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MOLECULAR ASPECTS

Comparison of transposon and deletion mutants in *Mycobacterium tuberculosis*: The case of *rv1248c*, encoding 2-hydroxy-3-oxoadipate synthase

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SUMMARY

We compared phenotypes of five strains of *Mycobacterium tuberculosis* (Mtb) differing in their expression of *rv1248c* and its product, 2-hydroxy-3-oxoadipate synthase (HOAS), with a focus on carbon source-dependent growth rates and attenuation in mice. Surprisingly, an *rv1248c* transposon mutant on a CDC1551 background grew differently than an *rv1248c* deletion mutant on the same background. Moreover, the same *rv1248c* deletion in two different yet genetically similar strain backgrounds (CDC1551 and H37Rv) gave different phenotypes, though each could be complemented. Whole genome re-sequencing did not provide an obvious explanation for these discrepancies. These observations offer a cautionary lesson about the strength of inference from complementation and sequence analysis, and commend consideration of more complex phenomena than usually contemplated in Mtb, such as epigenetic control.

1. Introduction

As tuberculosis (TB) continues to impose a devastating global burden, drug discovery efforts put a premium on characterization of annotated and orphan genes implicated in pathways vital for survival of *Mycobacterium tuberculosis* (Mtb) [1]. Transposon insertion mutagenesis is a powerful tool used to identify genetic elements that are essential for bacterial growth and pathogenesis [2]. Himar-1 transposon mutagenesis libraries generated in an Mtb H37Rv background suggested that *rv1248c (hoas)*, the gene that encodes 2-hydroxy-3-oxoadipate synthase (HOAS), may be essential for in vitro growth [3–5]. HOAS, a thiamin diphosphate (ThDP)-dependent enzyme, functions at a node linking the tricarboxylic acid (TCA) cycle and glutamate synthesis through its substrate, α-ketoglutarate (α-KG). HOAS is multifunctional through its ability to convert α-KG to succinyl-CoA, succinyl semialdehyde (SSA), and hydroxy-oxoadipate (HOA); HOA spontaneously gives rise to 5-hydroxylevulinc acid (HLA) [6]. Recombinant HOAS has been extensively studied in vitro, but characterization of the enzyme's function in the intact cell requires studies of cells bearing mutant alleles of *rv1248c*.

A transposon mutant, *hoas::tn*$_{CDC1551}$, was generated in a wild-type CDC1551 (WT$_{CDC1551}$) strain background as part of the Tuberculosis Animal Research and Gene Evaluation Taskforce (TARGET) initiative. Its existence suggested that HOAS is dispensable for growth of Mtb in vitro [7]. CDC1551 caused a TB outbreak near the Kentucky–Tennessee border in the mid 1990s and was initially considered hypervirulent in humans [8]. Studies of CDC1551 in mice found it to be less virulent than [9] or comparable in virulence to the laboratory strain H37Rv [10,11].

We began assessing the role of HOAS in Mtb by comparing the phenotype of WT$_{CDC1551}$ and *hoas::tn*$_{CDC1551}$. While that work was underway, we used the WT$_{CDC1551}$ parent to generate the deletion mutant described below, termed Δ*hoas*$_{CDC1551}$. We characterized the in vitro growth of Δ*hoas*$_{CDC1551}$ and *hoas::tn*$_{CDC1551}$ in media with defined carbon sources and compared it to the behavior of

