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The Wag31 protein interacts with AccA3 and coordinates cell wall lipid permeability and lipophilic drug resistance in *Mycobacterium smegmatis*

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ABSTRACT

Mycobacterium tuberculosis, especially drug resistant tuberculosis, is a serious threat to global human health. Compared with other bacterial pathogens, *M. tuberculosis* gains stronger natural drug resistance from its unusually lipid-rich cell wall. As a DivIVA homolog, Wag31 has been demonstrated to be closely involved in peptidoglycan synthesis, cell growth and cell division. Previous research rarely investigated the role of Wag31 in drug resistance. In this study, we found Wag31 knock-down in *Mycobacterium smegmatis* resulted in a co-decrease of the resistance to four lipophilic drugs (rifampicin, novobiocin, erythromycin and clofazimine) and an increase in the cell permeability to lipophilic molecules. Six proteins (AccA3, AccD4 and AccD5, Fas, InhA and MmpL3) that are involved in fatty acid and mycolic acid synthesis were identified in the Wag31 interactome through Co-Immunoprecipitation. The Wag31–AccA3 interaction was confirmed by the pull-down assay. AccA3 overexpression resulted in a decrease in lipid permeability and an increase in the resistance of rifampicin and novobiocin. It confirmed the close relationship of lipophilic drug resistance, lipid permeability and the Wag31–AccA3 interaction. These results demonstrated that Wag31 maintained the resistance to lipophilic drugs and that Wag31 could play a role in controlling the lipid permeability of the cell wall through the Wag31–AccA3 interaction.

1. Introduction

Tuberculosis remains one of the leading threats to global human health after its reemergence nearly twenty years ago. The geographically wide spread of multidrug resistant tuberculosis and extensively drug-resistant tuberculosis (XDR-TB) has become a serious problem facing global tuberculosis control efforts [1].

Compared with other bacterial pathogens, *Mycobacterium tuberculosis* gains stronger intrinsic drug resistance through its unusual multi-layer cell wall [2,3]. The cell wall of mycobacterium is relatively impermeable to most hydrophilic and lipophilic drugs, mainly because of the lipid-rich layer containing mycolic acids in the inner leaflet and long-chain fatty acids in the outer leaflet [4,5]. Defects in these lipids would damage the function of the cell wall as a barrier and increase the sensitivity to various mycobacterial drugs [5–9]. The enzymes involved in cell wall lipid metabolism play an important role in the permeability and drug resistance of the mycobacterium. The abnormal expression or the inactivation of enzymes, such as ACCase6 [10] and Ag85C [11], often result in changes in drug resistance.

In our former study, we found the overexpression of Wag31 in two clinical XDR strains through Isobaric tag for relative and absolute quantitation (data unpublished). As a DivIVA homolog, Wag31 has been shown to play an important role in cell wall synthesis, cell growth and cell division, mostly due to its important contribution to peptidoglycan synthesis [12,13]. The function of Wag31 depends on its ability to localize and bind to the inner side of curved membranes spontaneously, especially in the two poles of rod-shape cells [12]. In mycobacterium, the overexpression of Wag31 leads to multiseptation and significant changes in cell pleomorphism [12], and the expression of mutant Wag31 impairs its protection on peptidoglycan synthesis [13].

Few researchers focused on the role of Wag31 in drug resistance except Partha Mukherjee. He proved that Wag31 was involved in isoniazid resistance through binding and protecting PBP3 [14]. However, in consideration of the important role of the cell wall in natural multi-drug resistance and the important role of Wag31 in cell wall synthesis, there should be additional functions of Wag31 on drug resistance.

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In this study, we examined the changes in the MICs of 20 drugs resulting from decreased expression of Wag31. A co-decrease of the MICs of four lipophilic drugs was found. A new interaction between Wag31 and AccA3 was also found. AccA3 was shown to be closely involved in lipid and mycolic acid synthesis as the α subunit of Acyl-CoA carboxylase (ACCase) [15], but there was no direct evidence showing phenotypic changes resulting from AccA3 overexpression in the past. Our results showed that changes of Wag31 or AccA3 expression could result in MIC changes of lipophilic drugs and in changes of the accumulation rate of lipophilic molecules in the cytoplasm. The results suggested that Wag31 and AccA3 was closely involved in lipophilic drug resistance and lipid permeability.

