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Short Communication

Rhodococcus jostii RHA1 TadA-homolog deletion mutants accumulate less polyhydroxyalkanoates (PHAs) than the parental strain

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The Actinomycete bacterium *Rhodococcus jostii* RHA1 is capable of simultaneously accumulating multiple carbon storage compounds, which include polyhydroxyalkanoates (PHAs), triacyglycerols (TAGs) and glycogen (Hernandez et al., 2008). Accumulation of these storage compounds can be heavily influenced by the primary carbon growth substrate. Unbalanced growth conditions such as nutrient starvation (usually nitrogen) in the presence of excess carbon (Hernandez et al., 2008), normally induces an increased accumulation of these storage compounds. While many studies have examined either PHA or TAG accumulation in Actinomycete bacteria such as the *Rhodococcus* (Alvarez and Steinbuchel, 2002; Valappil et al., 2007), very little is known regarding the competition of these storage polymers for common metabolic precursors and/or cellular membrane components. For example, the biosynthesis of both PHAs and TAGs share the metabolite acetyl-CoA, and both of these storage polymers are synthesized as membrane-bound inclusion bodies (Alvarez and Steinbuchel, 2002; Valappil et al., 2007). Experimental evidence indicates that when RHA1 is grown on glucose, gluconate or acetate, TAGs represent the majority of storage compounds when compared to PHAs (Hernandez et al., 2008). Additional studies of *R. opacus* PD630 further demonstrated shifts in the composition of PHAs and TAGs during unbalanced growth that correlated with whether the cells were in an exponential or stationary phase (Alvarez et al., 2000). In contrast, a recent RHA1 genome-scale metabolic model attempted to predict the relative composition of the mixture of storage compounds, and concluded that PHAs represented the main storage pool when growing on glucose or acetate (Tajparast and Frigon, 2015).

To further explore these disparate observations and gain additional insights into PHA accumulation in the strain RHA1, we examined the effects of a putative TadA homolog (RHA1_ro02104) deletion on PHA accumulation under nitrogen rich, and nitrogen starved, growth conditions. *Rhodococcus* TadA mutants accumulate up to 30–40% fewer TAGs than the parental wild-type strains displaying defects in lipid body formation (Chen et al., 2014; Ding et al., 2012; MacEachran et al., 2010). Since the accumulation of both PHAs and TAGs manifest as intracellular inclusion bodies, we hypothesized that PHA accumulation in TadA mutants would also be reduced, suggesting that TadA is common to the biogenesis of both PHAs and TAGs.

An RHA1_ro02104 deletion was introduced into the strain RHA1 using a gene deletion construct consisting of the 500 bp flanking regions of RHA1_ro02104. The construct was synthesized (Celtek Biosciences) in the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). Gene deletion inserts liberated from pCR2.1 were subcloned into the BamHI site of the mobilizable plasmid pK18mob*sacB* (Schafer et al., 1994). Confirmation of the gene deletion was established via PCR analysis using the primers 5′ATCGCTCGCTGAGTCACG3′ and 5′GATGGCAAGCGATGATCGC3′. An expected band size differential of ~800 bp was observed between the wild-type (1841 bp) and mutant strains (1010 bp) (data not shown). The deletion construct was introduced into the strain RHA1 based on a conjugation strategy using a *sacB* counter selectable marker (van der Geize et al., 2001) with the exception that transformed clones were selected by growing on 25 mg/mL kanamycin and 15 mg/L nalidixic acid. DNA sequencing analysis using an ABI 3100 Genetic Analyzer (Life Technologies, Grand Island, NY USA) and Big Dye® Terminator v 3.1 Cycle Sequencing kit (Life Technologies #4337455) further confirmed deletion of RHA1_ro02104 (data not shown).

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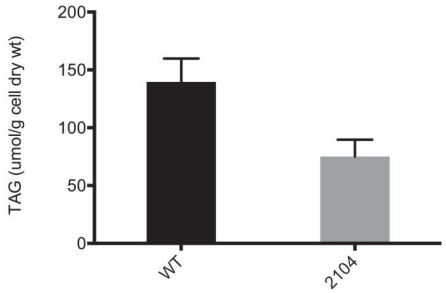

Fig. 1. Physiological confirmation of the RHA1_ro02104 mutant phenotype (TAG accumulation deficient) was established by subjecting the both the wild-type (black), and mutant (gray), strains to nitrogen starvation conditions in the presence of excess carbon (described in text) and quantifying the total TAGs per dry weight cell mass.