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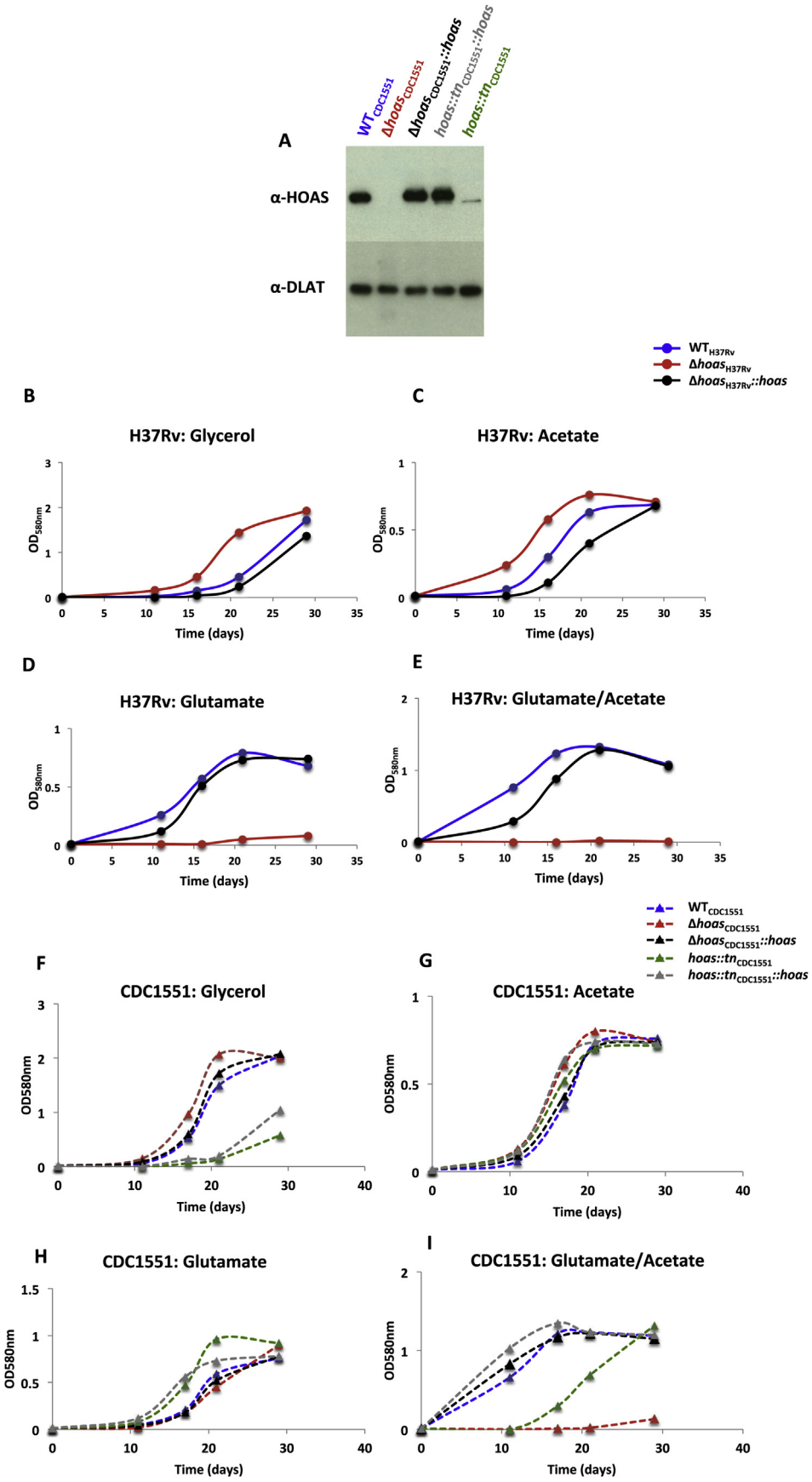

Figure 1. Characterization in vitro of Mtb strains with *hoas* deleted or disrupted by transposon insertion. **(A)** Immunoblot of HOAS in lysates of $WT_{CDC1551}$, *hoas*::$tn_{CDC1551}$, Δ*hoas*$_{CDC1551}$, *hoas*::$tn_{CDC1551}$::*hoas*, and Δ*hoas*$_{CDC1551}$::*hoas* using antiserum against HOAS. Antiserum against DlaT was used as a loading control. **(B–E)** Growth curves of WT_{H37Rv},

the *hoas* deletion mutant generated in an H37Rv WT background, termed Δ*hoas*$_{H37Rv}$, as reported elsewhere [12]. Surprisingly, the phenotypes of all three strains varied, even though all phenotypes but one were reverted to wild type by complementation with a full-length, wild type *hoas* allele. Whole-genome sequencing identified polymorphisms unique to each mutant and its WT parent but did not offer an apparent explanation for the divergent phenotypes.

