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Identification of a Novel Mycobacterial 3-Hydroxyacyl-Thioester Dehydratase, HtdZ (Rv0130), by Functional Complementation in Yeast▽

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We report on the identification of *Mycobacterium tuberculosis* HtdZ (Rv0130), representing a novel 3-hydroxyacyl-thioester dehydratase. HtdZ was picked up by the functional complementation of *Saccharomyces cerevisiae htd2Δ* cells lacking the dehydratase of mitochondrial type II fatty acid synthase. Mutant cells expressing HtdZ contained dehydratase activity, recovered their respiratory ability, and partially restored de novo lipoic acid synthesis.

The availability of the complete genome sequence of *Mycobacterium tuberculosis* (6) has substantiated the presumption that this organism contains all of the components of the type II fatty acid synthase (FASII) system (16). The latest FASII enzyme to be identified is 3-hydroxyacyl-acyl carrier protein dehydratase, represented by heterodimers of Rv0635-Rv0636 HadAB and Rv0636-Rv0637 HadBC (5, 15) but potentially also by Rv0130 (11) and Rv0098 (16). Here, we have exploited *Saccharomyces cerevisiae* as a surrogate host for expressing the latter two mycobacterial proteins (8). The yeast *htd2Δ* mutant used is devoid of the mitochondrial FASII enzyme 3-hydroxyacyl-thioester dehydratase Htd2p and therefore lacks the corresponding activity, contains abnormally small mitochondria, fails to assemble respiratory complexes or produce sufficient levels of lipoic acid, and is exclusively fermentative (12). Mutant cells expressing ectopic Rv0130 or Rv0098 were examined for hydratase activity, growth on glycerol medium, the assembly of cytochrome complexes, respiration, and lipoic acid production. We discuss our findings with reference to the identification of a novel *M. tuberculosis* FASII-like 3-hydroxyacyl-thioester dehydratase.

To assess whether these two mycobacterial proteins can compensate in vivo for the missing dehydratase activity in mitochondria of the yeast BY4741*htd2Δ* mutant strain (EUROSCARF), they were expressed as fusion constructs that were preceded by the Coq3p mitochondrial leader sequence (mit-Rv0130 and mit-Rv0098), which was demonstrated previously to be sufficient for targeting proteins to yeast mitochondria (10). As controls, isogenic *htd2Δ* cells were transformed with plasmids expressing either native mitochondrial Hdt2p (12) (mit-Hdt2p) or peroxisomal catalase A (per-Cta1p) (7). All four constructs were tethered behind the yeast oleic acid-inducible *CTA1* promoter on multicopy plasmids. The four transformants were propagated overnight in synthetic glucose medium lacking uracil for plasmid maintenance, and following adjustment of the cell concentration to an A_{600} of 0.5 and serial 10-fold dilution, cells were spotted onto solid 2% glucose or 3% glycerol medium, and the plates were incubated at 30°C until single colonies were detectable.

The results shown in Fig. 1 demonstrated that *htd2Δ* mutant cells expressing mit-Rv0130 resembled the self-complemented strain producing mit-Htd2p, since they could grow on glycerol as the sole carbon source. On the other hand, cells expressing mit-Rv0098 resembled the strain enriched for per-Cta1p in that they were completely incapable of growth on this medium. Therefore, we were able to show here for the first time that when expressed within the context of a heterologous FASII framework, Rv0130 can function as a physiological 3-hydroxyacyl-thioester dehydratase.

The recovery of *htd2Δ* cells from their respiratory deficiency phenotype by complementation is accompanied by the restoration of assembled cytochrome complexes and the regeneration of the electron transport chain (12). Hence, the above-mentioned yeast strains were monitored spectrophotometrically for cytochrome assembly using the cold-temperature cuvette method (13). The results presented in Fig. 2 showed that *htd2Δ* cells expressing mit-Rv0130 or mit-Htd2p were much more efficient than those expressing mit-Rv0098 or per-Cta1p at assembling cytochrome complexes c, c_1, b, a, and a_3. In addition, the application of 0.1% 2,4,5-triphenyltetrazolium chloride as a low-melting-temperature agarose overlay (3) to the four strains following growth on solid rich-glucose medium demonstrated that the former two strains were able to metabolize this compound to generate the red chromophore, whereas the latter two were not (not shown). The combined results showed that a mitochondrially localized Rv0130 can reverse the effects of the lesion caused by the *htd2Δ* mutation.

To characterize Rv0130 and Rv0098 biochemically, their enzyme activities were measured in yeast extracts. Following overnight propagation in liquid oleic acid medium, under which conditions the *CTA1* promoter driving the expression of the four open reading frames is highly induced, yeast cells from

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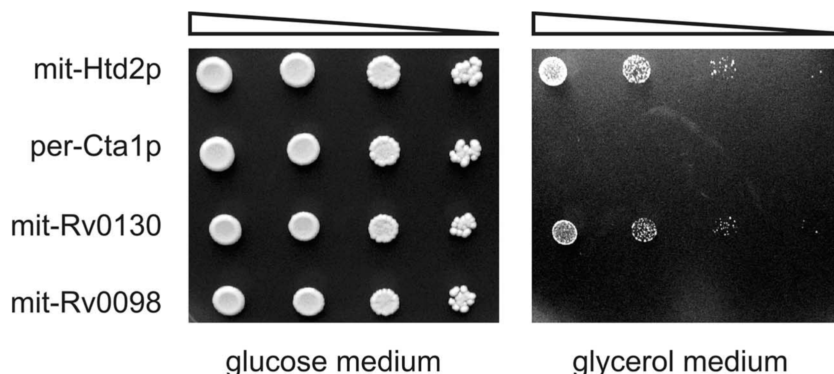

