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***Mycobacterium tuberculosis* gene Rv2136c is dispensable for acid resistance and virulence in mice**

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Summary

The gene *Rv2136c* is annotated to encode the *Mycobacterium tuberculosis* (*Mtb*) homologue of *Escherichia coli*'s undecaprenyl pyrophosphate phosphatase. In previous work, a genetic screen of 10,100 *Mtb* transposon mutants identified *Rv2136c* as being involved in acid resistance in *Mtb*. The *Rv2136c*:Tn strain was also sensitive to sodium dodecyl sulfate, lipophilic antibiotics, elevated temperature and reactive oxygen and nitrogen intermediates and was attenuated for growth and persistence in mice. However, none of these phenotypes could be genetically complemented, leading us to generate an *Rv2136c* knockout strain to test its role in *Mtb* pathogenicity. Genetic deletion revealed that *Rv2136c* is not responsible for any of the phenotypes observed in the transposon mutant strain. An independent genomic mutation is likely to have accounted for the extreme attenuation of this strain. Identification of the mutated gene will further our understanding of acid resistance mechanisms in *Mtb* and may offer a target for anti-tuberculosis chemotherapy.

Keywords

Mycobacterium; undecaprenyl pyrophosphate phosphatase; acid resistance

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is the most common cause of death worldwide due to a single bacterial pathogen, killing approximately two million people each year.¹ In the host, *Mtb* is found primarily within phagosomes of macrophages, where it encounters a relatively hostile environment. During the initial stages of infection, *Mtb* is capable of arresting phagosome fusion with acidic and hydrolytic lysosomes, allowing *Mtb* to reside in a mildly

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acidic environment (pH ~6.5) within non-activated macrophages.² However, this block in fusion is overcome upon macrophage activation by interferon gamma, resulting in acidification of the phagolysosome to pH ~4.5.^{3,4} The acidic, hydrolytic environment of the phagolysosome may eradicate some microorganisms. However, *Mtb* is not sterilized in macrophages and can persist in a non-replicative state indefinitely.

Although many acid resistance mechanisms have been elucidated for enteric pathogens that encounter extremely acidic environments, such as the stomach, little is known about *Mtb* and its mechanisms to resist phagolysosomal acidity. Few acid sensitive *Mtb* mutants have been identified. *Mtb* deficient in MgtC, a putative magnesium transporter, is attenuated for growth *in vitro* under mildly acidic conditions with low Mg²⁺ concentrations.⁵ *Mtb* mutants lacking OmpA_{tb}, an acid-dependent porin, are sensitive to low pH conditions *in vitro* and are attenuated in macrophages and mice.⁶ In a screen of 10,100 *Mtb* transposon mutants for impaired ability to recover from acid stress, 21 mutants were found to be hypersusceptible at pH 4.5.⁷ Killing of *Mtb* at pH 4.5 was largely dependent on the medium used. Most of the mutants only displayed enhanced susceptibility in 7H9 medium containing albumin and the dispersal agent Tween 80. At low pH, both Tween 80 and albumin can release fatty acids, which are toxic to *Mtb*.⁸ When a sodium phosphate-citrate buffer was used, albumin was omitted and Tween 80 was replaced with Tyloxapol, wild-type *Mtb* exhibited prolonged survival at pH 4.5. Of the 21 mutants, only two, *Rv3671c*:Tn and *Rv2136c*:Tn, remained acid sensitive under these conditions. Both strains were unable to maintain intrabacterial pH (pH_{IB}) homeostasis *in vitro* and within activated macrophages. Moreover, both mutants were also severely attenuated in mice, raising the possibility that pH_{IB} homeostasis is essential for *Mtb*'s growth and persistence *in vivo*. Studies are ongoing on the role of *Rv3671c*, a transmembrane serine protease, in acid resistance. In contrast, studies of *Rv2136c* have been delayed by prolonged and unsuccessful efforts to complement the phenotype of the transposon mutant with a wild-type copy of *Rv2136c* and/or members of the operon of which it is a part (O. Vandal, C. Darby, S. Ehrt, unpublished observations).⁷

Rv2136c is annotated as the *Mtb* homolog of the *Escherichia coli* *uppP* (also known as *bacA*), an undecaprenyl pyrophosphate phosphatase involved in synthesis of cell wall polymers including peptidoglycan, lipopolysaccharides, and teichoic acids. UppP dephosphorylates undecaprenyl pyrophosphate to generate undecaprenyl phosphate, the lipid carrier of biosynthetic intermediates for cell wall components. *E. coli* expresses four UppPs and a quadruple deletion mutant exhibits growth defects and abnormal cell morphology.⁹ *Staphylococcus aureus* and *Streptococcus pneumoniae* mutants lacking *uppP* are more susceptible than wild-type to bacitracin, an antibiotic that functions through sequestration of undecaprenyl pyrophosphate, and show reduced virulence in a mouse model of infection.¹⁰ Deletion of the *E. coli* *uppP* homolog in the non-pathogenic *Mycobacterium smegmatis* results in impaired biofilm formation as well as attenuated smegma development in an *in vivo* model.¹¹ The role of UppP in pathogenic mycobacteria has apparently not been explored.