2. Materials and methods

2.1. Mtb culture and generation of mutants

Standard liquid and agar culture methods, composition of Sauton's minimal medium (SMM), and growth experiments in carbon-defined Sauton's minimal medium were as described [12].

Procedures for engineering Δ*hoas*$_{H37Rv}$ and its complementation with a full length *hoas* allele (Δ*hoas*$_{H37Rv}$::*hoas*) were as described [12]. For Δ*hoas*$_{CDC1551}$, *hoas* was cloned downstream of an *hsp60* promoter into pDE43-MCS via Gateway Cloning Technology (Invitrogen) to yield pDE43h*sp60*hoas-MCS, which integrates into the attL5 site. This vector was electroporated into competent WT$_{CDC1551}$ to create merodiploid WT$_{CDC1551}$::*hoas*. This merodiploid strain was transformed with the nitrile-inducible recombineering plasmid pNIT(kan)::RecET-sacB, and colonies were selected on 7H10 agar plates with 10 ug/mL streptomycin and 15 ug/mL kanamycin. Replacement of native *hoas* with a hygromycin cassette was carried out by methods described in [12]. Knockout candidates (Δ*hoas*$_{CDC1551}$::pGMCS-hsp60*hoas*) were screened for kanamycin sensitivity, implicating loss of pNIT(kan)::RecET-sacB, and native *hoas* deletion was confirmed by Southern blot. To test essentiality of *hoas*, a plasmid harboring a zeocin resistance gene, pGMZq17-0X-0X, was transformed into competent Δ*hoas*$_{CDC}$::pGMCS-hsp60*hoas*. Δ*hoas*$_{CDC1551}$::pGMZq17-0X-0X (here called Δ*hoas*$_{CDC1551}$) candidates were isolated on 7H10 agar with 50 ug/mL hygromycin and 25 ug/mL zeocin and further screened by immunoblot. Plasmids pDE43-MCS and pGMZq17-0X-0X were a kind gift from Dirk Schnappinger, Weill Cornell Medical College and plasmid pNIT(-kan)::RecET-sacB was from Kenan Murphy and Christopher Sassetti, University of Massachusetts.

2.2. Other procedures

Protocols for immunoblotting and Mtb aerosol infection of mice were as reported in [12].

2.3. Whole genome sequencing

The genomes of the five strains described in this study were sequenced using next-generation sequencing technology. Three of the strains were sequenced using an Illumina HiSeq 2500 with a read length of 54 bp, and the other two were sequenced using an Illumina MiSeq with a read length of 150 bp. The machines were operated in paired end mode. All samples were prepared according to the standard Illumina genomic DNA sample preparation protocol (Illumina, Inc.). The sequencing statistics, including read-length and depth of coverage for each sample are given in Table S1. Genome sequences were assembled by a comparative method as described in [13], using published genomes for H37Rv and CDC1551 as reference sequences.

3. Results

3.1. Generation and complementation of hoas mutant strains on the CDC1551 background

Immunoblot with rabbit antiserum against purified recombinant HOAS demonstrated that *hoas::tn*$_{CDC1551}$ expresses a truncated variant of HOAS (Figure 1A). This was consistent with the distal insertion of Himar-1, which introduced a premature stop codon at nucleotide 3568, resulting in a putative 42 amino acid truncation at the C-terminus of this 1231-residue protein. To explore the essentiality of *hoas* in Mtb, we introduced a full-length *hoas* allele into the attL5 site of WT$_{CDC1551}$ Mtb, generating a merodiploid variant. We replaced native *hoas* with a hygromycin resistance cassette by allelic exchange via recombineering. By subsequently replacing the attL5 copy of *hoas* with a vector conferring zeocin resistance, we generated Δ*hoas*$_{CDC1551}$. Deletion of the gene was confirmed by Southern blot (Figure S1) and by absence of the protein on immunoblot (Figure 1A). Generation of Δ*hoas*$_{CDC1551}$ established that *hoas* is not essential for in vitro growth of Mtb in standard 7H9 complete medium.

