

# The *Mycobacterium tuberculosis* complex has a pathway for the biosynthesis of 4-formamido-4,6-dideoxy-D-glucose

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Received 9 March 2018; Accepted 10 May 2018

DOI: 10.1002/pro.3443

Published online 12 May 2018 proteinscience.org

**Abstract:** Recent studies have demonstrated that the O-antigens of some pathogenic bacteria such as *Brucella abortus*, *Francisella tularensis*, and *Campylobacter jejuni* contain quite unusual N-formylated sugars (3-formamido-3,6-dideoxy-D-glucose or 4-formamido-4,6-dideoxy-D-glucose). Typically, four enzymes are required for the formation of such sugars: a thymidyltransferase, a 4,6-dehydratase, a pyridoxal 5'-phosphate or PLP-dependent aminotransferase, and an N-formyltransferase. To date, there have been no published reports of N-formylated sugars associated with *Mycobacterium tuberculosis*. A recent investigation from our laboratories, however, has demonstrated that one gene product from *M. tuberculosis*, Rv3404c, functions as a sugar N-formyltransferase. Given that *M. tuberculosis* produces L-rhamnose, both a thymidyltransferase (Rv0334) and a 4,6-dehydratase (Rv3464) required for its formation have been identified. Thus, there is one remaining enzyme needed for the production of an N-formylated sugar in *M. tuberculosis*, namely a PLP-dependent aminotransferase. Here we demonstrate that the *M. tuberculosis* rv3402c gene encodes such an enzyme. Our data prove that *M. tuberculosis* contains all of the enzymatic activities required for the formation of dTDP-4-formamido-4,6-dideoxy-D-glucose. Indeed, the rv3402c gene product likely contributes to virulence or persistence during infection, though its temporal expression and location remain to be determined.

**Keywords:** aminotransferase; dTDP-4-amino-4,6-dideoxy-D-glucose; *Mycobacterium tuberculosis*; pyridoxal 5'-phosphate; Rv3402c

**Abbreviations:** dTDP, thymidine diphosphate; dTMP, thymidine monophosphate; ESI, electrospray ionization; HEPPS, N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid; HPLC, high-performance liquid chromatography; MBP, maltose-binding protein; NMR, nuclear magnetic resonance; ORF, open reading frame; PLP, pyridoxal 5'-phosphate; SCID, severe combined immunodeficiency; TEV, Tobacco Etch Virus; Tris, tris-(hydroxymethyl)aminomethane

Additional Supporting Information may be found in the online version of this article.

**Broader Statement:** Tuberculosis remains one of deadliest diseases worldwide. It has been estimated that approximately one-fourth of the world's population may be infected with *Mycobacterium tuberculosis*, the causative agent of the disease. Especially concerning is the rise of multi-drug resistant forms of the bacterium. Here we present data suggestive that *M. tuberculosis* produces an unusual N-formylated sugar at some point during its life cycle. Our results will encourage new lines of investigation into the complicated life cycle of this organism, which may ultimately lead to the development of novel therapeutics.

Grant sponsor: NIH Grant; Grant Number: GM115921 to H. M. H.

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## Introduction

The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is a facultative intracellular pathogen. It exhibits a complex life cycle with its human host that is still not fully understood. Indeed, the human immune response is often only partially effective, leading to bacteria that exist in nonproliferative persistent states.<sup>1</sup> These bacteria have apparently evolved a number of strategies to survive within human alveolar macrophages.<sup>2</sup> Strikingly, only 5–15% of those individuals with “latent” infections will develop active infections in the course of their lifetimes, and as a consequence they function as “repositories” for the bacterium.<sup>3</sup> This is especially concerning given that tuberculosis remains one of the top 10 causes of death worldwide according to the World Health Organization.

