ILVVDDDASLAEMLTIVLR. These peptides led to the identification of their parent protein, MtrA (Rv3246c).

and 17 spots were down-regulated. Based on the Pasteur Institute functional classification (http://genolist.pasteur.fr/TubercuList), the genes encoding the identified proteins belong to 6 classes (Table 1).

Proteins related to detoxification and adaptation

We identified 4 genes whose products are related to detoxification or adaptation (Table 1). Rv0251c and Rv2428 are up-regulated. It is reported that Rv0251c is induced by starvation and oxidative stress (Manganelli *et al.*, 2001; O'Toole *et al.*, 2003). Rv2428 could be induced by treatment of important anti-TB drugs isonizide (INH) or ethionamide (Wilson *et al*., 1999), which means that the higher expression of these genes is a characteristic response of *M. tuberculosis* to stress conditions and drug treatments. Hence the treatment of ATB107 can induce their expression.

Rv0440 and Rv0554 are down-regulated by ATB107 treatment. Rv0440 could prevent misfolding and promotes the refolding and proper assembly of generated unfolded polypeptides (Walters *et al.*, 2006). The decrease of its expression caused by ATB107 would weaken the self restoring function of bacteria under stress conditions, which may be a way for ATB107 to inhibit the growth of *M. tuberculosis.* Similarly, ATB107 could affect the function of Rv0554 involved in detoxification reactions (Sassetti *et al*., 2003).

Proteins involved in lipid metabolism

Seven proteins identified in our research (Table 1) are involved in lipid metabolism. Expression of Rv0905, Rv1094, Rv2243, and Rv3140 is up-regulated by ATB107 treatment. It is well known that these genes are related to the metabolism and biosynthesis of fatty acid and some amino acids (Xiong *et al.*, 2005), implying that ATB107 treatment promotes the metabolism and biosynthesis of fatty and amino acids, as well as other stress conditions like starvation, and that caused by treatment with tuberculosis drugs INH and ethionamide (Wilson *et al*., 1999).

Protein expression of Rv0468, Rv0642c, and Rv1484 is down-regulated by incubation with ATB107. Rv0468 is involved in the process of benzoate degradation and butanoate meta-

bolism (Fisher *et al*., 2002). The products of Rv1484 and Rv0642c are involved in mycolic acid biosynthesis and modification respectively, implying that ATB107 has an impact on these major constituents of the mycobacterial cell wall complex. The consequence of expression reduction of these 2 proteins could result in a decrease in mycolic acids.

Proteins involved in information pathways

Rv0685 is identified as the only gene that participates in information pathways in the present study. It encodes a probable iron-regulated elongation factor tu (EF-Tu) that promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. During Mg^{2+} starvation (Walters *et al*., 2006) this essential gene

Table 1. Protein variability between cell proteins of *M. tuberculosis* H37Rv strain with and without ATB107 incubation