If indeed the gene product of *Rv2136c* functions as a UppP, its disruption by transposon insertion could potentially result in altered membrane integrity, thereby rationalizing this strain's sensitivity to acid as well as to sodium dodecyl sulfate (SDS), lipophilic antibiotics, elevated temperatures, and reactive oxygen and nitrogen intermediates.¹² A *Streptococcus mutans* strain disrupted in *dgg*, an undecaprenyl kinase, was sensitive to acidic conditions, providing evidence that membrane integrity is important for acid resistance.¹³ Membrane modifications can also result in enhanced susceptibility to other host stresses. A known *Mtb* cell wall mutant disrupted in *lysX*, which exhibits altered lysinylation of phospholipid production, is attenuated in mice, with a 1 log₁₀ reduction in lung colony forming units (CFUs) at day 28.¹⁴ The *Rv2136c*:Tn mutant mouse phenotype was more severe than that of

the *lysX* mutant, with 2 log₁₀ reduction in lung CFUs by day 21 and no CFUs recovered at day 56 post infection, even though the *Rv2136c*:Tn mutant grows normally under replicating conditions *in vitro*.¹² Because none of the observed phenotypes of *Rv2136c*:Tn could be complemented by a wild-type copy of *Rv2136c* in *trans*, we sought to determine whether disruption of *Rv2136c* is responsible for the pleiotropic phenotypes and extreme attenuation seen in this strain. If not, it would be appropriate to launch a search for another *Mtb* gene that is presumably disrupted in this strain and that is conditionally essential during infection of the mouse.

2. Materials and methods

2.1. Strains and media

All strains were cultivated in Middlebrook broth 7H9 at pH 6.6 with 0.2% glycerol, 0.2% dextrose, 0.5% bovine serum albumin, 0.085% NaCl and 0.05% Tween-80 (7H9) or on Middlebrook 7H11 agar containing 0.5% glycerol and 10% OADC (oleic acid, albumin, dextrose, catalase supplement) (Difco). For pH_{IB} and acid sensitivity measurements, bacteria were washed and resuspended in sodium phosphate citrate buffer pH 4.5 with 0.02% Tyloxapol (Pcit-Tyl-4.5). Hygromycin 50 µg/mL and streptomycin 25 µg/mL were included when required for selection. The complemented strain contains a plasmid with a constitutively active *hsp60* promoter driving a wild-type copy of the *Rv2136c* gene, an *attB* site for chromosomal integration, and a streptomycin resistance cassette.

2.2. Mutant construction

Δ*Rv2136c* was constructed via allelic exchange using a specialized transducing phage phAE87.¹⁵ Briefly, ~500 bp fragments containing the upstream and downstream regions of the *Rv2136c* locus were amplified by PCR and cloned into pJSC284-loxP to flank the hygromycin resistance cassette. The plasmid was digested with PacI and packaged into the temperature-sensitive phage phAE87. The phage was amplified in *M. smegmatis* at 30 °C and used to infect *Mtb* as described.¹⁶

2.3. Southern blot analysis

To confirm deletion of *Rv2136c*, genomic DNA from wild-type H37Rv and knockout candidates was digested with AflIII, separated by agarose gel electrophoresis, and transferred to a nylon membrane that was probed with a ~ 500 bp fragment containing the upstream gene *Rv2137c*. Upon exposure with the ECL Direct Nucleic Acid Labeling and Detection System (Amersham) a 3.5 kb band for wild-type *Mtb* and a 5.3 kb band for *Rv2136c*-deficient *Mtb* would result.

2.4. Real-time PCR

Mtb cultures were grown in 7H9 medium to an optical density (OD_{580 nm}) of 2 and an equal volume of buffer containing guanidinium thiocyanate (4 M), sodium lauryl sulfate (0.5%), trisodium citrate (25 mM), and 2-mercaptoethanol (0.1 M) was added. Cultures were pelleted, resuspended in TRIzol, and bead beaten three times. RNA was extracted and qRT-PCR was performed using gene-specific Taqman probes and primers for *sigA* and *Rv2136c* (Biosearch Technologies).