2. Materials and methods

2.1. Strains and reagents

Mycobacterium smegmatis mc^2155, *Escherichia coli* DH5α and BL21 (DE3) were used in this study. Kanamycin (50 μg/ml) or ampicillin (250 μg/ml) was added to the broth for maintaining the plasmids, and 0.2%(w/v) acetamide or 20 ng/ml tetracycline was added to broth for inducing expression. The polyclonal antibody against recombinant $Wag31_{mtb}$ was developed in rabbits. The acetamide and the drugs were from Sigma Chemical Co. The RIPA buffer (#9806) was from Cell Signaling Technology. The enzymes were from New England Biolabs.

2.2. Construction of plasmids and strains

Pact and pMV306-act are two basic plasmids for *M. smegmatis*. Pact was constructed on the backbone of pMV261 as previously described [16]. pMV306-act was constructed on the backbone of pMV306. The entire length of the acetamide promoter was cut out from the *M. smegmatis* genome and inserted into pMV306. Pet28a and pEASY1-E1 (TransGen Biotech #CE101-01) are two plasmids used for protein expression in *E. coli*.

The mycobacterial genes were amplified from the genome of *M. tuberculosis* H37Rv and cloned into the plasmids. The plasmids and primers used for constructing each plasmid are shown in Tables S1 and S2.

The corresponding plasmids were transformed into *E. coli* or *M. smegmatis* to generate the strains used in the study (Table S2).

2.3. Minimum inhibitory concentration assay

The WT strain, Wag31 knock-down strain and AccA3 overexpressing strain were grown in 7H9/ADC at 37 °C until reaching an OD_{600} of 0.6–1.0. Acetamide (0.2%) was added to induce Wag31 antisense knock-down for 9 h. The bacterial suspensions were diluted until OD_{600} reached 0.5, and the suspensions were then diluted 1000 times in 7H9 broth with 0.4% acetamide. The final diluted broth was dispensed into each well of a 96-well cell culture plate (100 μl/well). The drug storage solutions were diluted to prepare twofold serial diluted solutions in 7H9 broth. The serial dilutions of the drugs were added to the wells of a 96-wall plate containing the bacterial suspensions. The 96-wall plate was incubated for 60–72 h at 37 °C. The wells containing bacterial pellets were the positive wells, and the wells containing clear broth were the negative wells. The minimum drug concentrations of the negative wells were the MICs of the drugs.

2.4. Nile red uptake assay

The assay was carried out as previously described [17]. The WT strain, Wag31 knock-down strain and AccA3 overexpressing strain were grown in 7H9/ADC at 37 °C until reaching an OD_{600} of 0.6–1.0. Acetamide (0.2%) was added to induce Wag31 knock-down or AccA3 overexpression for 9 h. The cells were collected, washed and resuspended in PBS buffer (containing 25 mM glucose). The final OD_{600} of the suspensions was 0.5. The suspensions were incubated with 2 μM Nile red at 37 °C in a Roche lightcycle® 480 II. The suspensions were excited by light (wavelength 533 nm), and the fluorescence data of the emission light (wavelength 610 nm) were collected.

2.5. Co-Immunoprecipitation (CoIP) assay

The Pierce® Crosslink Immunoprecipitation Kit (Thermo #26147) was used. WT and Wag31-His overexpression strains of *M. smegmatis* were grown in 7H9/ADC until reaching stationary phase (OD_{600} 1.5–2), then 0.2%(w/v) acetamide was added to induce Wag31-His expression for 6 h. The cells were collected and lysed in the lysis buffer from the kit. The cell lysates of the WT and Wag31-His strains were incubated with protein A/G agarose binding His antibody overnight at 10 °C. After the proper washing steps, the two samples were eluted and examined by SDS–PAGE. The gel from the SDS–PAGE was dyed, and the protein bands were cut out for analysis by mass spectrometry.

