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Deliverable D2.2

Report on thioesterases with confirmed functionality in cyanobacteria and microalgae for product chain-length control

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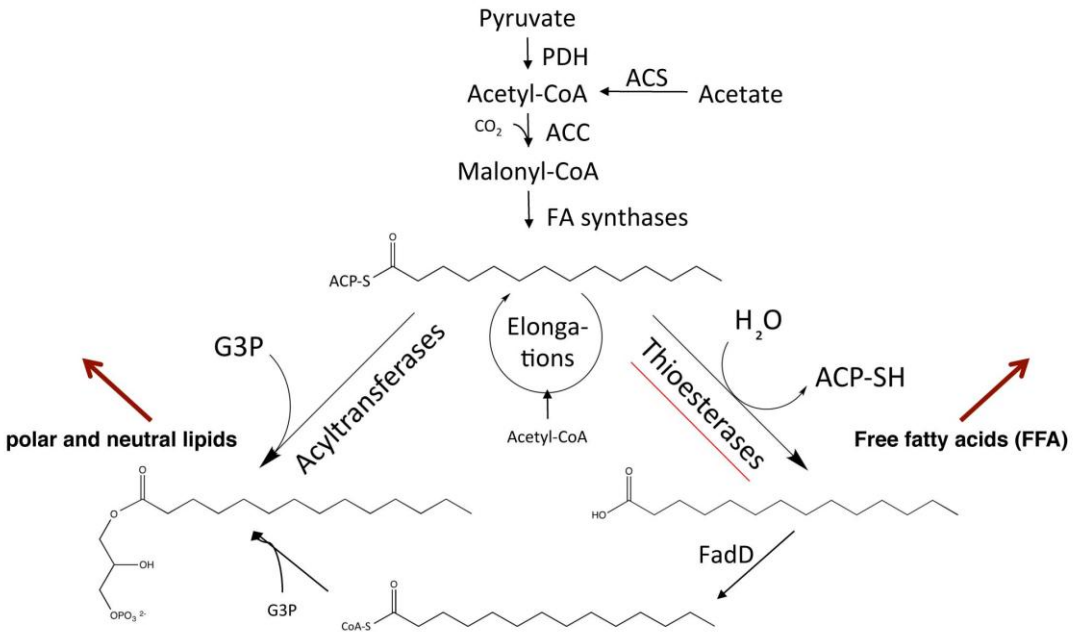
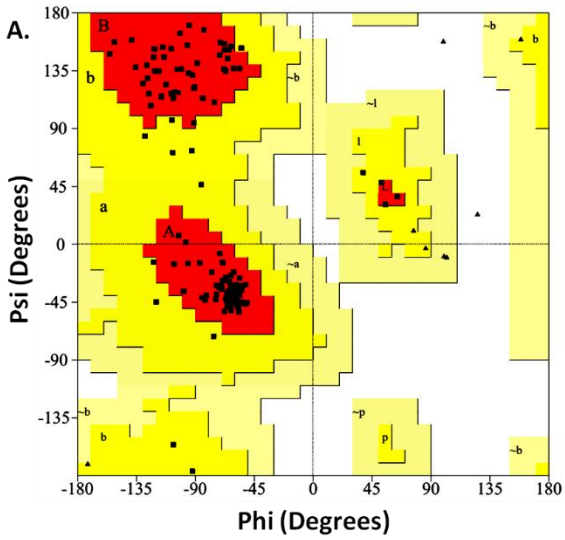
Publishable Summary

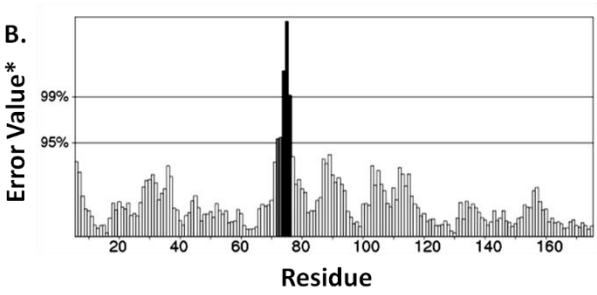
Fatty acids serve as an excellent precursor for fuel components, higher-value chemicals and nutrients. A challenge is how to effectively control the acyl chain-length and maximize productivity in order to direct fatty acid synthesis (FAS) towards useful end-products. Thioesterases (Tes) play an important role for deciding both key-parameters (acyl chain length profile and pathway flux). A wide range of thioesterase-encoding gene candidates exist thanks to genome sequencing. The question investigated here in this deliverable is which Tes to use in cyanobacteria, with the ultimate objective of producing both C8 *n*-octanol as well as C11 undecane.