Protein Spot No.[a]	ORF	Gene	Description	Functional class[b]	Density alteration[c]
Up-regulated					
1	Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	1	3.02
2	Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	1	1.40
5	Rv0281		Possible S-adenosylmethionine-dependent methyltransferase	10	2.47
9	Rv2971		Probable oxidoreductase	7	3.62
12	Rv2623	*TB31.7*	Conserved hypothetical protein	10	1.44
13	Rv2623	*TB31.7*	Conserved hypothetical protein	10	2.51
14	Rv2623	*TB31.7*	Conserved hypothetical protein	10	5.46
15	Rv2243	*fabD*	Malonyl CoA-acyl carrier protein transacylase	1	1.66
17	Rv0577	*TB27.3*	Conserved hypothetical protein	10	1.36
18	Rv1094	*desA2*	Possible acyl-(acyl-carrier protein) desaturase	1	1.37
19	Rv3099c		Conserved hypothetical protein	10	1.87
24	Rv0560c		Possible benzoquinone methyltransferase	7	5.55
	Rv3676		Probable transcriptional regulator belonging to crp/fnr family	9	
27	Rv0905	echA6	Possible enoyl-CoA hydratase	1	1.20
28	Rv2428	*ahpC*	Alkyl hydroperoxide reductase C	0	7.36
29	Rv3841	*bfrB*	Possible bacterioferritin bfrB	7	1.72
33	Rv0251c	acr2	Heat-stress-induced ribosome-binding protein A	0	1.37
Down-regulated					
3	Rv0685	*Tuf*	Iron-regulated elongation factor EF-Tu	2	0.64
4	Rv2629		Conserved hypothetical protein	10	0.41
6	Rv0363c	*Fba*	Fructose bisphosphate aldolase	7	0.62
7	Rv3224		Possible iron-regulated short-chain dehydrogenase/reductase	7	0.31
8	Rv0468	*fadB2*	Probable 3-hydroxybutyryl-CoA dehydrogenase	1	0.57
10	Rv0642c	*mmaA4*	Methoxy mycolic acid synthase 4	1	0.39
11	Rv2005c		Conserved hypothetical protein	10	0.62
16	Rv0440	*groEL*	60 kDa chaperonin 2	0	0.28
20	Rv2161c		Conserved hypothetical protein	7	0.45
21	Rv2624c		Conserved hypothetical protein	10	0.54
22	Rv1484	*inhA*	Enoyl-(acyl-carrier protein) reductase	1	0.56
	Rv1109c		Conserved hypothetical protein	10	
23	Rv0554	*bpoC*	Possible peroxidase bpoC	0	0.34
	Rv3400		Probable hydrolase	7	
25	Rv3246c	*mtrA*	DNA-binding response regulator MtrA	9	0.62
26	Rv0927c		Probable short-chain dehydrogenase/reductase	7	0.38
30	Rv1284		Beta-carbonic anhydrase	7	0.66
31	Rv2140c	*TB18.6*	Conserved hypothetical protein	10	0.48
32	Rv1284		Beta-carbonic anhydrase	7	0.44

[a] Protein spots numbered in underlined fonts were identified as products of the same gene.
Protein spots numbered in bold fonts were identified as the products of several different genes.

[b] According to TubercuList (http://genolist.pasteur.fr/TubercuList/)
0 Virulence, detoxification, adaptation
1 Lipid metabolism
2 Information pathways
7 Intermediary metabolism and respiration
9 Regulatory proteins
10 Conserved hypothetical proteins

[c] Density alteration is the ratio of density of protein spots in gel images of ATB107 treatment strains to density of the same protein spots in gel images of the strains without ATB107 treatment.

(Sassetti *et al*., 2003) presents a lower level of expression. The expression of this gene is down-regulated by ATB107 treatment. The reduction of this protein could affect protein biosynthesis and block global protein synthesis, which might be fatal for *M. tuberculosis*.

Proteins related to intermediary metabolism and respiration

Proteins encoded by 9 genes (Table 1) are involved in intermediary metabolism and respiration. Rv0560c encoding the 27-kDa protein which is uniquely induced in *M. tuberculosis* complex organisms by salicylate or para-amino-salicylate exposure (Sun *et al.*, 2001), is also induced by ATB107, indicating that ATB107 might have the similar mechanism of action with salicylate. Moreover, the product of Rv2971 is possibly involved in cellular metabolism (Mattow *et al.*, 2001; Sassetti *et al*., 2003). The product of Rv3841 would be regulated by stress conditions, such as hypoxia, temperature, and low-oxygen level (Rosenkrands *et al.*, 2002). The expression of these genes is also induced by ATB107 treatment.

The expression of 5 genes is down-regulated by ATB107 treatment. The products of Rv3400 and Rv0363c are probably lyases involved in the glycolysis pathway (Rosenkrands *et al*., 2002). The products of Rv0927c and Rv3224 are probable short-chain type dehydrogenase/reductase (Stewart *et al.*, 2002; Kendall *et al.*, 2007). Rv1284 is involved in nitrogen metabolism, reduction and fixation. Rv2161c is a conserved hypothetical protein with unknown functions (Rosenkrands *et al.*, 2000). It is speculated that ATB107 could affect some important cellular metabolism pathways by altering the expression of important enzymes.