To test if phenotypes of *hoas::tn*$_{CDC1551}$ and Δ*hoas*$_{CDC1551}$ were due to disruption of *hoas*, we complemented both strains with the full length *hoas* allele under the control of a constitutive *hsp60* promoter. Complementation restored HOAS protein to WT levels in both mutants (Figure 1A).

3.2. Growth on defined carbon sources

We next compared the in vitro growth profiles of *hoas::tn*$_{CDC1551}$, Δ*hoas*$_{CDC1551}$, and Δ*hoas*$_{H37Rv}$ in a modified Sauton's minimal medium supplemented with a single carbon source or a combination of two carbon sources. Deletion mutants Δ*hoas*$_{CDC1551}$ and Δ*hoas*$_{H37Rv}$ grew no less rapidly than their respective WT parental strains when cultured in either glycerol or acetate (Figure 1B and C, F and G). However, as reported elsewhere [12] and recapitulated here, glutamate, either as sole carbon source or in combination with glycerol or acetate in equal amounts by weight, greatly suppressed growth of Δ*hoas*$_{H37Rv}$ (Figure 1D and E). In striking contrast, Δ*hoas*$_{CDC1551}$ grew as well as WT$_{CDC1551}$ with glutamate as sole carbon source (Figure 1H). Nonetheless, growth of Δ*hoas*$_{CDC1551}$ was abolished when acetate, permissive for growth on its own, was added along with glutamate (Figure 1I). On the other hand, and again in striking contrast to results for Δ*hoas*$_{H37Rv}$, glycerol (as opposed to glutamate) stalled *hoas::tn*$_{CDC1551}$ growth for approximately 20 days (Figure 1F). Moreover, growth of *hoas::tn*$_{CDC1551}$, like growth of Δ*hoas*$_{CDC1551}$, was suppressed when glutamate was combined with acetate (Figure 1I). Complementation with full length HOAS in *hoas::tn*$_{CDC1551}$, Δ*hoas*$_{H37Rv}$, and Δ*hoas*$_{H37Rv}$, generating *hoas::tn*$_{CDC1551}$*::hoas*, Δ*hoas*$_{CDC1551}$::*hoas*, and Δ*hoas*$_{H37Rv}$::*hoas*, respectively, rescued all their observed growth defects, with one exception: the suppression of growth of *hoas::tn*$_{CDC1551}$*::hoas* in glycerol (Figure 1F).

3.3. Course of infection in mice

Next, we infected C57BL/6 mice with approximately 100 colony forming units of bacteria per lung of aerosolized WT$_{CDC1551}$, Δ*hoas*$_{CDC1551}$, or Δ*hoas*$_{CDC1551}$::*hoas*. At day 14, Δ*hoas*$_{CDC1551}$ grew to a level of CFU in lungs that was about 0.5 $\log_{10}$ units lower than the

Δ*hoas*$_{H37Rv}$, and Δ*hoas*$_{H37Rv}$::*hoas* in modified Sauton's minimal medium supplemented with either **(B)** glycerol, **(C)** acetate, **(D)** glutamate, or **(E)** glutamate and acetate, each at 0.2%. **(F–I)** Growth curves of W$_{CDC1551}$, Δ*hoas*$_{CDC1551}$, Δ*hoas*$_{CDC1551}$::*hoas*, *hoas::tn*$_{CDC1551}$, and *hoas::tn*$_{CDC1551}$*::hoas* in modified Sauton's minimal medium supplemented with either **(F)** glycerol, **(G)** acetate, **(E)** glutamate, or **(I)** glutamate and acetate, each at 0.2%. All results are representative of at least three experiments.