Within recent years, a large number of *M. tuberculosis* virulence genes have been identified, in part through the use of transposon mutant libraries and *in vivo* screening techniques.<sup>4</sup> Some of these genes encode putative enzymes involved in lipid biosynthetic pathways whereas others likely encode cell surface proteins or regulators of signal transduction systems.<sup>4</sup> The focus of this investigation is on the open reading frame *rv3402c*. This particular gene has been shown to be upregulated in *M. tuberculosis* cultures deficient in iron.<sup>5</sup> In addition, a recent report has demonstrated that the Rv3402c protein, when expressed in *Mycobacterium smegmatis*, enhances the organism’s intracellular persistence within macrophages.<sup>6</sup>

We recently demonstrated that another protein, Rv3404c from *M. tuberculosis* H37Rv, functions as a sugar *N*-formyltransferase by transferring a formyl group from *N*<sup>10</sup>-formyltetrahydrofolate to dTDP-4-amino-4,6-dideoxy-D-glucose.<sup>7</sup> Our results were especially intriguing given that there have been no reports in the literature regarding the existence of *N*-formylated sugars in *M. tuberculosis*. Typically, these unusual sugars are found on the lipopolysaccharides of pathogenic Gram-negative bacteria such as *Campylobacter jejuni*, *Francisella tularensis*, *Providencia alcalifaciens*, *Salmonella enterica*, and *Brucella melitensis*.<sup>8–15</sup>

Shown in Scheme 1 is the accepted biosynthetic pathway by which *N*-formylated sugars are produced in bacteria. Rv3404c catalyzes the last step in the pathway. The question then arises as to whether *M. tuberculosis* contains the additional enzymatic machinery required for the production of *N*-formylated sugars. As can be seen in Scheme 1, the first step involves the attachment of glucose-1-phosphate to a dTMP moiety via the action of a glucose-1-phosphate thymidyltransferase. There is an enzyme in *M. tuberculosis*, encoded by the *rv0334* gene that catalyzes such a reaction. It is specifically