Regulatory proteins

The products of Rv3246c and Rv3676 belong to the "regulatory proteins" class. Rv3246c is the transcriptional activator part of the MtrA-MtrB two-component regulatory system involved in tryptophan biosynthesis (Curcic *et al.*, 1994; Zahrt and Deretic, 2000) (Fig. 4). Rv3246c is required for survival in primary murine macrophages by transposon site hybridization (TraSH) in H37Rv (Rengarajan *et al.*, 2005). In the present study, it is down-regulated by treatment with ATB107, a potent inhibitor of IGPS belonging to the tryptophan biosynthesis pathway, implying that ATB107 would inhibit the activity of IGPS, causing accumulations of intermediate products. As a consequence, the overload of intermediate products could affect the expression of the MtrA-MtrB regulatory system; therefore, the bacteriostatic activity of ATB107 might be related to the down-regulation of the MtrA-MtrB two-component regulatory system which would reduce tryptophan production. This evidence suggests that ATB107 does affect tryptophan biosynthesis, which is consistent with the results of previous *in vitro* tests (Shen *et al*., 2009). No significant change in the expression of the downstream genes of MtrA was found, which may be because ATB107 could inhibit the enzyme activity of IGPS but not affect the expression of this enzyme. There is also the possibility that expression alterations of downstream genes are lower than the sensitivity of a 2-D gel.

The product of Rv3676 is a transcription factor belonging to the cAMP receptor protein (CRP) family (Rickman *et al.*, 2005). It can control transcription of the *rpfA* gene coding for a resuscitation promoting factor.

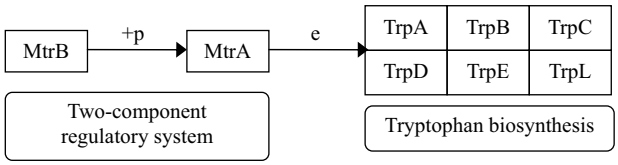

Fig. 4. Two-component regulatory system involved in tryptophan biosynthesis composed of MtrB and MtrA. MtrA, phosphorylated by MtrB, is the transcriptional activator part of the system. TrpA, TrpB, TrpC, TrpD, TrpE, and TrpL are involved in tryptophan biosynthesis. TrpC is known as the indole-3-glycerol phosphate synthase (IGPS). "+p": phosphorylation; "e": expression.

Conserved hypothetical proteins

Nine conserved hypothetical proteins are identified in our research (Table 1). Their functions remain unknown. Four are up-regulated proteins; 5 are down-regulated proteins (Table 1). The expression of 6 genes, Rv2623, Rv3099c, Rv2005c, Rv2624c, Rv2629, and Rv1109c, are regulated by stress conditions, such as hypoxia, starvation, and high temperature (Sherman *et al*., 2001; Betts *et al*., 2002; Rosenkrands *et al*., 2002). Rv2624c is reported as a universal stress protein in some bacteria such as *Saccharopolyspora* strains (Sherman *et al.*, 2001).

The results show that ATB107 can inhibit the activities of enzymes related to tryptophan biosynthesis *in vivo*, causing accumulations of intermediate products, resulting in the down-regulation of the regulator gene Rv3246c. This could eventually lead to a lack of tryptophan, which would decrease the biosynthesis of cell proteins. This is a type of stress condition for *M. tuberculosis* inducing the alteration of the protein expression profile.

Comparison of proteins regulated by ATB107 treatment in drug-sensitive strain H37Rv and drug-resistant strains

To investigate the difference in protein expression profiles between drug-sensitive and drug-resistant strains, protein expression profiles of *M. tuberculosis* 1620 (Fig. 5) and 1499 (Fig. 6) in response to ATB107 were monitored and compared with that of H37Rv (Table 2).

In drug-resistant strains, the altered expression of 9 protein spots (Table 2) is consistent with that in H37Rv. Protein spots of No. 1, 24, and 19 are up-regulated; the others are down-regulated, which may be the common response of virulent *M. tuberculosis* strains to ATB107 treatment.