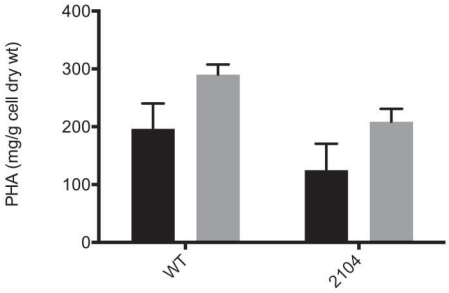

Fig. 2. A comparison of PHA accumulations under nitrogen limiting and nitrogen excess in the wild-type strain compared to the RHA1_ro02104 mutant.

PHA and total RNA samples for transcriptome studies were harvested following 48 hours of nitrogen starvation. Black bars: +N, Gray bars: –N.

Consistent with previous *Rhodococcus* TadA mutants (Chen et al., 2014; MacEachran et al., 2010), the RHA1_ro02104 mutant produced significantly less TAG (~46%) under nitrogen starvation conditions (Fig. 1). For this, and subsequent studies, *R. jostii* RHA1 was grown in M9 medium supplemented with 10 mM NH_4Cl, 20 mM each of glucose and succinate, and 0.04% (w/v) glycerol and yeast extract at 30°C with continuous shaking at 180 rpm. After 48 h, cultures were centrifuged at 8,000 × *g* for 10 min at 4°C, washed 2X with M9 salts solution, and resuspended in M9 medium with one set of cultures not limited for nitrogen and the other one limited for nitrogen. Complete depletion of NH_4Cl from the media was verified by analysis with an Aquanal ammonium assay kit (Sigma-Aldrich Corp., St. Louis, MO, USA). Total TAG content as a function of dry cell weight was determined with an ABcam Triglyceride Quantification Kit (ABcam, Cambridge, MA, USA).

Following physiological and genetic and confirmation of the TadA phenotype, PHA accumulation was determined under nitrogen rich, and nitrogen starved, conditions in both the wild-type, and mutant, strains. PHAs were purified and quantified following a protocol based on the method of Law and Slepecky (Law and Slepecky, 1961). A 32% increase in PHAs was observed in the wild-type strain at 48 h when comparing nitrogen starved, with nitrogen rich, growth conditions (Fig. 2). Similarly, a 40% increase in PHAs was observed in the mutant strain at 48 h when comparing nitrogen starved, with nitrogen rich, growth conditions. However, 28% and 36% decreases in the overall PHA content were observed at 48 h when comparing the RHA1_ro02104 mutant with the wild-type strain grown under nitrogen limiting, and nitrogen rich, conditions, respectively. Interestingly, when sacrificial samples were harvested at 72 h, differences in PHA accumulations between the mutants at all growth conditions were not significant (data not shown). Similarly, studies in *R. ruber* indicate that PHA accumulation peaks in the early stationary phase and then wanes as the cells age (Alvarez et al., 2000).

Table 1. Select nitrogen assimilation genes up regulated in response to nitrogen starvation in wild-type RHA1.

Locus	Function	Fold change	*P* value
RHA1_ro00573	glutamate dehydrogenase	5.4	0.0333
RHA1_ro00862	nitrite reductase large subunit	163.0	0.0000821
RHA1_ro00863	nitrite reductase small subunit	102.2	0.00092
RHA1_ro01815	sarcosine dehydrogenase	8.8	0.0117
RHA1_ro01821	sarcosine oxidase subunit beta	10.9	0.0601
RHA1_ro01822	sarcosine oxidase subunit delta	12.7	0.0137
RHA1_ro01823	sarcosine oxidase	17.9	0.0151
RHA1_ro01824	sarcosine oxidase subunit gamma	11.0	0.00589
RHA1_ro01828	L-serine ammonia-lyase	34.4	0.00196
RHA1_ro02135	urea carboxylase	13.1	0.0000936
RHA1_ro02820	glutamate-ammonia ligase	5.1	0.00117
RHA1_ro04515	xanthine/uracil permease	66.4	0.000664
RHA1_ro05149	cytosine/purines, uracil, allantoin permease	10.2	0.00287
RHA1_ro05151	cytosine deaminase	4.6	0.0119
RHA1_ro06169	ornithine-oxo-acid transaminase	4.7	0.00168
RHA1_ro06277	L-lysine aminotransferase	12.4	0.00119
RHA1_ro06366	nitrite reductase large subunit	272.4	0.0000194
RHA1_ro06367	nitrite reductase small subunit	81.8	0.00276