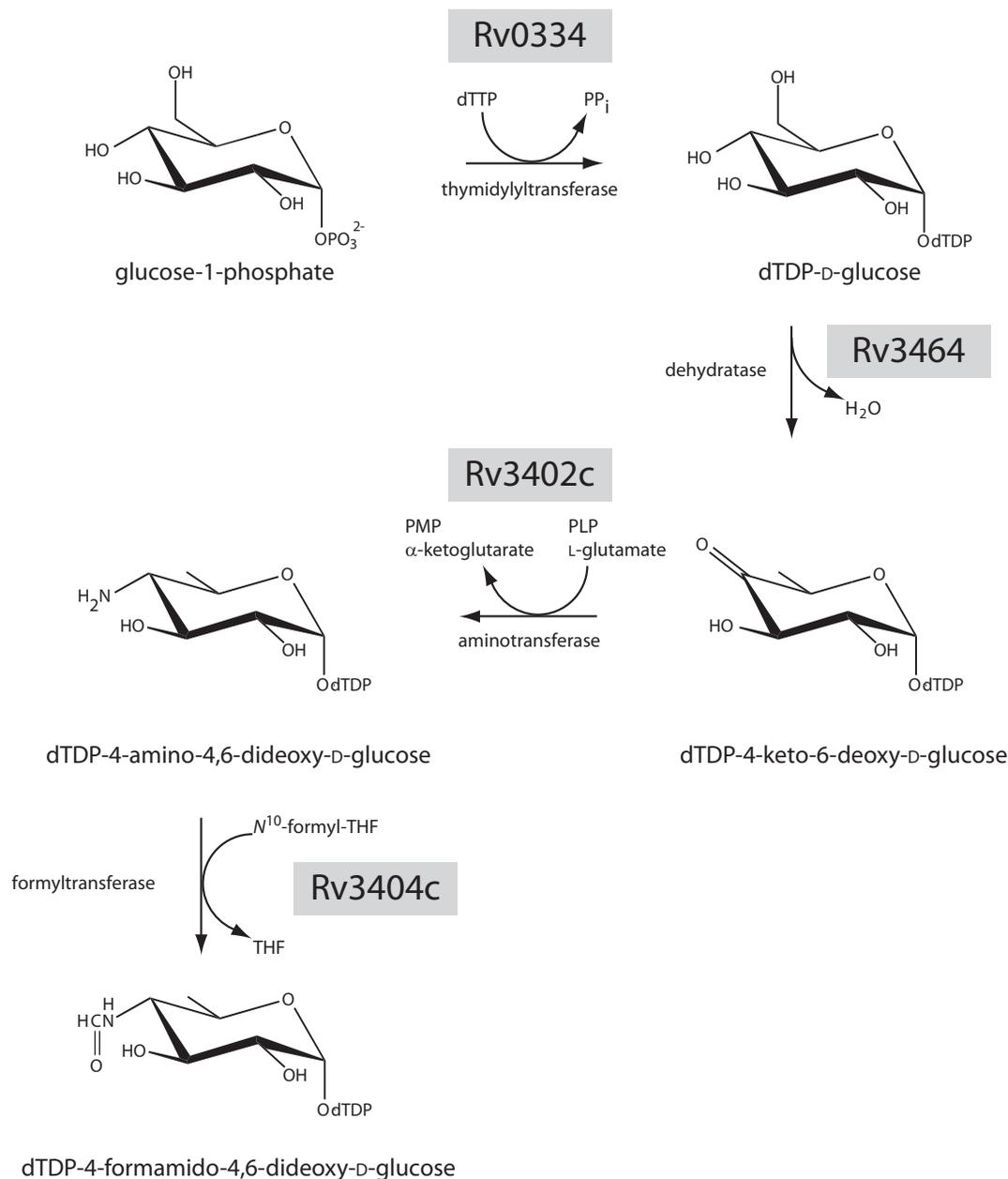
involved in the biosynthesis of L-rhamnose,<sup>16</sup> and its three-dimensional structure has now been determined to high resolution.<sup>17</sup> In the second step of the pathway, dTDP-D-glucose is dehydrated by the action of a 4,6-dehydratase to yield dTDP-4-keto-6-deoxy-D-glucose. Again, this reaction is also required for the production of L-rhamnose in *M. tuberculosis*, and not surprisingly the gene encoding for an enzyme with such activity has been identified, namely *rv3464*.<sup>16</sup>

Thus, the only enzyme missing from the pathway outlined in Scheme 1 is a pyridoxal 5'-phosphate or PLP-dependent aminotransferase. Our initial analysis of the *M. tuberculosis* H37Rv genome identified the Rv3402c protein as a potential candidate for such activity. A BLAST search of the Rv3402c amino acid sequence against the Protein Data Bank indicates that it belongs to the Type I aminotransferase superfamily.<sup>18</sup> The closest structural relative of Rv3402c that has been biochemically and structurally characterized is DesI from *Streptomyces venezuelae*.<sup>19</sup> Rv3402c and DesI demonstrate amino acid sequence identities and similarities of 29% and 39%, respectively (alignment provided in Supporting Information Material). Importantly, DesI catalyzes the formation of dTDP-4-amino-4,6-dideoxy-D-glucose from dTDP-4-keto-6-deoxy-D-glucose. It can thus be postulated that Rv3402c catalyzes the third reaction in Scheme 1. In keeping with this hypothesis, Rv3402c has been previously predicted to be involved in the biosynthesis of lipopolysaccharide-like molecules.<sup>5</sup>

Here we demonstrate that Rv3402c is, indeed, a PLP-dependent aminotransferase that functions on dTDP-4-keto-6-deoxy-D-glucose to produce dTDP-4-amino-4,6-dideoxy-D-glucose. Our results provide strong evidence that at some point in the life cycle of *M. tuberculosis*, *N*-formylated sugars are produced. Whether these sugars are important for virulence remains an open question. However, the loss of activity of a sugar *N*-formyltransferase in *Brucella abortus* results in a bacterial strain with attenuated pathogenicity.<sup>13</sup>