There are also 9 protein spots whose altered expression is different between drug-sensitive and drug-resistant strains. There are 8 gene products in these protein spots (Table 2). Among them, 4 proteins, encoded by Rv0242c, Rv1324, Rv1843c, and Rv2773c, are differently expressed in response to ATB107 treatment in clinical drug-resistant strains, while there are no significant expression changes in H37Rv. The product of Rv0242c is involved in the fatty acid biosynthesis pathway and is down-regulated by starvation (Betts *et al*., 2002; Malen *et al.*, 2007). The product of Rv1324 participates in various redox reactions and is also down-regulated by

starvation (Gu *et al*., 2003). The product of Rv1843c is involved in GMP biosynthesis and drug metabolism (Mawuenyega *et al*., 2005). The product of Rv2773c is induced in *M. tuberculosis* H37Rv in murine macrophages but not in H37Rv broth-cultures *in vitro* (Jungblut *et al.*, 1999; Mattow *et al.*, 2006). These genes might be related to the drug-resistance of *M. tuberculosis* strains; of course, this assumption is in need of further evaluation.

Comparative proteomic analysis of the response to ATB107 in *M. tuberculosis* strains H37Rv and H37Ra

We compared the protein expression profiles of avirulent *M. tuberculosis* strain H37Ra (Fig. 7) and virulent strain H37Rv after exposure to ATB107 (Table 3). Six protein spots are up-regulated and 4 protein spots are down-regulated in both strains. However, 30 protein spots in H37Ra are different in expression alteration from those in H37Rv.

Seven protein spots are differently expressed in H37Ra in response to ATB107, but show no obvious change in H37Rv (Table 3). Among them, 4 protein spots encoded by Rv1144, Rv1400c, Rv1856c, and Rv2095c, are mentioned in neither drug-sensitive strains nor drug-resistant strains. None of these genes are essential according to Himar 1-based transposon mutagenesis in the H37Rv strain (Sassetti *et al*., 2003). The products of Rv1144, Rv1400c, and Rv1856c are all involved in cellular metabolism (Gu *et al*., 2003; Deb *et al.*, 2006). The function of Rv2095c product is unknown (Lamichhane *et al.*, 2003). These results may be attributed to the difference in cellular metabolism between H37Ra and H37Rv strains. Moreover, proteins encoded by Rv0242c and Rv1324 are down-regulated not only in H37Ra but also in drug-resistant strains, whereas the product of Rv2773c is up-regulated.

