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.

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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
Figure 1. Characterization in vitro of Mtb strains with hoas deleted or disrupted by transposon insertion. (A) Immunoblot of HOAS in lysates of WT\textsubscript{CDC1551}, hoas::\textsubscript{tn}CDC1551, Δhoas\textsubscript{CDC1551}, hoas::\textsubscript{tn}CDC1551::hoas, and Δhoas\textsubscript{CDC1551}::hoas using antiserum against HOAS. Antiserum against DlaT was used as a loading control. (B–E) Growth curves of WT\textsubscript{H37Rv}.
the hoas deletion mutant generated in an H37Rv WT background, termed ΔhoasH37Rv, 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 ΔhoasH37Rv and its complementation with a full length hoas allele (ΔhoasH37Rv::hoas) were as described [12]. For ΔhoasCDC1551, hoas was cloned downstream of an hsp60 promoter into pDE43-MCS via Gateway Cloning Technology (Invitrogen) to yield pDE43hsp60hoas-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.

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::tnCDC1551 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 ΔhoasCDC1551. Deletion of the gene was confirmed by Southern blot (Figure S1) and by absence of the protein on immunoblot (Figure 1A). Generation of ΔhoasCDC1551 established that hoas is not essential for in vitro growth of Mtb in standard 7H9 complete medium.

To test if phenotypes of hoas::tnCDC1551 and ΔhoasCDC1551 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::tnCDC1551, ΔhoasCDC1551, and ΔhoasH37Rv, in a modified Sauton’s minimal medium supplemented with a single carbon source or a combination of two carbon sources. Deletion mutants ΔhoasCDC1551 and ΔhoasH37Rv 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 ΔhoasH37Rv (Figure 1D and E). In striking contrast, ΔhoasCDC1551 grew as well as WT CDC1551 with glutamate as sole carbon source (Figure 1H). Nonetheless, growth of ΔhoasCDC1551 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 ΔhoasH37Rv, glycerol (as opposed to glutamate) stalled hoas::tnCDC1551 growth for approximately 20 days (Figure 1F). Moreover, growth of hoas::tnCDC1551, like growth of ΔhoasCDC1551, was suppressed when glutamate was combined with acetate (Figure 1I). Complementation with full length HOAS in hoas::tnCDC1551, ΔhoasH37Rv, and ΔhoasCDC1551, generating hoas::tnCDC1551::hoas, ΔhoasCDC1551::hoas, and ΔhoasH37Rv::hoas, respectively, rescued all their observed growth defects, with one exception: the suppression of growth of hoas::tnCDC1551::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, ΔhoasCDC1551, or ΔhoasCDC1551::hoas. At day 14, ΔhoasCDC1551 grew to a level of CFU in lungs that was about 0.5 log10 units lower than the
CFU of WT<sub>CDC1551</sub> or Δhoa<sub>cdc1551</sub> rose no further between days 14 and 28 and then declined, so that by day 62, no Δhoa<sub>cdc1551</sub> 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, Δhoa<sub>hs37rv</sub> was attenuated in the mouse as well [12], but not as severely as seen here for Δhoa<sub>cdc1551</sub>. Moreover, WT<sub>hs37rv</sub> established much higher CFU levels than WTCDC1551, for which only about 10<sup>4</sup> 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<sub>CDC1551</sub>, Δhoa<sub>cdc1551</sub>, and Δhoa<sub>hs37rv</sub>, and the low bacillary loads of WT<sub>CDC1551</sub> in mouse lungs, we re-sequenced the genomes of five strains: WT<sub>CDC1551</sub>, Δhoa<sub>cdc1551</sub>, and Δhoa<sub>hs37rv</sub> (Table 1). WT<sub>hs37rv</sub> and Δhoa<sub>hs37rv</sub> 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<sub>hs37rv</sub> and Δhoa<sub>hs37rv</sub> share a non-synonymous mutation M69L in Rv0516c (possible anti–anti sigma factor). In addition, the WT<sub>hs37rv</sub> 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 recombinase plasmid. The only unique mutation identified in the Δhoa<sub>hs37rv</sub> 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 Δhoa<sub>hs37rv</sub> 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.