2.6. Pull-down assay

The pull-down assay was performed as previously described [18,19]. The bait protein with His tag and the target proteins with tags other than His tag were expressed in *E. coli*. Cells of expressing strains were collected and lysed with RIPA buffer. The cell lysate with the target protein was mixed with cell lysate of *E. coli* strain with empty plasmid to generate the control mixture. The cell lysate containing the bait protein Wag31-His was mixed with cell lysates containing target proteins AccA3-HA, AccD4-Flag or AccD5-cMyc separately to study the interaction of Wag31–AccA3, Wag31–AccD4 and Wag31–AccD5. Mixtures were incubated overnight at 4 °C. Cell lysate containing the bait protein AccA3-His was mixed with cell lysates containing the target protein Wag31 to confirm the interaction of Wag31–AccA3. Mixtures were incubated for 2 h at room temperature. After the incubation, the mixture was passed through 25 μl Ni–NTA agarose column. The agarose was washed with RIPA buffer containing 0 mM, 20 mM and 50 mM imidazole and eluted with the same buffer containing 500 mM imidazole. The elutions were examined by SDS–PAGE and Western blotting.

3. Results

1. Wag31 knock-down decreases the minimum inhibitory concentrations (MICs) of several antituberculosis drugs, especially four lipophilic drugs.
   For the MIC assay, an inducible Wag31 antisense knock-down strain, AA2, was constructed with *M. smegmatis*, using pMV261 with an acetamide promoter instead of the HSP60 promoter [16]. After the Wag31 expression decrease was confirmed (Fig. 1A), we examined the change of the growth rate when Wag31 expression decreased. The results showed that lower Wag31 expression resulted in a lower growth rate (Fig. 1B) but did not cause significant cell death (Table S1).
   We then tested the MICs of 20 drugs, including most first-line and second-line drugs, on strain AA2 (Table 1). Wag31