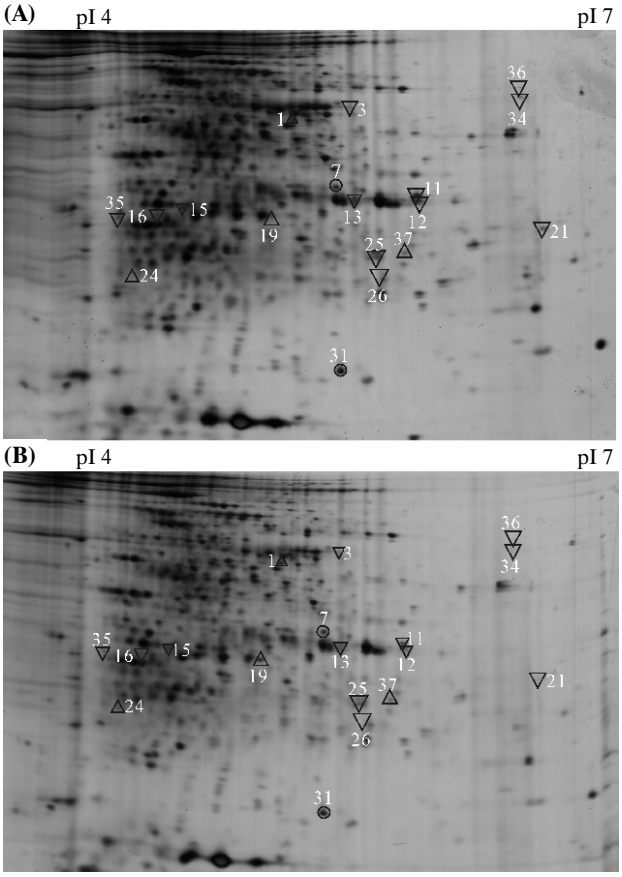

Fig. 5. Two-dimensional electrophoresis patterns of cell total proteins of clinical drug-resistant *M. tuberculosis* strain 1620 (INH-resistant strain) without ATB107 treatment (A) and with ATB107 (B). Spots of the same response as clinical drug-resistant *M. tuberculosis* strain 1499 (INH- and RIF-resistant strain) to ATB107 treatment marked on each gel image as (△) an increase in the spot intensity after ATB107 treatment; (▽) a decrease in spot intensity after ATB107 treatment; (○) no significant change in spot intensity.

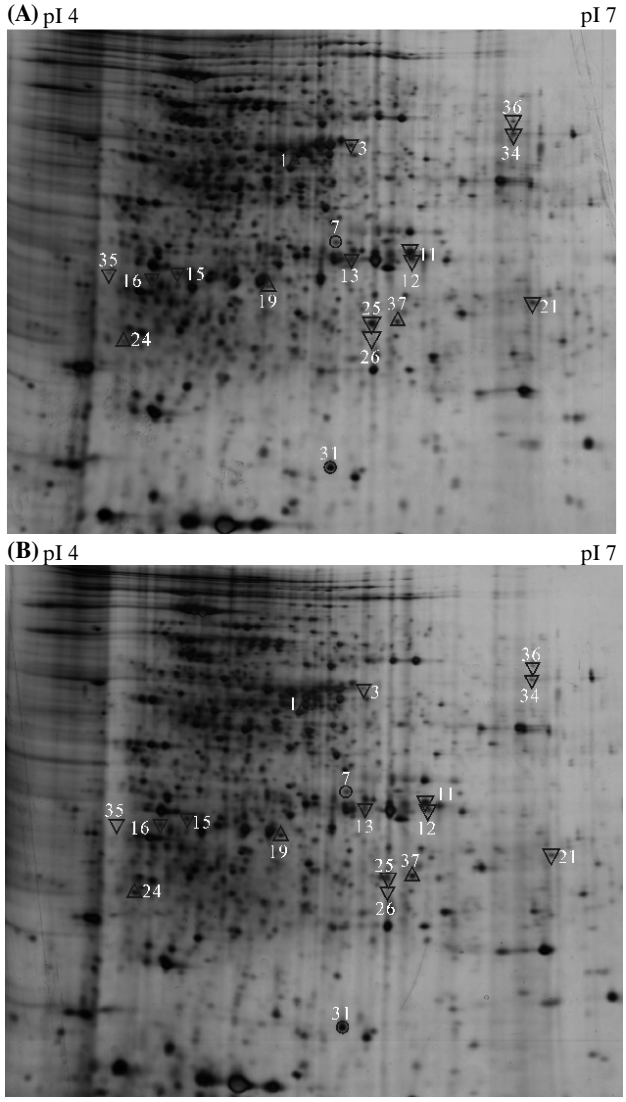

Fig. 6. Two-dimensional electrophoresis patterns of cell total proteins of clinically drug-resistant *M. tuberculosis* strain 1499 (INH- and RIF-resistant strain) without ATB107 treatment (A) and with ATB107 (B). The spots of the same response as clinically drug-resistant *M. tuberculosis* strain 1620 (INH-resistant strain) to ATB107 treatment marked on each gel image as, (△) an increase in the spot intensity after ATB107 treatment; (▽) a decrease in spot intensity after ATB107 treatment; (○) no significant change in spot intensity.