CFU of $WT_{CDC1551}$. CFU of $\Delta hoas_{CDC1551}$ rose no further between days 14 and 28 and then declined, so that by day 62, no $\Delta hoas_{CDC1551}$ colonies were recovered from mouse lungs after minimal dilution of the organ homogenate, such that the limit of detection was only 10 CFU (Figure 2). Thus, deletion of *hoas* in the CDC1551 background severely attenuated Mtb in vivo, and this defect was complemented by the WT allele. As noted elsewhere, $\Delta hoas_{H37Rv}$ was attenuated in the mouse as well [12], but not as severely as seen here for $\Delta hoas_{CDC1551}$. Moreover, WT_{H37Rv} established much higher CFU levels than $WT_{CDC1551}$, for which only about 10^4 CFU were recovered, similar to results in another study [9].

3.4. Whole genome re-sequencing

In an effort to understand the discrepancies in the growth phenotypes observed between *hoas::tn*$_{CDC1551}$, $\Delta hoas_{CDC1551}$, and $\Delta hoas_{H37Rv}$, and the low bacillary loads of $WT_{CDC1551}$ in mouse lungs, we re-sequenced the genomes of five strains: $WT_{CDC1551}$, $\Delta hoas_{CDC1551}$, *hoas::tn*$_{CDC1551}$, WT_{H37Rv} and $\Delta hoas_{H37Rv}$ (Table 1). WT_{H37Rv} and $\Delta hoas_{H37Rv}$ harbored mutations relative to the published H37Rv sequence (NC_000962.3) that were also shared among five other laboratory strains, and, in particular, were most similar to the strain H37RvCO [13]. In terms of novel mutations, both WT_{H37Rv} and $\Delta hoas_{H37Rv}$ share a non-synonymous mutation M69L in Rv0516c (possible anti–anti sigma factor). In addition, the WT_{H37Rv} strain had two other unique mutations not found in the deletion mutant. These included a frameshift in residue 1242 of *rv2932* (*ppsB*, component of type I polyketide synthase) and H80Q in Rv0505c (*serB1*, phosphoserine phosphatase). These were most likely acquired in the parental stock subsequent to generation of the deletion mutant, as H37Rv/pNIT(kan):RecET-SacB [12] was plated on sucrose to ensure the loss of the recombineering plasmid. The only unique mutation identified in the $\Delta hoas_{H37Rv}$ mutant, other than deletion of *rv1248c*, was a novel insertion of the transposable element IS6110 in *rv1358* (supplementary to the 16 known insertion sites in H37Rv). *rv1358* is a transcription factor whose role is currently unknown. The growth impairment observed on glutamate for the $\Delta hoas_{H37Rv}$ strain, compared to the parental strain (H37Rv), is most likely explained by the deletion of the HOAS gene, because it could be complemented with the WT allele of *rv1248c*.

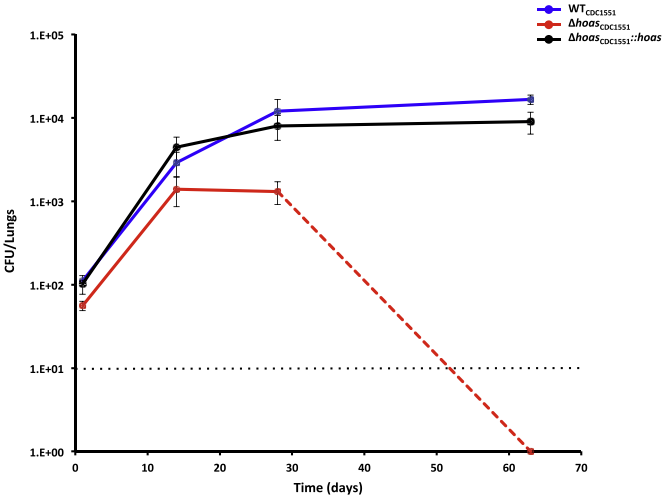

Figure 2. Phenotype in mice of WT, *hoas*-deleted and *hoas*-disrupted Mtb strains on the CDC1551 background. Bacterial CFU counts in lungs of mice infected with $WT_{CDC1551}$, $\Delta hoas_{CDC1551}$, or $\Delta hoas_{CDC1551}$::*hoas*. Data are means ± SEM for five mice per time point (four on day 1). Dashed line indicates the limit of detection. For $\Delta hoas_{CDC1551}$ no colonies were recovered at day 62.