Collectively, the physiological data above indicate that the RHA1_ro02104 mutant not only leads to reductions in TAG accumulation as shown previously, but also to reductions in PHA accumulation. This finding suggests that RHA1_ro02104 contributes to both the biogenesis of PHAs and TAGs. To gain additional insights into the molecular mechanisms behind these physiological observations, microarray experiments were carried out using samples from the experiments described above. Total RNA was extracted using the Qiagen RNeasy extraction kit following the manufacturer's protocol with the addition of bead beating (MP Biomedicals Fastprep 24 at 6.0 M/s for 30 s) prior to extraction with Qiagen Lysis tubes S. The Agilent One-Color Microarray Hybridization protocol with the Low Input Quick Amp kit was utilized for microarray hybridizations following the manufacturer's recommendations. Microarrays were analyzed using in-house Agilent Technologies, High-Resolution Microarray Scanner (Model G2505C, Agilent Technologies, Santa Clara, CA, USA) and Agilent's Feature Extraction software. Expression data across multiple transcriptomes were log2 transformed and statistical significance was determined by Moderated t test (*P* value = 0.05, four-fold induction). Pathway enrichment analysis of differentially expressed genes was determined using the online Biocyc analysis database and tools (http://biocyc.org/). Pathway enrichments were determined via the Fisher Exact algorithm with no corrections, *P* value < 0.1. The microarray

Table 2. Select differentially expressed genes involved in storage molecule synthesis/degradation in response to nitrogen starvation in wild-type RHA1.

Locus	Function	Fold change	*P* value
Upregulated genes			
RHA1_ro00054	acyl-CoA synthetase	4.9	0.0212
RHA1_ro00057	3-oxoacyl-ACP synthase	4.9	0.0077
RHA1_ro00058	3-oxoacyl-ACP synthase	8.8	0.00445
RHA1_ro02258	acyl-ACP desaturase	12.6	0.00375
RHA1_ro03244	acyl-CoA dehydrogenase	7.2	0.00645
RHA1_ro03422	fatty acid desaturase	63.7	0.00153
RHA1_ro04283	acyl-CoA dehydrogenase	4.6	0.0378
RHA1_ro04513	esterase	19.6	0.0064
Downregulated genes			
RHA1_ro01115	1-acyl-sn-glycerol-3-phosphate acyltransferase	5.9	0.0143
RHA1_ro01447	glycogen phosphorylase	5.3	0.000069
RHA1_ro01455	acetyl-CoA acetyltransferase	5.3	0.0282
RHA1_ro01538	enoyl-CoA hydratase	5.7	0.0147
RHA1_ro01932	acyl-CoA dehydrogenase	4.3	0.00498
RHA1_ro02121	3-hydroxybutyryl-CoA dehydrogenase	6.6	0.0356
RHA1_ro02340	3-oxoacyl-ACP reductase	4.0	0.0211
RHA1_ro04591	acyl-CoA dehydrogenase	5.3	0.0636
RHA1_ro05257	acetyl-CoA acetyltransferase	6.1	0.00204
RHA1_ro06006	esterase	6.4	0.0282
RHA1_ro06598	acetate-CoA ligase	11.0	0.000495
RHA1_ro06885	acyl-CoA thioesterase	6.7	0.0618

Table 3. Subset of RHA1 transcripts that were induced and repressed as a result of deletion of RHA1_ro02104.