Lipid precursor metabolism. After elongation, a growing acyl-ACP is transferred to a G3P backbone by acyltransferases to produce glycerol lipid molecules. Thioesterases (underlined in red), in contrast, cleave the ACP molecule at the thiol bond releasing free fatty acids which can be used for various biotechnological purposes. G3P – glycerol-3-phosphate, PDH – pyruvate dehydrogenase, ACC – Acetyl-CoA carboxylase, ACS – Acetyl-CoA synthase, ACP – acyl carrier protein, FA – fatty acids, FadD – long-chain acyl-CoA synthetase.

A computational workflow was developed to assist in narrowing the choice of gene candidates to evaluate. The functionality of the workflow was verified by analyzing the variation in catalytic specificity of a handful of previously studied Tes enzymes and comparing predicted affinities with assay data from real-world experiments. For example, the change in chain-length specificity afforded by a single-residue mutation (L109P) to TesA'.





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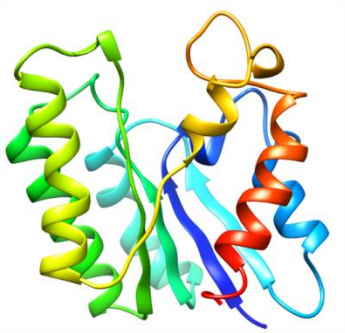


Model	GDT-HA	RMSD	MolProbity
Unrefined TesA"	1.0000	0.000	2.296
Refined TesA"	0.9874	0.281	1.046

Homology modelling and structural refinement of TEs. A The Ramachandran plot for refined predicted TesA' model revealed that 92.8% of residues were in the most favourable region, while 7.2% were in allowed region, confirming that the predicted refined model is of good quality; **B** ERRAT is a so-called "overall quality factor" for non-bonded atomic interactions, with higher score implies higher quality. The generally accepted range is >50 for a high quality model. For the current refined TesA' model, the overall quality factor predicted was 97.059. The 3D verify analysis predicted that 99.98% of the residues in refined TesA' model had an average 3D-1D score>0.2, thereby verifying the model. **C** 3D verified refined TesA' structure for molecular docking studies.)

With the hypothesis that the outcome of Tes choice also depends on the context, i.e. an interaction between Tes and FAS, we further evaluated the computational workflow in comparison to *in vivo* measured substrate specificities in 4 different organisms (*E. coli*, *Chlamydomonas reinhardtii*, *Synechocystis sp.* PCC 6803 and *Synechococcus sp.* PCC 7002). The analysis is ongoing. Results so far suggest that the outcome is indeed dependent on an interaction between thioesterase and host metabolism.