The *hoas::tn*$_{CDC1551}$ and $\Delta hoas_{CDC1551}$ mutants each exhibited several unique polymorphisms compared to the parental $WT_{CDC1551}$ sequence. The transposon-insertion mutant, *hoas::tn*$_{CDC1551}$, had unique SNPs in *rv0472c* (A24A), *MT1802* (P167A), and *rv1812c* (V279L). The mutation in *rv0472c* is synonymous and hence presumably silent. Rv1812c is annotated as a dehydrogenase but its substrates are unknown. MT1801, annotated as a molybdopterin oxidoreductase, is encoded in a region of the CDC1551 genome that is deleted from H37Rv. It is not apparent how mutations in either enzyme might impart sensitivity to glycerol or avoid the growth inhibitory effect of glutamate, as compared to $\Delta hoas_{H37Rv}$.

As noted, the recombineered deletion mutant, $\Delta hoas_{CDC1551}$, was also able to grow on glutamate (unlike $\Delta hoas_{H37Rv}$), and its growth defect on glutamate plus acetate was only rescued by complementation after a delay that was not observed in $\Delta hoas_{H37Rv}$. However, after filtering out mutations shared between the parental CDC1551 strain and the deletion mutant, and discounting the effect of the frameshift in *ppsB* (which should only affect PDIM biosynthesis), the only unique mutation left in the $\Delta hoas_{CDC1551}$ mutant was G37R in *rv3919c* (*gidB*). The latter SNP is unlikely to explain the growth phenotype, as GidB is an rRNA methyltransferse. The mutation is potentially due to selection on streptomycin during the mutant generation protocol, as mutations in *gidB* are often found to confer streptomycin resistance [14].

Table 1
Results of whole genome re-sequencing. Identified mutations in $WT_{CDC1551}$, $\Delta hoas_{CDC1551}$, *hoas::tn*$_{CDC1551}$, WT_{H37Rv} and $\Delta hoas_{H37Rv}$ that differ from their respective reference strains. Boldface indicates polymorphisms that are unique; non-boldfaced polymorphisms are shared between two or more strains.

Strain	Polymorphisms	Annotation
$WT_{CDC1551}$	*rv2672*:R320Q	Possible secreted protease
	rv3303c: +A (1 bp ins) in T410	LpdA: NAD(P)H quinone reductase
$\Delta hoas_{CDC1551}$	***rv1248c***	HOAS
	***rv2932*: -T (frameshift in aa 420/1538)**	PpsB: Pthiocerol synthesis type-I polyketide synthase
	***rv3919c*: G37R**	GidB: rRNA methyltransferase
	rv2672:R320Q	Possible secreted protease
	rv3303c: +A (1 bp ins) in T410	LpdA: NAD(P)H quinone reductase
hoas::tn$_{CDC1551}$	***rv1248c*::Tn (in aa 1189/1242)**	HOAS
	***rv0472c*:A24A**	Probable TetR-family transcription factor
	***rv1812c*:P167A**	Probable dehydrogenase
	MT1801:V279L	Molybdopterin Oxidoreductase (not present in H37Rv)
WT_{H37Rv}	*rv0516c*:M69L	Possible anti–anti sigma factor
	***rv0505c*:H80Q**	SerB1: Phosphoserine phosphatase
	***rv2932*: -C (frameshift in aa 1242/1538)**	PpsB: Phthiocerol synthesis type-I polyketide synthase
$\Delta hoas_{H37Rv}$	***rv1248c***	HOAS
	rv0516c:M69L	Possible anti–anti sigma factor
	***rv1358*:IS6110 insertion**	Probable transcription factor

Boldface indicates polymorphisms that are unique; non-boldface polymorphisms are shared between two or more strains. Italics is a standard way to indicate genes.