## Results and Discussion

Rv3402c was cloned and over-expressed in *Escherichia coli* utilizing a MBP tag to aid in the *in vivo* protein folding and solubility. The tag was ultimately removed using TEV protease, and Rv3402c was first tested for activity using an HPLC assay that included it and dTDP-D-glucose, *E. coli* RmlB (which is functionally equivalent to *M. tuberculosis* *rv3464*), PLP, and glutamate (Scheme 1). As a negative control, the assay was also conducted in the absence of Rv3402c. Shown in Figure 1(a,b) are the corresponding HPLC traces without and with the addition of Rv3402c to the reaction mixture. As can be seen, in the absence of Rv3402c, one major peak

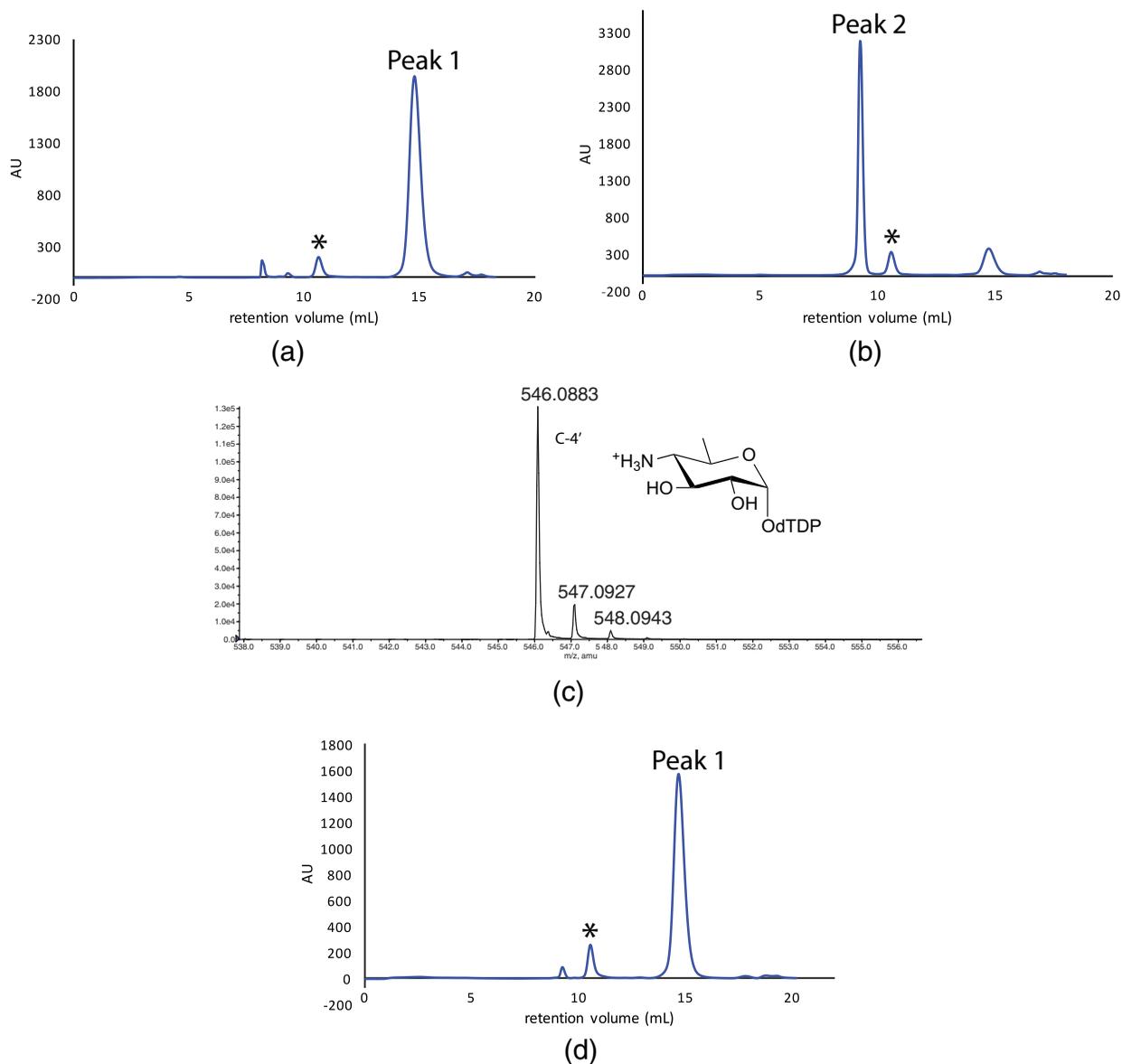


## Possible pathway for the *in vivo* production of dTDP-4-formamido-4,6-dideoxy-D-glucose

**Scheme 1.** Possible pathway for the *in vivo* production of dTDP-4-formamido-4,6-dideoxy-D-glucose.

is observed at 14.8 mL. On the basis of our laboratory standards, this peak corresponds to either dTDP-D-glucose or dTDP-4-keto-6-deoxy-D-glucose. The HPLC trace of the reaction mixture in the presence of Rv3402c, however, shows a new peak at 9.2 mL [Fig. 1(b)]. A sample from this peak was subjected to electrospray ionization mass spectrometry in the negative ion mode. The spectrum is shown in Figure 1(c). There is a major peak at a  $m/z = 546.0883$ , which would correspond to a dTDP-4-aminosugar in its protonated state.

Rv3402c was difficult to purify to homogeneity so in order to ensure that the production of the aminosugar was not due to the presence of contaminating *E. coli* aminotransferases, a variant of Rv3402c was produced via site-directed mutagenesis. Specifically, on the basis of amino acid sequence homology and the known catalytic properties of the Type I aminotransferases, Lys 227 in Rv3402c was predicted to be the conserved residue required for formation of the internal aldimine.<sup>18</sup> Indeed, the HPLC trace of the reaction mixture containing the K227A