Table 2. Comparison of protein spots regulated by ATB107 treatment in drug-sensitive strain (H37Rv) and drug-resistant strains (1620, 1499)

Protein Spot No.[a]	ORF	Gene	Description	Functional class[b]	Density alteration[c]		
					H37Rv	1620	1499
Consistent in all drugR and drugS strains							
1	Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	1	3.02	1.96	2.48
3	Rv0685	*tuf*	Iron-regulated elongation factor EF-Tu	2	0.64	0.76	0.53
11	Rv2005c		Conserved hypothetical protein	10	0.62	0.74	0.61
16	Rv0440	*groEL*	60 kDa chaperonin 2	0	0.28	0.56	0.54
19	Rv3099c		Conserved hypothetical protein	10	1.87	2.21	1.28
21	Rv2624c		Conserved hypothetical protein	10	0.54	0.45	0.69
24	Rv0560c		Possible benzoquinone methyltransferase	7	5.55	1.75	2.53
	Rv3676		Probable transcriptional regulator belonging to crp/fnr family	9			
25	Rv3246c	*mtrA*	DNA-binding response regulator MtrA	9	0.62	0.57	0.54
26	Rv0927c		Probable short-chain dehydrogenase/reductase	7	0.38	0.66	0.52
Discriminated between drugR and drugS strains							
7	Rv3224		Possible iron-regulated short-chain dehydrogenase/reductase	7	0.31	NS	NS
12	Rv2623	*TB31.7*	Conserved hypothetical protein	10	1.44	0.80	0.60
13	Rv2623	*TB31.7*	Conserved hypothetical protein	10	2.51	0.63	0.62
15	Rv2243	*fabD*	Malonyl CoA-acyl carrier protein transacylase	1	1.66	0.50	0.79
31	Rv2140c	*TB18.6*	Conserved hypothetical protein	10	0.48	NS	NS
34	Rv0242c	*fabG*	3-oxoacyl-(acyl-carrier protein) reductase	1	NS[d]	0.43	0.50
35	Rv1324		Possible thioredoxin	7	NS	0.67	0.62
36	Rv1843c	*guaB1*	Probable inosine-5'-monophosphate dehydrogenase	7	NS	0.47	0.43
37	Rv2773c	*dapB*	Dihydrodipicolinate reductase	7	NS	1.27	1.53

[a] Protein spots numbered in underlined fonts were identified as products of the same gene.
Protein spots numbered in bold fonts were identified as products of several different genes.
[b] According to TubercuList (http://genolist.pasteur.fr/TubercuList/)
0 Virulence, detoxification, adaptation
1 Lipid metabolism
2 Information pathways
7 Intermediary metabolism and respiration
9 Regulatory proteins
10 Conserved hypothetical proteins
[c] Density alteration is the ratio of density of protein spots in gel images of ATB107 treatment strains to density of the same protein spots in gel images of the strains without ATB107 treatment.
[d] No significant change

Conclusion

To explore the mechanism of ATB107 action, we have identified the alteration of protein expression profiles in *M. tuberculosis* strains through a 2D gel electrophoresis method coupled with MALDI-TOF-MS analysis. We found that ATB107 could affect the biosynthesis of tryptophan by decreasing the expression of protein encoded by Rv3246c, which is the transcriptional regulatory protein MtrA of the MtrA-MtrB two-component regulatory system, in both drug-sensitive and drug-resistant virulent strains. This protein however, is up-regulated in the H37Ra strain with exposure to ATB107, which may be attributed to differences between virulent and avirulent strains. This is consistent with the conclusion of Zahrt and Deretic who have identified that *mtrA*, as an essential response regulator gene in *M. tuberculosis*, is differentially expressed in virulent *M. tuberculosis* H37Rv and avirulent *M. bovis* BCG strains during growth in macrophages (Zahrt and Deretic, 2000).

ATB107 might provide a similar stress like INH or ethionamide for *M. tuberculosis* since the altered expression in response to ATB107 of some genes, such as Rv3140, Rv2243, and Rv2428, is consistent with INH or ethionamide treatment (Wilson *et al*., 1999). Furthermore, ATB107 can also inhibit the growth of clinical isolates of drug-resistant *M. tuberculosis* strains.

After incubation with ATB107, the expression of 2 proteins encoded by Rv0685 and Rv2624c is down-regulated while that of protein encoded by Rv3140 is up-regulated in all *M. tuberculosis* strains used in this study, which could be the common response to tryptophan absence. Their relations to ATB107 are unknown and further evaluation is warranted.