Locus	Function	Fold change	*P* value
Nitrogen limitation			
up regulated			
RHA1_ro01602	probable esterase	5.4	0.00523
RHA1_ro02361	probable lipase	4.6	0.0594
RHA1_ro06647	phosphoesterase	5.5	0.0239
RHA1_ro06885	acyl-CoA thioesterase II	6.2	0.0108
RHA1_ro11078	possible acyl carrier protein	5.2	0.0012
down regulated			
RHA1_ro02306	possible long-chain-fatty-acid-CoA ligase	4.4	0.0453
RHA1_ro02708	long-chain-fatty-acid-CoA ligase	4.1	0.0461
RHA1_ro06074	2,4-dienoyl-CoA reductase	7.9	0.0215
RHA1_ro03213	3-oxoacyl-[acyl-carrier-protein] reductase	5.1	0.000334
RHA1_ro11022	fatty-acid-CoA ligase	5.3	0.00804
RHA1_ro02104	hypothetical protein	177.0	1.4E-08
RHA1_ro05077	probable CoA-transferase	5.2	0.00244
RHA1_ro05815	probable thiolase	5.4	0.0288
RHA1_ro05294	reductase	5.0	0.0447

data set obtained from this effort is available from the NCBI gene expression and hybridization array data repository (www.ncbi.nlm.nih.gov/geo) under accession GSE78828.

Transcriptome studies confirm the nitrogen-limited physiological state of both the wild-type and mutant cells. For example in the wild-type cells, significant increases in genes involved in nitrogen assimilation as well as multiple nitrogen generating catabolic processes such as creatinine, amino acid, nucleoside, and urea degradation were observed under all nitrogen-limited growth conditions (Table 1). A total of 194 differentially expressed genes were identified, of which 67 were increased in expression and 127 were decreased in expression under nitrogen starved conditions. Substantial increases in gene expression were observed for key nitrogen assimilation like nitrite reductase genes (Table 1). In some cases, nitrite reductase genes and the genes involved in creatinine degradation are arranged in an operon based on their contiguous proximity. In addition, multiple differentially expressed genes involved in the synthesis and consumption of pools of acetyl-CoA, acetoacetyl-CoA and 3-hydroxybutyryl-CoA, key metabolites for PHA and TAG synthesis, were identified (Table 2). Genes involved in both fatty acid oxidation (RHA1_ro00054, RHA1_ro03244, RHA1_ro04283) and fatty acid elongation (RHA1_ro00057 and RHA1_ro00058) were up-regulated, collectively providing acetyl-CoA substrates and activated fatty acid substrates for PHA biosynthesis and TAG biosynthesis, respectively. By contrast, there were a number

of down regulated genes observed involved in the generation of acetoacetyl CoA (RHA1_ro01455, RHA1_ro05257, RHA1_ro02121) and 3-hydroxybutyryl-CoA (RHA1_ro01538) key steps in the biosynthesis of PHAs. Additional genes involved in TAG biosynthesis (RHA1_ro01115) and glycogen degradation (RHA1_ro01447) were also down regulated. These findings provide some pathway-based insights for the differential PHA accumulation presented in Fig. 2, as well as for previous experimented evidence which demonstrate that RHA1 produces TAGs and, to a lesser extent, PHAs under nitrogen limiting conditions (Hernandez et al., 2008).

An enrichment analysis of the 50 differentially expressed genes in the nitrogen starved wild-type, versus the isogenic RHA1_ro02104 mutant strain identified, genes in the mutant involved in fatty acid and lipid biosynthesis (RHA1_ro03213, RHA1_ro05294, RHA1_ro02306, RHA1_ro11022, RHA1_ro02708, *P*-value 0.065)) as well as genes involved in acyl-CoA hydrolysis (RHA1_ro06885, *P*-value 0.071) and triacylglycerol degradation (RHA1_ro02361, *P*-value 0.097). However, with the exception of RHA1_ro06885, these genes, while functionally redundant, were different from those observed in the wild-type strain (Table 2). Interestingly, RHA1_ro06885, an acetyl-CoA producing gene, was up regulated in the RHA1_ro02104 mutant. In addition, a gene involved in the generation of acetoacetyl-CoA from acetate (RHA1_ro05815), a key step in PHA synthesis, was down regulated, providing at least a partial explanation for the reduced PHA accumulation observed in the mutant (Fig. 2). Transcriptome comparisons between the wild type and the mutant further confirmed the lack of expression of the deleted gene RHA1_02104 with a ~200-fold reduction in expression. Collectively, these observations indicate a significant redundancy and diversity in the number of genes and their associated expression that synthesize and consume cellular pools of acetyl-CoA, acetoacetyl-CoA and 3-hydroxybutyryl-CoA. As a result, additional sophisticated gene deletion studies combined with physiological and genetic studies and flux balance analysis based modeling will be required to better predict the relative composition of storage compounds in response to nitrogen starvation as a result of deletion of RHA1_ro02104 (Tajparast and Frigon, 2015).