**Figure 1.** HPLC assay of Rv3402c activity and mass spectrum of the Rv3402c product. Aminotransferase activity was assessed in the absence (a) and presence (b) of Rv3402c. (a) Peak 1 (14.8 mL) corresponds to either dTDP-D-glucose or dTDP-4-keto-6-deoxy-D-glucose. (b) Peak 2 (9.2 mL) corresponds to a dTDP-aminosugar, although the actual stereochemistry about the C-4' carbon cannot be determined from these data. The peaks in (a), (b), and (d), marked by the asterisks, indicate the presence of dTMP, which is often a contaminant of the dTDP-D-glucose preparations. Shown in (c) is the ESI mass spectrum corresponding to Peak 2 in the elution profile displayed in (b). Finally, the elution profile for the activity assay of the K227A protein variant is presented in (d).

variant [Fig. 1(d)] is comparable to that of the reaction with no added aminotransferase. Whereas a small amount of aminosugar was produced by the K227A variant, there are reports of other aminotransferases that still show activity when their catalytic lysines are mutated. Indeed, PseC from *Helicobacter pylori* exhibited 12% activity when its catalytic lysine was mutated to an arginine.<sup>20</sup> By comparison, the K227A Rv3402c variant exhibited approximately 2% activity of the wild-type protein, strongly suggesting that Lys 227 is necessary for catalysis, and that aminosugar formation is due to Rv3402c and not to a

contaminating protein. Furthermore, contaminating proteins that persisted during purification were subjected to mass spectrometry analysis and determined to be the *E. coli* chaperones DnaJ and the large subunit of GroEL. Finally, the only known enzyme in the laboratory-strain of *E. coli* that uses dTDP-4-keto-6-deoxy-D-glucose as a substrate is WeeE, which synthesizes an aminosugar having a different stereochemistry about the C-4' carbon position as compared to the Rv3402c product.<sup>21</sup>