The hoas::tn<sub>CDC1551</sub> and Δhoa<sub>cdc1551</sub> mutants each exhibited several unique polymorphisms compared to the parental WT<sub>CDC1551</sub> sequence. The transposon-insertion mutant, hoas::tn<sub>CDC1551</sub>, had unique SNPs in rv0472c (A24A), MTB1802 (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. MTB1801, annotated as a molybdothionin 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 Δhoa<sub>hs37rv</sub>

As noted, the recombinated deletion mutant, Δhoa<sub>hs37rv</sub>, was also able to grow on glutamate (unlike Δhoa<sub>hs37rv</sub>), and its growth defect on glutamate plus acetate was only rescued by complementation after a delay that was not observed in Δhoa<sub>hs37rv</sub>. 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 Δhoa<sub>hs37rv</sub> mutant was G37R in rv3919c (gidB). The latter SNP is unlikely to explain the growth phenotype, as GidB is an rRNA methyltransferase. 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**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polymorphisms</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT&lt;sub&gt;CDC1551&lt;/sub&gt;</td>
<td>rv2672/R320Q</td>
<td>Possible secreted protease</td>
</tr>
<tr>
<td>Δhoa&lt;sub&gt;cdc1551&lt;/sub&gt;</td>
<td>rv1248c</td>
<td>LpdA: NAD(P)H quinone reductase</td>
</tr>
<tr>
<td>hoas::tn&lt;sub&gt;CDC1551&lt;/sub&gt;</td>
<td>rv2932:: -T (frameshift in aa 420/1538)</td>
<td>PpsB: Phthiocerol synthesis type-I polyketide synthase</td>
</tr>
<tr>
<td>WT&lt;sub&gt;hs37rv&lt;/sub&gt;</td>
<td>rv3919c: G37R</td>
<td>GidB: rRNA methyltransferase</td>
</tr>
<tr>
<td>Δhoa&lt;sub&gt;hs37rv&lt;/sub&gt;</td>
<td>rv6272/R320Q</td>
<td>Possible secreted protease</td>
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<tr>
<td>hoas::tn&lt;sub&gt;CDC1551&lt;/sub&gt;</td>
<td>rv2932:: -T (frameshift in aa 420/1538)</td>
<td>Possible secreted protease</td>
</tr>
<tr>
<td>WT&lt;sub&gt;hs37rv&lt;/sub&gt;</td>
<td>rv1812c::P167A</td>
<td>Probable TetR-family transcription factor</td>
</tr>
<tr>
<td>Δhoa&lt;sub&gt;hs37rv&lt;/sub&gt;</td>
<td>MTB1801::V279L</td>
<td>Probable dehydrogenase</td>
</tr>
<tr>
<td>hoas::tn&lt;sub&gt;CDC1551&lt;/sub&gt;</td>
<td>rv0516c::M69L</td>
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<td>Probable TetR-family transcription factor</td>
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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 Δhoas\textsubscript{CDC1551} and WT\textsubscript{CDC1551} also had several unique polymorphisms not observed in other stocks of CDC1551, such as the transposon insertion mutant hoas\textsubscript{::Tn\textsubscript{CDC1551}} (which was generated in a different lab). These include a SNP in \textit{Rv2672} (possible secreted protease) and a frameshift in \textit{LpdA}. It is possible that these SNPs are related to the relative attenuation of the WT\textsubscript{CDC1551} and Δhoas\textsubscript{CDC1551} strains in mice, compared to the bacillary loads typically achieved by H\textit{H}37\textit{R}v-derived strains. However, since these SNPs are shared by \textit{Δh\textit{o}tas\textsubscript{CDC1551}} and WT\textsubscript{CDC1551}, they should not have any bearing on the in vitro growth phenotype of Δhoas\textsubscript{CDC1551}. The genome sequence we determined for WT\textsubscript{CDC1551} had hundreds of differences from the published CDC1551 reference sequence, NC\textsubscript{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 \textit{hoas}::\textsubscript{Tn\textsubscript{CDC1551}}, \textit{hoas::Tn\textsubscript{CDC1551}}, \textit{hoas::Tn\textsubscript{CDC1551}}, and \textit{hoas::Tn\textsubscript{CDC1551}} before testing the phenotype of \textit{WT\textsubscript{CDC1551}} and \textit{Δhoas\textsubscript{CDC1551}} in the mouse. The marked attenuation of WT\textsubscript{CDC1551} in the mouse was unanticipated. That observation led us to conclude that a study of the phenotype of WT\textsubscript{CDC1551} and \textit{Δhoas\textsubscript{CDC1551}} was warranted. We therefore recapitulated our studies in the CDC1551 background. We completed our in vitro studies of \textit{WT\textsubscript{CDC1551}} and \textit{Δhoas\textsubscript{CDC1551}}. This gave us the opportunity to compare and contrast the phenotypic consequences of identical gene deletions in two different M\textit{tb} strains – \textit{Δhoas\textsubscript{CDC1551}} and \textit{Δhoas\textsubscript{CDC1551}} – 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 glutaconate: glutamate was suppressive for \textit{Δhoas\textsubscript{CDC1551}}, while \textit{Δhoas\textsubscript{CDC1551}} was non-suppressive for \textit{Δhoas\textsubscript{CDC1551}}. Glutamate also abolished growth of \textit{Δhoas\textsubscript{CDC1551}} when added to acetate. For \textit{Δhoas\textsubscript{CDC1551}}, the combination of glutamate and acetate was growth suppressive, but as noted, glutamate alone supported growth.