2.5. pH_{IB} measurements

All *Mtb* strains were transformed with a pH-sensitive ratiometric GFP plasmid to monitor pH_{IB} and selected using hygromycin. Cultures were grown to mid log phase in 7H9, washed twice, resuspended in Pcit-Tyl-4.5 to an OD_{580 nm} = 0.1 (~5 × 10⁷ CFU/mL). At 2 and 6 days, the fluorescence of each well was read on a Molecular Dynamics SpectraMax M5

spectrofluorometer at excitation 395 nm, emission 510 nm and at excitation 475 nm, emission 510 nm. pH_{IB} was measured using the ratio of reading 1 (excitation 395 nm) to reading 2 (excitation 475 nm) and converting to pH_{IB} in reference to a calibration curve.

2.6. Measurement of acid sensitivity

Mid-log phase cultures were washed with Pcit-Tyl-4.5 buffer and centrifuged at 120 g for 10 minutes. Single cell suspension were adjusted to an OD₅₈₀ of 0.1 and incubated at 37 °C. After 2 and 6 days, samples were serially diluted and plated on 7H11 agar. CFUs were enumerated after 2 weeks.

2.7. Measurement of sensitivity to SDS

Mtb strains were grown to mid-log phase, a 10-fold dilution series was made from OD_{580 nm} of 0.1 to 0.001, and 5 µL was spotted onto 7H11 agar plates containing OADC with or without 0.0085% SDS.

2.8. Mouse infection

C57BL/6 8-week-old female mice (Jackson Laboratories) were infected by aerosol using a Middlebrook inhalation exposure system (Glas-Col). Early-log-phase *Mtb* cultures were aerosolized as single-cell suspensions in PBS to deliver ~100-200 CFU per mouse. Serial dilutions of lung homogenates from four mice per data point were plated onto 7H11 agar plates to quantify CFUs..

3. Results

3.1. Generation of an *Mtb* strain lacking *Rv2136c*

Our inability to complement the phenotypes observed in the *Rv2136c*:Tn mutant led us to generate an *Mtb* knockout strain, $\Delta Rv2136c$, in which the *Rv2136c* ORF was deleted and replaced with a hygromycin resistance cassette (Figure 1A). The first 20 base pairs and the last 48 base pairs of *Rv2136c* were retained in the knock out construct in an effort to maintain expression of the downstream gene *Rv2135c*, which is in a putative operon with *Rv2136c*. Deletion of *Rv2136c* was confirmed by Southern blot (Figure 1B) and quantitative real-time PCR (Figure 1C). Generation of this mutant confirms that *Rv2136c* is non-essential *in vitro*, as predicted by TraSH analysis.¹⁷

3.2. *Rv2136c* is not responsible for acid or SDS sensitivity phenotype

We initially examined whether deletion of *Rv2136c* resulted in sensitivity to acid and SDS, as was observed in the *Rv2136c*:Tn mutant. We transformed $\Delta Rv2136c$ and *Rv2136c*:Tn with a plasmid expressing a pH-sensitive ratiometric GFP. This pHGFP differentially fluorescences at two excitation wavelengths depending on the proton milieu, thus allowing for the non-invasive measurement of pH_{IB} over a dynamic range of pH 5.5 to 8.5. $\Delta Rv2136c$ maintained pH_{IB} and viability after 2 and 6 days at pH 4.5, and was indistinguishable from wild-type *Mtb* in these respects. In contrast, *Rv2136c*:Tn was unable to maintain pH_{IB} and exhibited a 1 log₁₀ reduction in CFUs after 2 days at pH 4.5 (Figure 2A and B). Also, $\Delta Rv2136c$ was not hypersensitive to SDS, while the *Rv2136c*:Tn mutant was attenuated for growth in the presence of 0.0085% SDS (Figure 2C). Additional phenotypes of *Rv2136c*:Tn, including sensitivity to heat (45 °C) and to various lipophilic antibiotics, were also not observed in $\Delta Rv2136c$ (data not shown).

3.3. *Rv2136c* is dispensable for *Mtb*'s virulence in mice

Although *Rv2136c* does not appear to be involved in acid resistance mechanisms, we sought to determine if it contributed to *Mtb*'s virulence. After infection of mice, $\Delta Rv2136c$

exhibited the same logarithmic growth and subsequent persistence as wild-type (H37Rv) *Mtb* (Figure 2D). This is in contrast to the *Rv2136c*:Tn strain, which was highly attenuated for growth and persistence in the mouse model of infection.¹² Therefore, *Rv2136c* is not essential for establishment or persistence of infection in mice.

4. Discussion

In this study, construction and characterization of an *Rv2136c*-deficient *Mtb* strain allowed us to establish that *Rv2136c* does not contribute to acid resistance in *Mtb* and is not required for virulence in mice.