Many studies found that a protein spot from 2-D gel could contain more than one protein (Florczyk *et al.*, 2001; Jungblut *et al.*, 2001). In our research, MS results show that protein spots No. 22, 23, and 24 contained 2 proteins, respectively. Sequence analysis results indicate that there is no significant sequence similarity between the proteins in the same spot. There is little difference in the predicted molecular weight and pI value between the proteins, which made it difficult to resolve them by 2-DE. We also identified several protein spots as the same protein (Tables 1, 2, and 3). There is little difference

Table 3. Comparison of protein spots regulated by ATB107 treatment in virulent strain (H37Rv) and avirulent strain (H37Ra)

Protein spot No.[a]	ORF	Gene	Description	Functional class[b]	Density alteration[c]	
					H37Rv	H37Ra
Consistent in virulent and avirulent strains						
1	Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	1	3.02	3.68
2	Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	1	1.40	1.28
3	Rv0685	*tuf*	Iron-regulated elongation factor EF-Tu	2	0.64	0.57
4	Rv2629		Conserved hypothetical protein	10	0.41	0.80
12	Rv2623	*TB31.7*	Conserved hypothetical protein	10	1.44	2.75
14	Rv2623	*TB31.7*	Conserved hypothetical protein	10	5.46	1.43
15	Rv2243	*fabD*	Malonyl CoA-acyl carrier protein transacylase	1	1.66	2.59
20	Rv2161c		Conserved hypothetical protein	7	0.45	0.21
21	Rv2624c		Conserved hypothetical protein	10	0.54	0.31
33	Rv0251c	*hsp*	Heat-stress-induced ribosome-binding protein A	0	1.37	1.89
Discriminated in virulent and avirulent strains						
5	Rv0281		Possible S-adenosylmethionine-dependent methyltransferase	10	2.47	0.47
6	Rv0363c	*fba*	Fructose bisphosphate aldolase	7	0.62	1.42
7	Rv3224		Possible iron-regulated short-chain dehydrogenase/reductase	7	0.31	2.53
8	Rv0468	*fadB2*	Probable 3-hydroxybutyryl-CoA dehydrogenase	1	0.57	NS[d]
9	Rv2971		Probable oxidoreductase	7	3.62	NS
10	Rv0642c	*mmaA4*	Methoxy mycolic acid synthase 4	1	0.39	1.23
11	Rv2005c		Conserved hypothetical protein	10	0.62	1.95
13	Rv2623	*TB31.7*	Conserved hypothetical protein	10	2.51	0.24
16	Rv0440	*groEL*	60 kDa chaperonin 2	0	0.28	NS
17	Rv0577	*TB27.3*	Conserved hypothetical protein	10	1.36	0.57
18	Rv1094	*desA2*	Possible acyl-(acyl-carrier protein) desaturase	1	1.37	NS
19	Rv3099c		Conserved hypothetical protein	10	1.87	NS
22	Rv1484	*inhA*	Enoyl-(acyl-carrier protein) reductase	1	0.56	NS
	Rv1109c		Conserved hypothetical protein	10		
23	Rv0554	*bpoC*	Possible peroxidase bpoC	0	0.34	1.59
	Rv3400		Probable hydrolase	7		
24	Rv0560c		Possible benzoquinone methyltransferase	7	5.55	0.58
	Rv3676		Probable transcriptional regulator belonging to crp/fnr family	9		
25	Rv3246c	*mtrA*	DNA-binding response regulator MtrA	9	0.62	1.42
26	Rv0927c		Probable short-chain dehydrogenase/reductase	7	0.38	2.04
27	Rv0905	*echA6*	Possible enoyl-CoA hydratase	1	1.20	NS
28	Rv2428	*ahpC*	Alkyl hydroperoxide reductase C	0	7.36	NS
29	Rv3841	*bfrB*	Possible bacterioferritin bfrB	7	1.72	NS
30	Rv1284		Beta-carbonic anhydrase	7	0.66	NS
31	Rv2140c	*TB18.6*	Conserved hypothetical protein	10	0.48	NS
32	Rv1284		Beta-carbonic anhydrase	7	0.44	NS
34	Rv0242c	*fabG*	3-oxoacyl-(acyl-carrier protein) reductase	1	NS	0.79
35	Rv1324		Possible thioredoxin	7	NS	0.67
37	Rv2773c	*dapB*	Dihydrodipicolinate reductase	7	NS	1.40
38	Rv1856c		Possible oxidoreductase	7	NS	0.78
39	Rv2095c		Conserved hypothetical protein	10	NS	0.63
40	Rv1144		Probable short-chain dehydrogenase/reductase	7	NS	0.70
41	Rv1400c	*lipI*	Possible lipase	7	NS	0.33