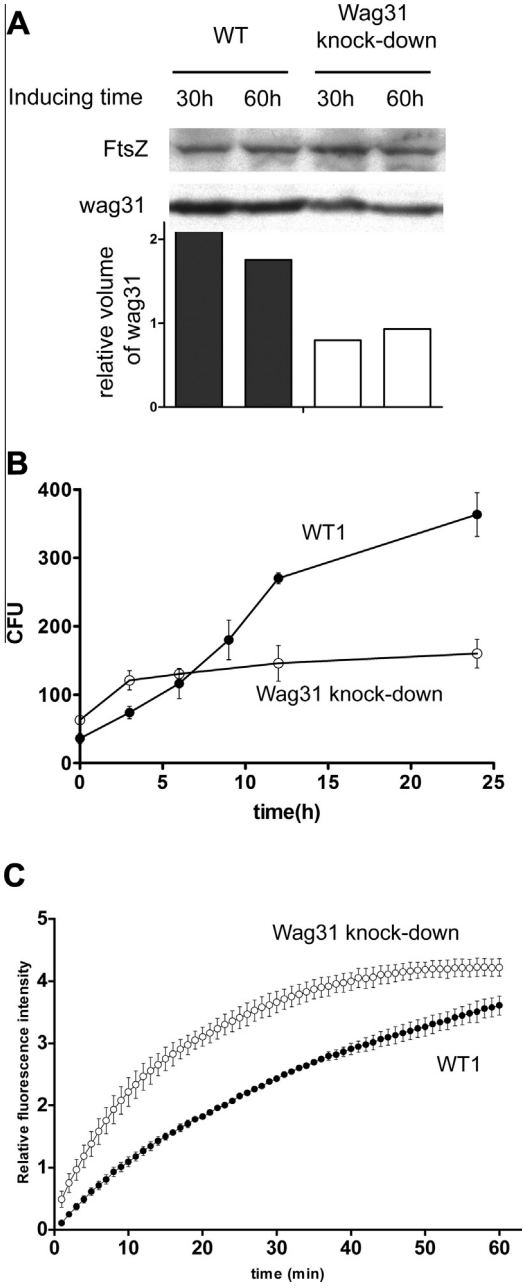

Fig. 1. The construction of the Wag31 knock-down strain of *M. smegmatis*. (A) The Western blotting results showed an almost 50% decrease in Wag31 expression in the Wag31 knock-down strain AA2. The WT1 and AA2 strains were incubated in 7H9/ADC broth at 37 °C until they reached an OD_{600} of 0.6–1.0. Acetamide was added into the incubations at 0.2% (w/v), and the induction was started. FtsZ expression was detected as the control. (B) The growth curves determined by CFU counts. The WT1 and AA2 strains were incubated in the same manner as described in (A). The induction was started at 0 h by adding 0.2% (w/v) acetamide. (C) Wag31 knock-down led to higher accumulation of the lipophilic molecule Nile red in the cytoplasm. Strain WT1: ●; strain AA2: ○.

knock-down broadly influenced the drug resistance of *M. smegmatis*. The co-decrease of the MICs of four lipophilic drugs, rifampicin, erythromycin, novobiocin and clofazimine, drew our attention.

The main drug targets of those four drugs are different [20–23]. Based on previous research, the PBP3–Wag31 interaction was shown to be involved in isoniazid resistance [14], but it could not explain the changes of the MICs of the four lipophilic drugs.

Table 1
The MICs of 20 drugs of WT and Wag31 knock-down strains. The bold entities show the co-decrease of the MICs of four lipophilic drugs in the Wag31 knock-down strain.

Drug (μg/ml)	WT	Wag31 knock-down
Rifampicin	**32**	**8**
Erythromycin	**40**	**10**
Novobiocin	**25**	**12.5**
Clofazimine	**2.7**	**1.35**
Penicillin G	500	250
Ampicillin	128	64
Isoniazid	64	32
Ethionamide	240	120
Amikacin	2	1
Capreomycin	8	4
Sparfloxacin	0.1	0.1
Norfloxacin	3.2	3.2
Ciprofloxacin	0.2	0.2
Levofloxacin	0.25	0.25
Ofloxacin	0.5	0.5
Ethambutol	0.25	0.25
Chloramphenicol	24	24
Tetracycline	0.25	0.25
Streptomycin	0.5	0.5
Cycloserine	125	125

Based on the diversity of drug targets and the function on cell wall synthesis of Wag31, the reason for the co-decrease of the MICs was likely to be the change of lipophilic drug accumulation in the cytoplasm.

2. Wag31 knock-down leads to higher accumulation of lipophilic molecules in the cytoplasm.
   Previous research suggested a close relationship between lipid permeability and susceptibility against rifampicin and erythromycin [7,24]. To examine the influence of wag31 on the lipid permeability, we analyzed the accumulation of lipophilic molecules in the cytoplasm when Wag31 expression was decreased. A lipophilic fluorescence molecule, Nile red, was used to represent lipophilic drugs. The emission wavelength of Nile red mostly depends on the buffer [25] and changes when Nile red crosses the cell wall from PBS buffer into the cytoplasm. In the cytoplasm, the excitation/emission wavelengths are 535/630 nm, and higher fluorescence intensity suggests there is more Nile red in the cytoplasm [17]. Based on that observation, WT and AA2 strains were incubated in PBS buffer with 2 μM Nile red at 37 °C the fluorescence data were collected. It indicated that the decrease of Wag31 expression resulted in higher accumulation of Nile red in the cytoplasm (Fig. 1C), suggesting the lipid permeability of the cell wall was increased.
3. Six enzymes involved in cell wall lipid synthesis, especially AccA3, AccD4 and AccD5, are identified in the Wag31 interactome through CoIP.
   Liu reported that a mutant strain of *M. smegmatis* with a defective mycolic acid layer had higher lipid permeability and higher sensitivity to rifampicin, erythromycin and novobiocin [4]. Korycka-Machala demonstrated that the free lipids in the outer leaflet of the cell wall lipid layer influence the drug resistance against rifampicin and erythromycin [9]. These results revealed the close relationship among the cell wall lipid, lipid permeability and lipophilic drug resistance. We suspected that Wag31 might play a role in the synthesis of the cell wall lipid layer. Because Wag31 was predicted to be a scaffolding protein and to have no catalytic activity itself [26], we decided to examine Wag31 interactome through Co-Immunoprecipitation (CoIP). Wag31 from the H37Rv genome was expressed in *M.*