The mutant strain Δ*hoas*$_{CDC1551}$ and WT$_{CDC1551}$ also had several unique polymorphisms not observed in other stocks of CDC1551, such as the transposon insertion mutant *hoas::tn*$_{CDC1551}$ (which was generated in a different lab). These include a SNP in Rv2672 (possible secreted protease) and a frameshift in LpdA. It is possible that these SNPs are related to the relative attenuation of the WT$_{CDC1551}$ and Δ*hoas*$_{CDC1551}$ strains in mice, compared to the bacillary loads typically achieved by H37Rv-derived strains. However, since these SNPs are shared by Δ*hoas*$_{CDC1551}$ and WT$_{CDC1551}$, they should not have any bearing on the in vitro growth phenotype of Δ*hoas*$_{CDC1551}$. The genome sequence we determined for WT$_{CDC1551}$ had hundreds of differences from the published CDC1551 reference sequence, NC_002755.2, which are assumed to be due to corrections in original sequencing errors, including many ambiguous nucleotides and short 1 bp indels.

4. Discussion

We began our studies of the biology of HOAS by taking advantage of the availability of the transposon mutant in the CDC1551 background. We completed our in vitro studies of *hoas::tn*$_{CDC1551}$, *hoas::tn*$_{CDC1551}$*::hoas*, Δ*hoas*$_{CDC1551}$ and Δ*hoas*$_{CDC1551}$*::hoas* before testing the phenotype of WT$_{CDC1551}$ and Δ*hoas*$_{CDC1551}$ in the mouse. The marked attenuation of WT$_{CDC1551}$ in the mouse was unanticipated. That observation led us to conclude that a study of the biology of HOAS in intact Mtb in vitro might be compromised in the CDC1551 background. We therefore recapitulated our studies in the WT$_{H37Rv}$ background after generating Δ*hoas*$_{H37Rv}$ and Δ*hoas*$_{H37Rv}$*::hoas* [12]. This gave us the opportunity to compare and contrast the phenotypic consequences of identical gene deletions in two different Mtb strains −Δ*hoas*$_{CDC1551}$ and Δ*hoas*$_{H37Rv}$ − that were engineered with the same genetic tools. We believe that the discrepancies we observed, while unexplained, have cautionary value.

First, we observed a startling difference in growth profiles for the deletion mutants in glutamate: glutamate was suppressive for Δ*hoas*$_{H37Rv}$, but supportive for Δ*hoas*$_{CDC1551}$. Glutamate also abolished growth of Δ*hoas*$_{H37Rv}$ when added to acetate. For Δ*hoas*$_{CDC1551}$, the combination of glutamate and acetate was growth suppressive, but as noted, glutamate alone supported growth.

Second, growth of *hoas::tn*$_{CDC1551}$, a strain expressing a truncated version of HOAS, was defective in glycerol (as opposed to glutamate) and this defect was not rescued by complementation. This result was starkly different from what was observed with both deletion mutants, whose phenotypes were all complemented by the wild type allele.

Third, in mixtures of glutamate and acetate, *hoas::tn*$_{CDC1551}$ and Δ*hoas*$_{CDC1551}$ had opposing phenotypes, yet in each case these particular phenotypes were normalized by genetic complementation. The genome sequences of WT$_{H37Rv}$ and WT$_{CDC1551}$ have a considerable number of differences, given that strains H37Rv and CDC1551 are derived from different TB lineages (i.e. T clade versus X clade, respectively [15,16]), including approximately 1300 SNPs between them, as well as several inserted/deleted genes and genomic regions. Thus there are many potential reasons why there might be differences in phenotypes between mutants derived from different genetic backgrounds.

In other respects, both deletion mutants exhibited similar properties. As reported elsewhere, Δ*hoas*$_{H37Rv}$ was much more susceptible to reactive nitrogen intermediates in vitro than WT$_{H37Rv}$ [12]. Likewise, Δ*hoas*$_{CDC1551}$ was far more susceptible to reactive nitrogen intermediates in vitro than WT$_{CDC1551}$ (data not shown). Exposure to glutamate induced selective increases in intracellular α-ketoglutarate and succinic semialdehyde levels within Δ*hoas*$_{H37Rv}$ [12]. Levels of these same metabolites were also selectively and extensively elevated when Δ*hoas*$_{CDC1551}$ was grown in the presence of glutamate, despite this strain's lack of a growth defect with glutamate as sole carbon source (data not shown). Finally, both deletion mutants were attenuated in mice, although attenuation was more severe on the CDC1551 background.