The HPLC assay and the mass spectrophotometric data indicate that Rv3402c catalyzes a PLP-

**Table I.** NMR Spectroscopic Data of dTDP-4-Amino-4,6-Dideoxyglucose (dTDP-Qui4N) Produced Using Rv3402c

	H/C-1 (ppm)	$J_{n,n+1}$ (Hz)	H/C-2 (ppm)	$J_{n,n+1}$ (Hz)	H/C-3 (ppm)	$J_{n,n+1}$ (Hz)	H/C-4 (ppm)	$J_{n,n+1}$ (Hz)	H/C-5 (ppm)	$J_{n,n+1}$ (Hz)	H/C-6 (ppm)
<b>Qui4N</b>	5.59	3.48	3.62	9.6	3.93	10.4	3.02	10.4	4.29	6.3	1.36
	96.2		73.0		69.8		58.1		66.8		18.0
<b>2dRib</b>	6.34		2.37 <sup>a</sup>		4.62		4.19		4.17 <sup>a</sup>		
	86.2		39.8		72.2		86.5		66.6		
<b>Thymine</b>											7.73
											138.5

<sup>a</sup> Identical shifts were determined for the two protons at these positions.

dependent amination of dTDP-4-keto-6-deoxy-D-glucose. The final dTDP-sugar product was analyzed by 2D NMR (Table I). The resulting monosaccharide had all vicinal coupling constants characteristic for the  $\alpha$ -glucopyranose configuration and the <sup>13</sup>C chemical shifts expected for 4-amino-4,6-dideoxy- $\alpha$ -glucopyranoside, thus confirming that the Rv3402c product is dTDP-4-amino-4,6-dideoxy- $\alpha$ -D-glucopyranose.

As noted in the introduction, previous biochemical studies have utilized a transposon library made in *M. tuberculosis* H37Rv to determine the genetic requirements for mycobacterial growth *in vitro* and for bacterial survival during infection.<sup>22</sup> Both Rv0334 and Rv3464 (Scheme 1) were found to be essential for growth, which is consistent with their conservation across *Mycobacterium* spp. and with their role in the synthesis of L-rhamnose, an essential component of the mycobacterial cell envelope. Rv3402c and Rv3404c, however, were found to be nonessential for both *in vitro* and *in vivo* growth. It should be noted that Rv3402c and Rv3404c have a more restricted distribution than Rv0334 and Rv3464, being absent in rapidly growing *Mycobacterium* spp. as well as in *M. leprae*, *M. simiae*, and *M. kansasii*. Rv3402c and Rv3404c are conserved within the *M. tuberculosis* complex (Table II), except for strains with RD16 (region of difference 16) such as *M. bovis* BCG-Moreau where there is a deletion going from Rv3400 to Rv3405c.<sup>23</sup> A nonsense mutation is observed in Rv3402c from lineages 5- and 6- as well as in animal lineages of the *M. tuberculosis* complex, causing it to be present as a pseudogene in these strains.<sup>24</sup> Notably

a significant homologue (71–75% identity) of Rv3402c is found in the *M. avium* complex whereas Rv3404c is absent in that group of strains.

While it is not possible to conclude if the presence of Rv3402c and/or Rv3404c contributes to specific clinical presentations, there are a few studies suggesting that they have a role in determining the extent of virulence. Indeed, the *rv3402c* gene has been shown experimentally to be regulated by IdeR, an iron-responsive DNA-binding protein known to control iron acquisition.<sup>5</sup> Notably, the mRNA level of the *rv3402c* gene was induced 17-fold in cultures of *M. tuberculosis* starved for iron, and it was induced 25-fold during infection of human THP-1 macrophages. Although Rv3402c clearly does not play a role in iron metabolism, it was postulated that *M. tuberculosis* might alter its membrane structure under iron-limiting conditions such as those found during intracellular growth or infection. Rv3402c was also found to enhance mycobacterial survival within macrophages and to modulate host pro-inflammatory cytokine production.<sup>6</sup> Indeed, heterologous expression of the *rv3402c* gene in the non-pathogenic *M. smegmatis* strain showed that Rv3402c enhanced its intracellular survival in human and mice macrophages.