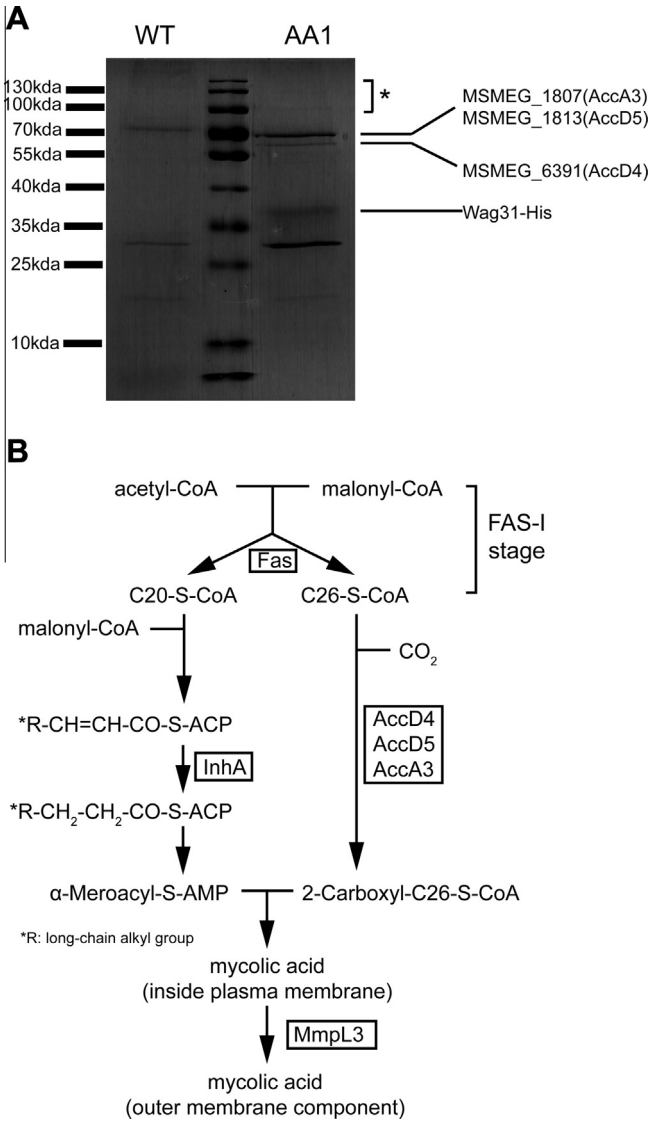

Fig. 2. Analysis of the Wag31 interactome. (A) The SDS–PAGE results of the Wag31 interactome and the WT control from the CoIP. The Wag31 interactome was from the cell lysates of the Wag31-His overexpression strain AA1. The WT control included the cell lysates of the WT1 strain. The bands of AccA3, AccD4 and AccD5 were analyzed by MALDI-TOF mass spectrometry. The bands marked with an * in Fig. 3A were analyzed by HPLC–MS/MS. (B) The functions of the six proteins identified in the Wag31 interactome.

smegmatis with a 6* His tag (strain AA1). A rabbit anti-His antibody was used to immunoprecipitate Wag31-His from the cell lysates. The results from SDS–PAGE of the Wag31 interactome are shown in Fig. 2A. Three proteins, MSMEG_1807 (AccA3), MSMEG_1813 (AccD5) and MSMEG_6391 (accD4), were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. They are three major subunits of a protein complex named biotin-dependent acyl-CoA carboxylase, which has acetyl-CoA carboxylase and propionyl-CoA carboxylase activities [27].