In conclusion, we have examined the effects of a gene deletion of a TadA homolog (RHA1_ro02104) on PHA accumulation under nitrogen rich, and nitrogen starved, growth conditions. It was determined that RHA1_ro02104 mutants accumulate ~46% less TAGs than the parental wild-type strains. Therefore, we hypothesized, and subsequently demonstrated, that PHA accumulation in the RHA1_ro02104 mutant is also reduced suggesting that TadA shares a common role in the biogenesis of PHAs and TAGs. The mechanism by which TadA influences PHA accumulation is not known, but transcriptome data suggests a dynamic balance between pathways that synthesize and consume key metabolic precursors like acetyl-CoA, acetoacetyl-CoA and 3-hydroxybutyryl-CoA. Previous studies with TAGs further indicate that TadA is similar to apolipoproteins, structural proteins of plasma lipoproteins (Chen et al., 2014; Ding et al., 2012; MacEachran et al., 2010). TadA mutants were first identified in *R. opacus* PD630 during a genetic screen for transposon mutants that accumulate reduced amounts of TAGs. In contrast, overexpression of TadA resulted in the formation of larger lipid bodies (MacEachran et al., 2010). We speculated that TadA may work in concert with other proteins/molecules to mediate lipid body formation. Further *in vitro* analysis of TadA indicated that its C-terminus binds to heparin and facilitates the aggregation of lipids bodies. The authors further demonstrated *in vivo* that TadA C-terminal deletion mutants were no longer able to complement the mutant phenotype. As such, it is tempting to speculate that TadA serves a structural role as a downstream gatekeeper for the aggregation of PHAs and TAGs, and that a deletion in this gene could represent a partial bottleneck whereby upstream metabolic intermediates accumulate or are siphoned off to other cellular needs.

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References

Alvarez, H. M. and Steinbuchel, A. (2002) Triacylglycerols in prokaryotic microorganisms. *Appl. Microbiol. Biotechnol.*, **60**, 367–376.

Alvarez, H. M., Kalscheuer, R., and Steinbuchel, A. (2000) Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. *Appl. Microbiol. Biotechnol.*, **54**, 218–223.

Chen, Y., Ding, Y., Yang, L., Yu, J., Liu, G. et al. (2014) Integrated omics study delineates the dynamics of lipid droplets in *Rhodococcus opacus* PD630. *Nucleic Acids Res.*, **42**, 1052–1064.

Ding, Y., Yang, L., Zhang, S., Wang, Y., Du, Y. et al. (2012) Identification of the major functional proteins of prokaryotic lipid droplets. *J. Lipid Res.*, **53**, 399–411.

Hernandez, M. A., Mohn, W. W., Martinez, E., Rost, E., Alvarez, A. F. et al. (2008) Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. *BMC Genomics*, **9**, 600–613.

Law, J. H. and Slepecky, R. A. (1961) Assay of poly-beta-hydroxybutyric acid. *J. Bacteriol.*, **82**, 33–36.

MacEachran, D. P., Prophete, M. E., and Sinskey, A. J. (2010) The *Rhodococcus opacus* PD630 heparin-binding hemagglutinin homolog TadA mediates lipid body formation. *Appl. Environ. Microbiol.*, **76**, 7217–7225.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierback, G. et al. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, **145**, 69–73.

Tajparast, M. and Frigon, D. (2015) Genome-scale metabolic model of *Rhodococcus jostii* RHA1 (iMT1174) to study the accumulation of storage compounds during nitrogen-limited condition. *BMC Syst. Biol.*, **9**, 43–57.

Valappil, S. P., Boccaccini, A. R., Bucke, C., and Roy, I. (2007) Polyhydroxyalkanoates in Gram-positive bacteria: insights from the genera Bacillus and Streptomyces. *Antonie Van Leeuwenhoek*, **91**, 1–17.

van der Geize, R., Hessels, G. I., van Gerwen, R., van der Meijden, P., and Dijkhuizen, L. (2001) Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid Delta1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker. *FEMS Microbiol. Lett.*, **205**, 197–202.