Second, growth of \textit{hoas::Tn\textsubscript{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, \textit{hoas::Tn\textsubscript{CDC1551}} and \textit{Δhoas\textsubscript{CDC1551}} had opposing phenotypes, yet in each case these particular phenotypes were normalized by genetic complementation. The genome sequences of WT\textsubscript{H37\textsubscript{R}v} and WT\textsubscript{CDC1551} have a considerable number of differences, given that strains H\textit{H}37\textit{R}v 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, \textit{Δh\textit{o}tas\textsubscript{H37\textsubscript{R}v}} was much more susceptible to reactive nitrogen intermediates in vitro than WT\textsubscript{H37\textsubscript{R}v} [12]. Likewise, \textit{Δh\textit{o}tas\textsubscript{CDC1551}} was far more susceptible to reactive nitrogen intermediates in vitro than WT\textsubscript{CDC1551} (data not shown). Exposure to glutamate induced selective increases in intracellular \textit{α}-ketoglutarate and succinic semialdehyde levels within \textit{Δhoas\textsubscript{H37\textsubscript{R}v}} [12]. Levels of these same metabolites were also selectively and extensively elevated when \textit{Δhoas\textsubscript{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 \textit{hoas::Tn\textsubscript{CDC1551}}, as well as to understand why WT\textsubscript{CDC1551} was so attenuated on its own, we sequenced the genomes of the three mutant strains and their WT parents. We discovered that both \textit{Δhoas\textsubscript{CDC1551}} and WT\textsubscript{H37\textsubscript{R}v} genomes contained deletions in \textit{rv2932}, the gene encoding the phthiocerol synthesis type-I polyketide synthase, PpsB, in the \textit{ppSA}–\textit{E} operon that encodes enzymes vital for synthesis of phthiocerol dimyococerosate (PDIM). PDIM is a wax in the cell wall necessary for full virulence of M\textit{tb} 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\textsubscript{H37\textsubscript{R}v} in mouse lungs compared to other reports using WT\textsubscript{H37\textsubscript{R}v} [12]. However, PDIM biosynthesis was not genetically disrupted in WT\textsubscript{CDC1551}, whose sequence hardly differed from that of WT\textsubscript{CDC1551} used in other laboratories. Thus PDIM deficiency did not seem likely to account for our fourth surprising observation, the marked attenuation of WT\textsubscript{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 \textit{hoas} deletion strains. The deletion of HOAS in \textit{Δhoas\textsubscript{H37\textsubscript{R}v}} explains the sensitivity to glutamate in an H\textit{H}37\textit{R}v background [12]; Relative to this, the ability of \textit{Δhoas\textsubscript{CDC1551}} to grow on glutamate was unexpected; however, the sole unique secondary mutation observed in \textit{Δhoas\textsubscript{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 \textit{Δhoas\textsubscript{CDC1551}}, \textit{Δhoas\textsubscript{CDC1551}}, and \textit{hoas::Tn\textsubscript{CDC1551}}. The \textit{hoas::Tn\textsubscript{CDC1551}} genome harbors only three unique mutations compared to \textit{Δhoas\textsubscript{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 M\textit{tb}, 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 M\textit{tb}, 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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Ethical approval: The mouse infections were approved by Weill Cornell’s Institutional Animal Care and Use Committee (IACUC), protocol #2012-0035.

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