The protein bands higher than 70 kda in the SDS–PAGE gel (marked with * in Fig. 2A) were analyzed by high-performance liquid chromatography–mass spectrometry (HPLC–MS/MS). The band in the gel containing the band in the same position as the WT strain lane was cut out and analyzed as the control. The optimization of the results was based on protein PEP value (less than 0.01) and protein molecular mass (more than 70 kda). Three more proteins involved in mycolic acid synthesis, inhA, Fas and MmpL3, were identified (Fig. 2B, Table 2). The function of those three proteins in the synthesis pathway of mycolic acid was shown.

From the genes identified in the Wag31 interactome, we concluded that Wag31 might play a role in cell wall lipid synthesis by binding key enzymes in the pathway. Based on the band volume, we selected AccA3, AccD4 and AccD5 as Wag31-binding candidates to examine.

4. Wag31 binds to AccA3 but not to AccD4 or AccD5.

To examine the interaction between Wag31 and each of three candidates, Ni–NTA pull-down assays were performed. Four genes were cloned from the *M. tuberculosis* genome. The bait protein Wag31-His and three target proteins (AccA3-HA, AccD4-Flag and AccD5-cMyc) were expressed in *E. coli* (*E. coli*). The cell lysate containing Wag31-His and the cell lysate containing one target protein were mixed together and incubated with Ni–NTA for 2 h in room temperature. After the proper washing steps, the bait protein and the target protein were eluted and analyzed (Fig. 3A–C). The results demonstrated that Wag31 interacted with AccA3 instead of AccD4 or AccD5. To confirm the interaction between Wag31 and AccA3, AccA3-His and Wag31 (no tag) were expressed as the bait protein and the target protein. Positive results from the pull-down assay are shown in Fig. 3D, and these results confirmed the interaction.

The reason that AccD4 and AccD5 were identified in the Wag31 interactome might be the interaction between AccA3 and either AccD4 or AccD5. Tae-Jin Oh proved that

Table 2
Proteins identified in by liquid chromatography–mass spectrometry in the * region are shown in Fig. 3. (The genes marked in bold have been shown to be involved in mycolic acid synthesis.)

ORF No. in *M. smegmatis*	ORF No. in *M. tuberculosis*	Posterior error probability (PEP)	Mol. weight (kDa)	No. of peptides matched	Peptide matched with highest score	Peptide score		Proteins identified
MSMEG_4757	Rv2524c	0.00031812	329.53	2	APGNPLFIVSQK	80.318	**fas**	Fatty acid synthase
MSMEG_3596		2.5628E−219	115.29	20	AVASGNAIAVQNR	247.67		ATPase
MSMEG_0250	Rv0206c	0.0090476	109.4	1	VDALGVTTLLK	96.229	**MmpL3**	Membrane protein, MmpL family protein
MSMEG_6917	Rv0041	0.0013071	106.56	1	VLYDLGYVSSR	102.52		Leucyl-tRNA synthetase
MSMEG_1881	Rv3240c	1.4001E−67	106.49	16	NVLLNVIDRK	212.21		Preprotein translocase, SecA subunit
MSMEG_4323	Rv2241	0.00014763	103.08	2	SAQILASGVAMPEALR	89.484		Pyruvate dehydrogenase E1 component
MSMEG_3151	Rv1484	1.7206E−11	102.17	3	NGGILQYVLR	111.65	**InhA**	Putative aconitate hydratase
MSMEG_3641	Rv1836c	4.0963E−42	80.66	10	SLVTSPVVLATSPELK	203.27		Predicted integral membrane protein

interaction [15]. We repeated the pull-down assay using the cell lysates of *E. coli* expressing AccA3-His, AccD4-Flag and AccD5-cMyc separately, and the results confirmed the interaction (Fig. S3).

5. Overexpression of AccA3 causes a decrease in the lipid permeability of the cell wall and an increase in the resistance to rifampicin and erythromycin.