FIG. 1. Growth of *S. cerevisiae htd2*Δ mutants expressing *M. tuberculosis* Rv0130. Yeast *htd2*Δ cells expressing native mitochondrial 3-hydroxyacyl-thioester dehydratase (mit-Htd2p) (positive control), native peroxisomal catalase A (per-Cta1p) (negative control), mitochondrially targeted Rv0130 (mit-Rv0130), or mitochondrially targeted Rv0098 (mit-Rv0098) were applied to solid glucose or glycerol medium following a serial 10-fold dilution (◺).

three independent inductions were broken with glass beads, and their contents were examined for hydratase activity (14) using 2-*trans*-butyryl (crotonyl-)-, -hexenoyl-, and -decenoyl-coenzyme A (CoA) as substrates. The results of the enzyme assays showed that any potential hydratase activity in soluble protein extracts made from cells overexpressing either mit-Htd2p, per-Cta1p, or mit-Rv0098 was below the detection limit of the assay used, i.e., <0.05 nmol substrate metabolized per mg protein per min. On the other hand, soluble protein extracts produced from mutant cells overexpressing mit-Rv0130 contained a 3-hydroxyacyl-thioester hydratase activity equivalent to 1.81 ± 0.39 nmol/mg · min^{-1} (mean ± standard deviation) ($n = 3$) using 2-*trans*-butyryl-CoA as a substrate, 5.80 ± 0.49 nmol/mg · min^{-1} using 2-*trans*-hexenoyl-CoA, and 0.36 ± 0.07 nmol/mg · min^{-1} using 2-*trans*-decenoyl-CoA. Hence, in agreement with previously published data on the hydratase activity of recombinant Rv0130 (11), the overexpression of Rv0130 in mutant *htd2*Δ cells was coincidental with soluble protein extracts containing detectable levels of 3-hydroxyacyl-thioester hydratase activity.

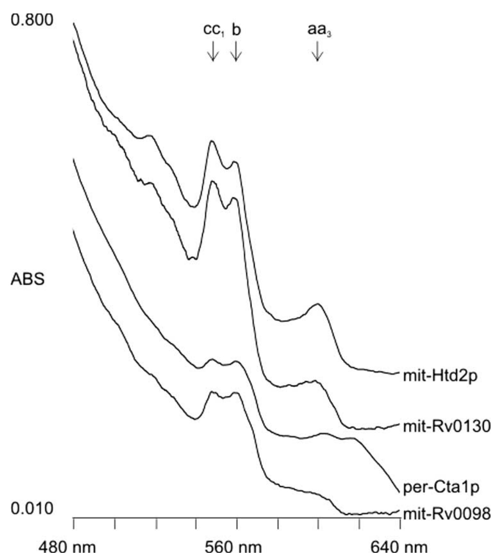

FIG. 2. Spectrophotometric assay for mitochondrial cytochrome complexes. Cell pastes from the indicated strains were collected from colonies grown on solid rich-glucose medium that were left for 4 days at room temperature and applied to the glass face of otherwise aluminum cold-temperature cuvettes that were immersed in liquid nitrogen prior to being placed into a spectrophotometer for analysis. The pastes were scanned at wavelengths of between 480 nm and 640 nm. Numbers to the left indicate units of absorbance (ABS); those at the bottom refer to wavelengths in nm. Peaks corresponding to known cytochromes are indicated (letters with arrows).

To link Rv0130 expression with fatty acid biosynthesis in yeast mitochondria, the production of lipoic acid (4, 9) was examined in the four strains, and the values reported represent an average of data for three independent bacterial growth responses. The results showed that extracts derived from *htd2*Δ mutant cells expressing mit-Htd2p supported a level of bacterial growth that was equivalent to 233 ± 5 ng lipoic acid per gram (wet weight) yeast cells (mean ± standard deviation) ($n = 3$), whereas those enriched for per-Cta1p supported only background-level growth corresponding to 10 ± 1 ng lipoic acid per gram. The production of mit-Rv0130 or mit-Rv0098 in these cells resulted in bacterial growth that correlated with 43 ± 4 and 11 ± 2 ng lipoic acid per gram, respectively. In a separate representative experiment for lipoic acid production, BY4741 wild-type cells contained 254 ng lipoic acid per gram (duplicates). Hence, the expression of mit-Rv0130 gave rise to a fourfold-greater amount of lipoic acid than did the expression of mit-Rv0098 or per-Cta1p. Since previous expressions of heterologous dehydratases in the *htd2*Δ mutant successfully corrected the mutant's defective respiration, including bacterial FabZ and FabA (12) as well as human (2) and *Trypanosoma brucei* (1) HTD2, we suggest that due to its similar success at restoring respiration to this mutant, Rv0130 represents a bona fide *M. tuberculosis* FASII-like 3-hydroxyacyl-thioester dehydratase, and we propose naming it HtdZ.

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