The previous report that initially characterized *Rv2136* in *Mtb* described a transposon mutant strain wherein the transposon inserted within the last 80 nucleotides of the *Rv2136c* gene. This proximity to the 3' end of the gene may lead to the production of a truncated protein with different functions, a dominant negative dysfunctional protein, or no protein at all. In an attempt to implicate *Rv2136c* in the phenotypes observed, the authors tried to complement the defects, but were unsuccessful in doing so.⁷ The inability to complement the *Rv2136c*:Tn phenotypes by supplying a wild-type copy of *Rv2136c* in *trans* indicated that *Rv2136c* may not be involved in acid resistance. By constructing a genetic knockout strain, we have confirmed this. The absence of parallel phenotypes among *Rv2136c*:Tn and $\Delta Rv2136c$ suggests that a genomic mutation independent of the transposon insertion is responsible for the severe attenuation of this strain.

In addition to its dispensability towards acid resistance, *Rv2136c* is also not required for the virulence of *Mtb*. Although *Rv2136c* is the only *Mtb* gene with amino acid similarity to the *E. coli* UppP, additional UppP(s) may exist that can compensate for the loss of *Rv2136c*. The majority of *E. coli*'s UppP activity is attributed to BacA; however, three additional UppPs (YbjG, PgpB, and YeiU) have been identified, each having unique substrate specificities.^{18,19} Similar redundancy in UppP activity may exist in *Mtb* and the importance of each enzyme may depend on the environmental conditions encountered by the bacterium. Enzymatic studies are required to determine if *Rv2136c* does in fact function as a UppP.

There could be several explanations for the defects in the *Rv2136c*:Tn strain. Polar effects of the transposon on other genes in the *Rv2136c* operon are unlikely to have caused the phenotype, as the transposon mutant was not complemented even with the entire operon. Quantitative real-time PCR was performed on the *Rv2136c*:Tn strain to evaluate levels of *Rv2135c* transcript. We did not detect expression of *Rv2135c* in the *Rv2136c*:Tn strain, although it was readily detectable in wild type *Mtb* (data not shown). Complementation of *Rv2136c*:Tn strain with a construct containing *Rv2136c*, *Rv2135c*, *Rv2134c*, and *Rv2133c* resulted in over-expression of *Rv2135c*. Given that the phenotypes of these two *Rv2136c*:Tn strains were identical whether *Rv2135c* was undetectable or over-expressed, it appears that *Rv2135c* is not involved in the observed phenotypes. Generation of a dominant negative protein was another unlikely explanation, as transformation of wild-type *Mtb* with a plasmid encoding the putative truncated protein conferred no phenotype (data not shown). Most likely, the phenotype is due to an independent mutation at another locus. We have confirmed that there is no mutation within the *Rv3671c* gene, eliminating the possibility that a mutation in at least one gene known to control acid sensitivity could produce the observed phenotypes in the *Rv2136c*:Tn strain (data not shown). We are currently sequencing the *Rv2136c*:Tn genome to identify genetic mutations that may account for the acid sensitivity and the extreme attenuation in mice.

In conclusion, we do not think Rv2136c itself warrants further attention as a potential target for TB drug discovery. However, the product of the gene responsible for acid sensitivity could prove to be an attractive target for chemotherapy.

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Figure 1. Generation of *Rv2136c*-deficient *Mtb*

(A) Genomic region of wild-type *Rv2136c* and design of disrupted allele. Dashed lines indicate AflIII restriction products.

(B) Southern blot of AflIII restricted DNA from wild-type (WT) and $\Delta Rv2136c$ (KO) strains.

(C) Level of *Rv2136c* transcripts assessed by quantitative Real-time PCR in WT and KO strains. Results show means $\pm$ SEM of one sample from three individual experiments. Samples in which transcript was not detected were labeled n. d.

Figure 2. *Rv2136c* is dispensable for acid and SDS resistance and for virulence in mice
 (A) Intrabacterial pH (pH_{IB}) measurements of wild-type (H37Rv) and mutants after 2 and 6 days post exposure to phosphate citrate buffer plus tyloxapol pH 4.5. Means ± SEM of triplicate experimental samples are shown from one experiment, representative of two.
 (B) Survival of wild-type and mutant strains after 2 and 6 days post exposure to pH 4.5. Means ± SEM of triplicate experimental samples are shown from one experiment, representative of two.
 (C) Sensitivity of wild-type and mutant bacteria to 0.0085% SDS. Data are representative of results from two independent experiments.
 (D) Growth and persistence of wild-type, Δ*Rv2136c*, and Δ*Rv2136c* Complemented (COMP) strains in mice. Data are means ± SEM from four mice per time point and group and are the results of one experiment.