[a] Protein spots numbered in underlined fonts were identified as products of the same gene.
Protein spots numbered in bold fonts were identified as products of several different genes.
[b] According to TubercuList (http://genolist.pasteur.fr/TubercuList/)
0 Virulence, detoxification, adaptation
1 Lipid metabolism
2 Information pathways
7 Intermediary metabolism and respiration
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10 Conserved hypothetical proteins
[c] Density alteration is the ratio of density of protein spots in gel images of ATB107 treatment strains to density of the same protein spots in gel images of the strains without ATB107 treatment.
[d] No significant change

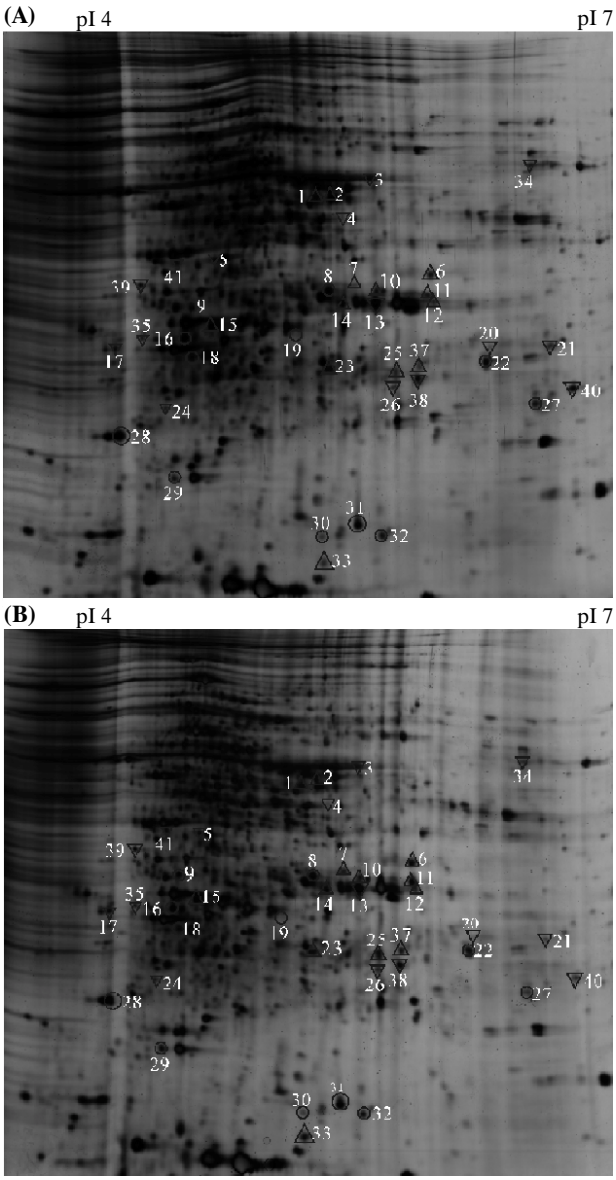

Fig. 7. Two-dimensional electrophoresis patterns of cell total proteins of *M. tuberculosis* avirulent strain H37Ra without ATB107 treatment (A) and with ATB107 (B), (△) an increase in the spot intensity after ATB107 treatment; (▽) a decrease in spot intensity after ATB107 treatment; (○) no significant change in spot intensity.

in the molecular weights of proteins identified as the same protein according to gel images (Figs. 2, 5, 6, and 7), and their differences are mainly in pI values, which may be related to variations in maturation states of proteins in the cells.

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