Importantly, the gene encoding Rv3404c was identified as one of 56 *M. tuberculosis* genes preferentially expressed in mouse lung in an investigation using a promoter trap strategy.<sup>25</sup> This regulation may indicate a role for growth or persistence in that particular environment. In another study, McAdam *et al.*<sup>26</sup> generated insertional mutants in 351

**Table II.** Summary of the Occurrence of dTDP-4-Formamido-4,6-Dideoxy-D-Glucose Biosynthetic Genes in *Mycobacterium* spp

ORF# <sup>a</sup>	Activity	Rapidly growing <i>Mycobacterium</i> spp.	<i>M. tuberculosis</i> complex	Comments
Rv0334	Glucose-1-phosphate thymidyltransferase	Conserved	Conserved	Essential for growth <sup>b</sup>
Rv3464	dTDP-D-glucose 4,6-dehydratase	Conserved	Conserved	Essential for growth <sup>b</sup>
Rv3402c	4-aminotransferase	Absent	Conserved	Impact on pathogenicity <sup>c</sup>
Rv3404c	4-N-formyltransferase	Absent	Conserved	Impact on pathogenicity <sup>d</sup>

<sup>a</sup> According to *M. tuberculosis* H37Rv.

<sup>b</sup> Ref. 22.

<sup>c</sup> Ref. 6.

<sup>d</sup> Ref. 26.

different ORFs of *M. tuberculosis* H37Rv in order to identify virulence factors. Strikingly, SCID mice infected with a mutant form of Rv3404c presented significant increases in survival times when compared to mice infected with the wild-type strain, thus identifying Rv3404c as a virulence candidate for further investigation. The *rv3404c* gene was also distinguished as one of 21 genes whose expression was able to discriminate between isoniazid-, thioacetamycin-, or triclosan-treated *M. tuberculosis*.<sup>27</sup> The exact role of Rv3404c in the response to drug treatment was not characterized, however.

The locations and structural characterizations of putative glycolipids or other glycoconjugates containing *N*-formylated sugars in *M. tuberculosis* remain major experimental challenges due to the complexity of the cell wall envelope. Nevertheless, the conservation of the genes involved in the bioproduction of dTDP-4-formamido-4,6-dideoxy-D-glucose in the *M. tuberculosis* complex and their impact on pathogenicity certainly support the targeting of this biosynthetic pathway as a strategy to develop novel anti-tuberculosis therapeutics.

## Materials and Methods

### Cloning, expression, and purification of Rv3402c

The gene encoding Rv3402c from *M. tuberculosis* H37Rv was synthesized by Integrated DNA Technologies using codons optimized for protein expression in *E. coli*. The gene was placed into plasmid pKLD116, a pET31b derivative containing a His<sub>6</sub>-tagged maltose-binding protein (MBP) followed by a TEV protease cleavage site.<sup>28</sup> The pKLD116-*rv3402c* plasmid was utilized to transform Rosetta2(DE3) *E. coli* cells (Novagen). The cultures were grown in lysogeny broth supplemented with ampicillin and chloramphenicol (100 and 25 mg/L concentration, respectively) at 37°C with shaking until an optical density of 0.9 was reached at 600 nm. The flasks were cooled in ice water, and the cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and allowed to express protein at 21°C for 20 h.