In an attempt to clarify phenotypic discrepancies between both deletion mutants and between them and *hoas::tn*$_{CDC1551}$, as well as to understand why WT$_{CDC1551}$ was so attenuated on its own, we sequenced the genomes of the three mutant strains and their WT parents. We discovered that both Δ*hoas*$_{CDC1551}$ and WT$_{H37Rv}$ genomes contained deletions in *rv2932*, the gene encoding the phthiocerol synthesis type-I polyketide synthase, PpsB, in the *ppsA-E* operon that encodes enzymes vital for synthesis of phthiocerol dimycocerosate (PDIM). PDIM is a wax in the cell wall necessary for full virulence of Mtb strains in mice, and is frequently lost during in vitro passage [17,18]. This polymorphism most likely contributed to the slightly reduced CFU count we observed for WT$_{H37Rv}$ in mouse lungs compared to other reports using WT$_{H37Rv}$ [12]. However, PDIM biosynthesis was not genetically disrupted in WT$_{CDC1551}$, whose sequence hardly differed from that of WT$_{CDC1551}$ used in other laboratories. Thus PDIM deficiency did not seem likely to account for our fourth surprising observation, the marked attenuation of WT$_{CDC1551}$.

Contrary to our expectations, whole genome re-sequencing results did not appear to explain the sole phenotypic discrepancy observed in vitro (growth in glutamate) between the two *hoas* deletion strains. The deletion of HOAS in Δ*hoas*$_{H37Rv}$ explains the sensitivity to glutamate in an H37Rv background [12]. Relative to this, the ability of Δ*hoas*$_{CDC1551}$ to grow on glutamate was unexpected; however, the sole unique secondary mutation observed in Δ*hoas*$_{CDC1551}$ (compared to the parental CDC1551) was unable to explain this anomalous phenotype. Nor did any one of the unique polymorphisms seem likely to explain the phenotypic disparities among Δ*hoas*$_{CDC1551}$, Δ*hoas*$_{CDC1551}$, and *hoas::tn*$_{CDC1551}$. The *hoas::tn*$_{CDC1551}$ genome harbors only three unique mutations compared to Δ*hoas*$_{CDC1551}$, despite its unique growth profile, which suggests caution in interpreting further results with this transposon-insertion strain.

Our observations appear to challenge the underlying premise of genetic complementation: that restoration of WT behavior upon supplying the WT allele establishes that a mutant's phenotype is due specifically to disruption or deletion of the corresponding gene. Our observations also illustrate that re-sequencing of the genome of a mutant may not always reveal potential explanations for its phenotype. Among speculative explanations for these observations we suggest the following. (i) The observed polymorphisms, acting in concert, may have epistatic effects that would be unanticipated from the annotated function of each polymorphic gene considered separately. (ii) There may be polymorphisms in regions of the genome that were not well covered by sequencing. (iii) There could be copy-number variation in tandem-repeat sequences (e.g. MIRU sequences), which are difficult to resolve with short-read sequencing data. (iv) Single nucleotide polymorphisms that are silent in the annotated coding region may change sense in an unannotated open reading frame or change function in an unannotated intergenic regulatory sequence on the opposite strand. (v) RNA editing may occur in Mtb, so that the sequence of some transcripts may differ from the sequence encoded in the DNA. (vi) Epigenetic changes, for example differences in DNA methylation patterns, may affect gene expression in Mtb, leading to different growth phenotypes [19].

5. Conclusions

This study illustrates three findings that reveal limitations in what is currently the standard approach to mycobacterial genetics:

(i) a transposon mutant's growth profile differed from that of a deletion mutant in the same strain background, and both could be complemented; (ii) the same gene deletion in two genetically similar strain backgrounds gave different phenotypes, though each could be complemented; and (iii) whole genome re-sequencing did not provide an obvious explanation for (i) or (ii).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.08.009.

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