Based on the Wag31–AccA3 interaction, we concluded that Wag31 knock-down might impair cell wall permeability by impairing the function of AccA3. Though the importance of AccA3 in fatty acid and mycolic acid synthesis was confirmed by several groups [15,28], it was not known whether AccA3 was involved in lipid permeability and lipophilic drug resistance. To examine this question, we overexpressed $AccA3_{mtb}$-HA in *M. smegmatis* using pMV306 with an acetamide promoter (Strain AA3). We did not use knock-out or knock-down because AccA3 is essential for *M. tuberculosis* [29], and we do not have an AccA3 antibody to confirm the knock-down efficiency.

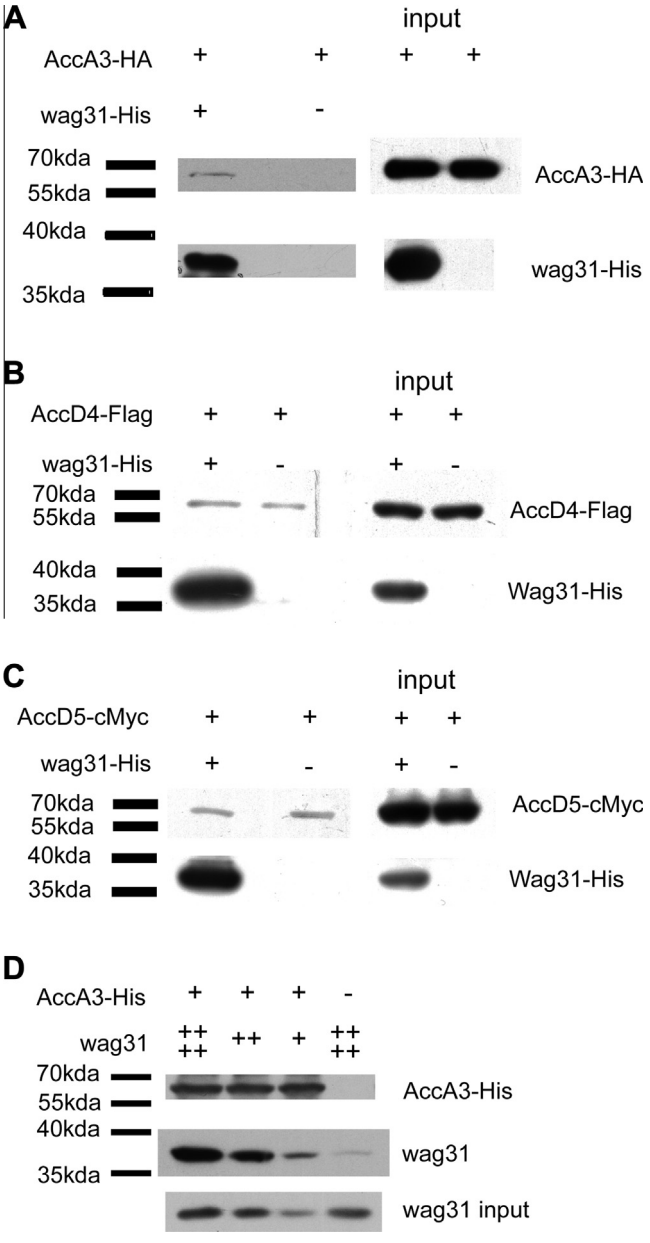

Fig. 3. Examination of the Wag31–AccA3, Wag31–AccD4 and Wag31–AccD5 interactions through the Ni–NTA pull-down assay. (A) The interaction between Wag31-His and AccA3-HA was shown. (B and C) There were no significant differences between the bands of the experimental mixtures and the control mixtures for Wag31–AccD5 or Wag31–AccD4. (D) Wag31 with no tag and AccA3-His were expressed and used to confirm the Wag31–AccA3 interaction.

Table 3
The MICs of four lipophilic drugs of WT and AccA3 overexpression strains. The bold entities show the changes of the MICs brought by AccA3 overexpression.