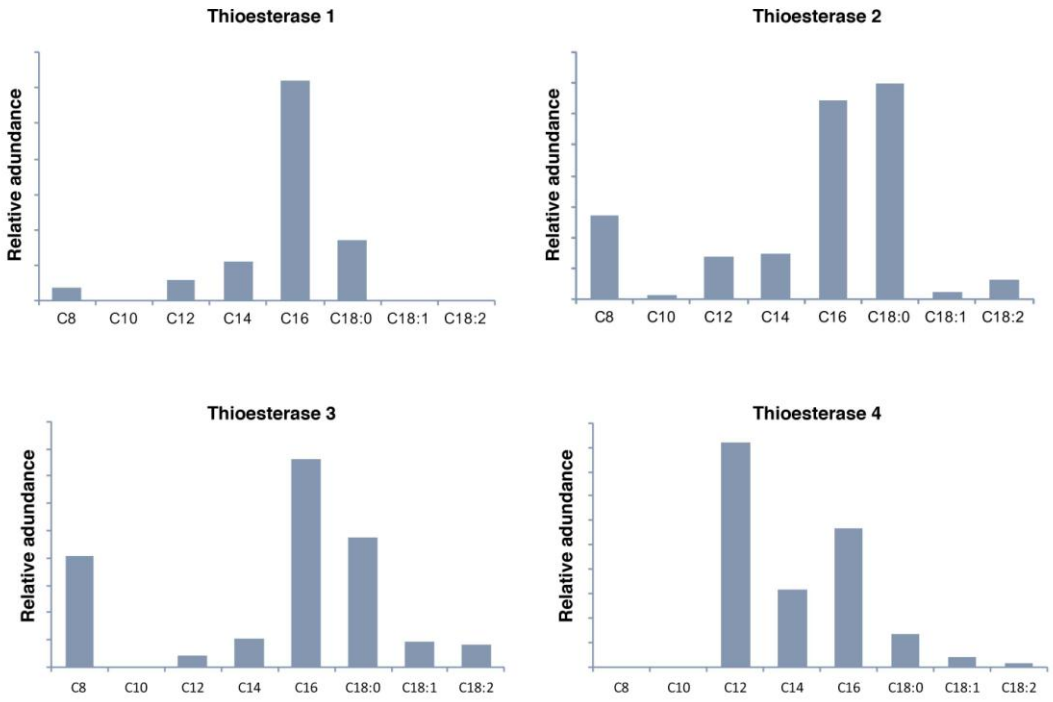
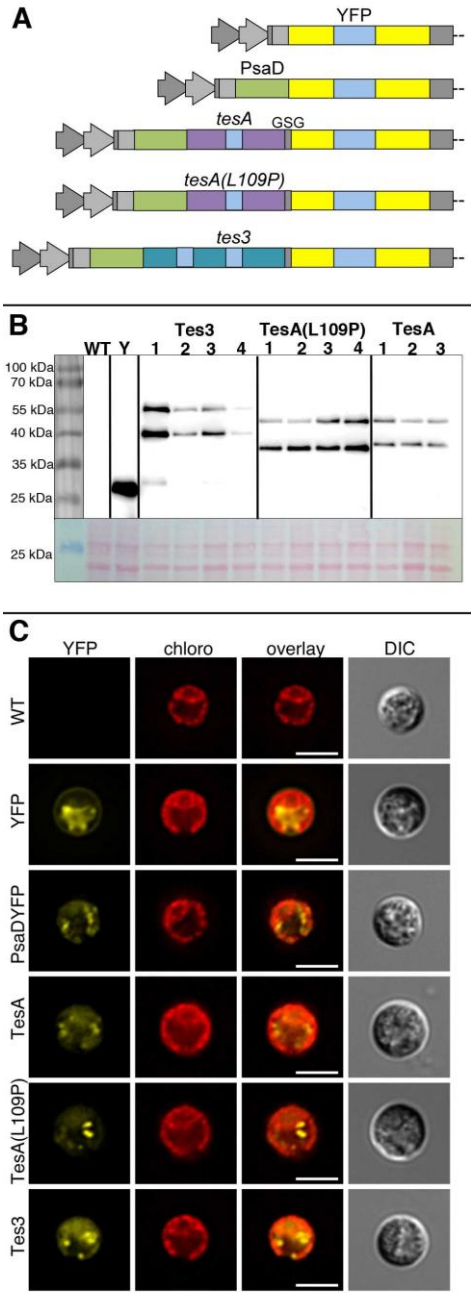


Figure 15. The fatty acids profiles obtained from strains carrying different thioesterases. Target chain-length fatty acids are highlighted in green (C8) and red (C12).

In *C. reinhardtii* three bacterial thioesterases with preferences for C16:0, C14:0, and C8:0 fatty acid chain lengths were expressed from the nuclear genome and subsequently targeted to the algal chloroplast.

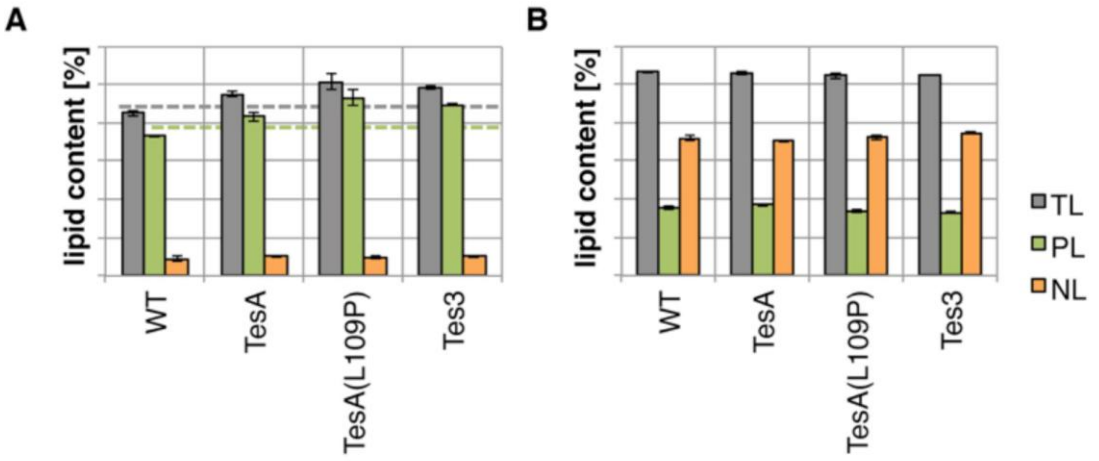
Thioesterase	Organism	Carbon chain length preference	Reference
TesA’	*E. coli*	C16:0 some C14:0	(Cho and Cronan, 1995)
TesA’(L109P)	Point mutation of TesA’	C14:0 some C12:0	(Choi and Lee, 2013)
Tes3	*Anaerococcus tetradius*	C8:0	(Jing et al., 2011)