The cells were harvested by centrifugation and frozen as pellets in liquid nitrogen. The pellets were subsequently disrupted by sonication on ice in a lysis buffer composed of 50 mM sodium phosphate, 20 mM imidazole, 20% ethylene glycol, and 300 mM sodium chloride, pH 8.0. The lysate was cleared by centrifugation, and MBP-Rv3402c was purified at 4°C utilizing nickel nitrilotriacetic acid resin (Qia-gen) according to the manufacturer's instructions. All buffers were adjusted to pH 8.0 and contained 50 mM sodium phosphate, 300 mM sodium chloride, and imidazole concentrations of 25 mM for the wash buffer and 250 mM for the elution buffer. The column was first washed in buffer containing 20% ethylene glycol and subsequently washed with buffer

lacking it. The protein was pooled and mixed in a 30:1 molar ratio (MBP-Rv3402c protein:TEV protease) based on a calculated extinction coefficient for the MBP-Rv3402c protein of 0.76 (mg/mL)<sup>-1</sup>cm<sup>-1</sup>. The recombinant protein was allowed to digest at 4°C for 48 h. Uncleaved protein, His<sub>6</sub>-MBP and the TEV protease were removed by two passages over Ni-nitrilotriacetic acid resin. Cleaved protein was collected and dialyzed against 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl at 4°C and concentrated to ~6 mg/mL based on a calculated extinction coefficient of 0.93 (mg/mL)<sup>-1</sup> cm<sup>-1</sup>.

### Site-directed mutagenesis

The K227A variant of MBP-Rv3402c was generated via the QuikChange method of Stratagene. It was expressed and purified as described for the wild-type MBP-Rv3402c construct.

### Enzymatic assay

Reactions containing 50 mM HEPES (pH 8.0), 4 mM dTDP-D-glucose, 0.1 mM PLP, 40 mM sodium glutamate, and 0.5 mg/mL *E. coli* RmlB (a 4,6-dehydratase) were set up with or without 1 mg/mL Rv3402c or the K227A variant and allowed to incubate for 24 h at room temperature. The reactions were stopped by removing the enzyme(s) via filtration through a 30 kDa cutoff filter. The mixtures were diluted 30× with water and examined by HPLC. Initially, small samples of the mixtures were examined with a 12-column volume, 0.0–1.0 M gradient of ammonium acetate (pH 4.0) on a 1 mL Resource-Q column. The reaction mixture containing Rv3402c demonstrated a decrease in the ketosugar (retention volume of 14.8 mL) concomitant with the appearance of a peak at 9.2 mL (approximately 300 mM ammonium acetate), corresponding to the expected dTDP-aminosugar. The reaction lacking Rv3402c resulted in no decrease in the peak at 14.8 mL. dTMP contamination was present in both samples (retention volume of 10.5 mL). Subsequently, the remainder of the Rv3402c reaction mixture was separated on a 6 mL Resource-Q column using the same gradient described above. Fractions containing the expected dTDP-aminosugar were pooled and lyophilized for <sup>1</sup>H NMR analysis. The required RmlB enzyme for the assay was purified in the laboratory.

### Sugar product analysis

Prior to lyophilization of the dTDP-aminosugar, a 300 μL sample was removed for mass spectrometry analysis. The identity of the Rv3402c product was confirmed as a dTDP-aminosugar by electrospray ionization mass spectrometry in the negative ion mode (Mass Spectrometry/Proteomic Facility at the University of Wisconsin).

## NMR spectroscopy

NMR spectra ( $^1\text{H}$ , gCOSY, TOCSY,  $^1\text{H}$ - $^{13}\text{C}$  HSQC,  $^1\text{H}$ - $^{31}\text{P}$  HSQC) were recorded using a Bruker AVANCE III 600 MHz spectrometer at 25°C in  $\text{D}_2\text{O}$ . Spectra were referenced to an internal acetone standard ( $\delta\text{H}$  2.23 ppm and  $\delta\text{C}$  31.45 ppm).

## Conflict of Interest

The authors have no competing financial interests.

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