Drug (μg/ml)	WT	AccA3 overexpression
Rifampicin	**16**	**32**
Erythromycin	80	80
Novobiocin	**25**	**100**
Clofazimine	4	4

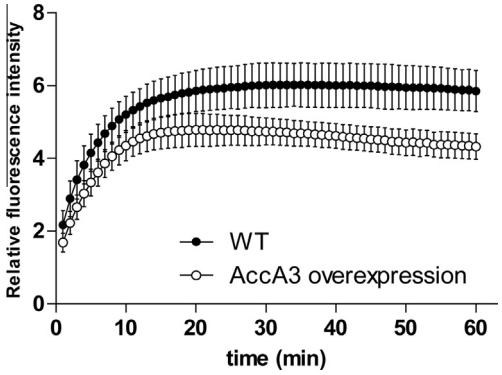

Fig. 4. AccA3 overexpression resulted in lower accumulation of the lipophilic molecule Nile red in *M. smegmatis*. The WT strain WT2: ●; the AccA3 overexpression strain AA3: ○.

After AccA3 overexpression was confirmed, we tested the MICs of rifampicin, erythromycin, novobiocin and clofazimine. AccA3 overexpression in *M. smegmatis* resulted in increased MICs of rifampicin and erythromycin (Table 3). The same Nile red uptake assay was applied on the AccA3 overexpressing strain. AccA3 overexpression increased the rate of lipophilic molecule accumulation in the cytoplasm (Fig. 4). These results suggested that AccA3 overexpression resulted in a decrease in the lipid permeability and an increase of resistance to rifampicin and erythromycin. The results confirmed our speculation that AccA3 plays a role in maintaining lipophilic drug resistance through adjusting the lipid permeability of the cell wall.

4. Discussion

The changes in the MICs and in lipid permeability caused by Wag31 knock-down or AccA3 overexpression highlights the fact that Wag31 and AccA3 are important in lipophilic drug resistance by adjusting cell wall permeability.

AccA3 has been shown to be closely involved in lipid and mycolic acid synthesis as the α subunit of ACCase. ACCase is an important enzyme for fatty acid and mycolic acid synthesis, and it catalyzes acetyl-CoA into malonyl-CoA, which is utilized for the elongation of growing acyl chains [30]. AccD4, AccD5 and AccD6 are three β subunits that interact with AccA3. There are three α subunits and six β subunits in *M. tuberculosis*. The ortholog of AccA3, Ml0726, is the only α subunit in *Mycobacterium leprae*. The quantitative real-time PCR analysis showed that the AccA3 expression was at least 10 times higher than the expression of the other two α subunits in the exponential growth stage of *M. tuberculosis*, and AccD4, AccD5 and AccD6 were the only three β

subunits with high expression levels in exponential growth stage [28]. Those evidences highlight the importance of AccA3, AccD4 and AccD5.

Until now, there was no direct evidence showing that phenotypic changes resulted from AccA3 overexpression. Previous studies about ACCase mostly focused on the β subunits instead of AccA3, which was simply treated as a carboxyl donor. This does not mean that AccA3 is not important: as the only carboxyl donor in this complex, AccA3 seems to be the only irreplaceable molecule. The quantitative real-time PCR analysis showed that the AccA3 expression was at least ten times lower than AccD4, AccD5 and AccD6, and the AccA3 expression peak was in the exponential stage of growth [28]. These results suggest the AccA3 might be one of the regulatory enzymes of lipid carboxylation. Compared with AccA1 and AccA2 in *M. tuberculosis*, AccA3 might be the one controlling proteins of the fitness of lipid synthesis. The conclusions are more reasonable in light of our results that AccA3 overexpression in *M. smegmatis* resulted in an increase of resistance to rifampicin and novobiocin and in a decrease in the lipid permeability.

Because Wag31 could naturally assemble in cell poles, the interaction between Wag31 and AccA3 might facilitate the complex forming to obtain higher activity in the cell poles or might facilitate the stabilization of the complex that is already formed. When Wag31 expression is impaired, the ACCase might become unstable or invalid, damaging the function of cell wall as a lipid barrier, which leads to decreased drug resistance.

Our results highlight the importance of Wag31 in drug resistance. With the interaction of Wag31 and AccA3, it is necessary to re-examine Wag31. We conclude that Wag31 is a potential drug target because of its functions on cell wall synthesis and cell wall permeability.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.116.

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