Each independent thioesterase expressed and localized appropriately. The thioesterase expressing strains exhibited increased total lipid contents under normal growth conditions compared to the parental due to significant increases of cellular polar lipids, largely found as C18:1n9c. After 96 hours of nutrient deprivation, no difference in relative fatty acid contents were observed between the parental and mutant strains, however, two strains exhibited increased volumetric biomass productivities under these conditions.

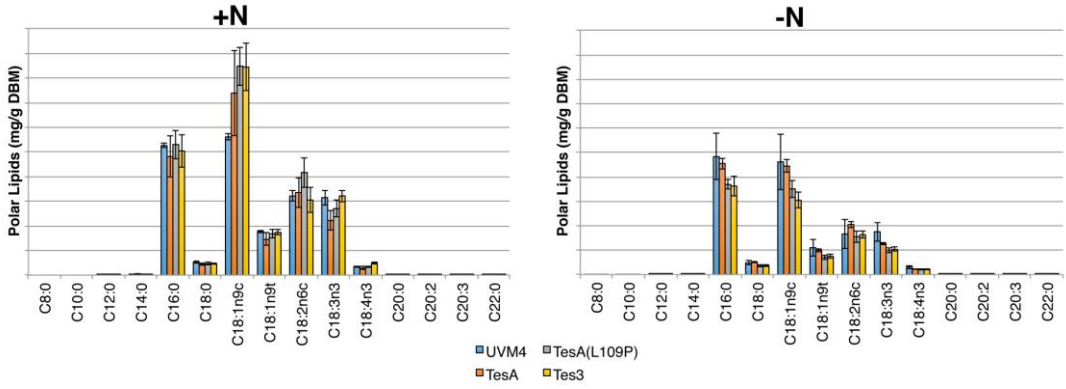


Heterologous thioesterase expression may have de-regulated fatty acid biosynthesis, however, the target chain lengths of fatty acids from each thioesterase were not present in neutral or polar lipid fractions. Investigations are on-going to determine if free fatty acids are lost to the culture media rather than being incorporated into cellular lipids.

Thioesterase expression in *C. reinhardtii* (A) Genetic constructs for thioesterase expression from the nuclear genome of *C. reinhardtii*. Under control of the HSP70-RBCS2(i1) promoter, the pOpt_YFP_Paro vector was a template to add the PsaD chloroplast localization peptide. All thioesterases were targeted to the chloroplast by insertion between the targeting peptide and the YFP reporter. Light blue rectangles represent the RBCS2 introns 1 and 2 (in the YFP). (B) Full-length protein expression was confirmed by Western blotting of several mutants from each construct that exhibited YFP fluorescence. The double band is the result of a site-specific cleavage in the middle of YFP by an unknown chloroplast protease. (C) Representative microscopic images of thioesterase-YFP fusions targeted to the algal chloroplast, a cytosolic and chloroplast (PsaD) targeted YFP are shown in addition to the parental control (WT). YFP – signal in the yellow emission channel. chloro – chlorophyll autofluorescnce is used to orient the cells and an overlay with the YFP signal is shown. DIC – differential interference contrast. Scale bars represents 10 µm.



Thioesterase expressing *C. reinhardtii* strains have an increased polar lipid content under replete conditions. However, have neutral and polar lipid contents comparable with the parental strain after 96 hours of nitrogen deprivation. Lipid contents are presented as a percent of cell dry biomass. (A) Under nitrogen replete conditions, the three thioesterase strains have significantly increased total lipid (TL) contents (dashed grey line) due to significant increases in the polar lipid (PL) fraction (dashed green line). The TesA'(L109P) as well as Tes3 strain have total lipid contents comparable to that of nitrogen starved cells after 96 h nitrogen deprivation. (B) Under nitrogen limitation, neutral lipids (NL) increase significantly for all strains, however, no significant difference was found in total lipid contents under –N for any of the thioesterase expressing strains compared to the parental. Error bars represent standard deviation.



Fatty acid compositions of the polar lipid fraction of parental and three thioesterase expressing strains under nutrient replete (+N) and nitrogen deficient (-N) conditions.