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Engineering of Fatty Acid Production and Secretion in *Saccharomyces cerevisiae*

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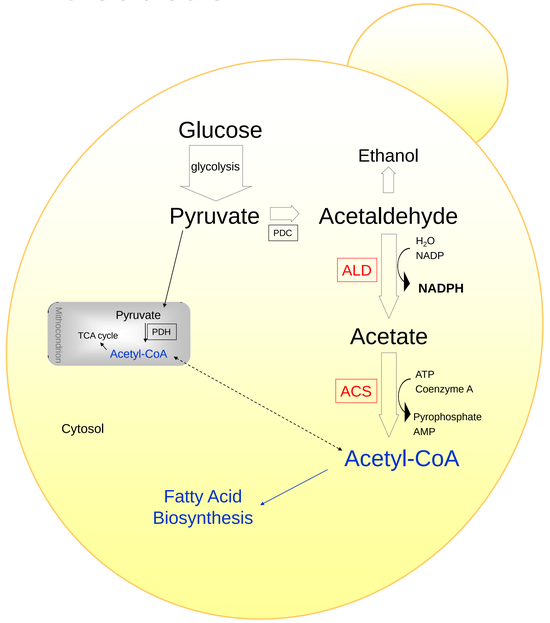
Abstract

Production of renewable liquid biofuels that can substitute fossil fuel, has emerged as a major challenge for applied biology.
Biodiesel, in the form of fatty acid esters, produced by oleaginous microorganisms could be an attractive alternative, since the utilization of diesel fuel is more efficient than for example ethanol.
Oleaginous yeasts may accumulate very high (60%) levels of intracellular lipids but two drawbacks are the relatively complicated extraction process and the subsequent transesterification with the accompanying glycerol by-product formation.
The objective of this work is to apply metabolic engineering of fatty acid synthesis and secretion in the model yeast *S. cerevisiae* in order to create a microorganism able to produce and secrete free fatty acids or fatty acid esters. *S. cerevisiae* is a proper model, since lipid metabolism has been studied extensively and all genes encoding enzymes directly involved in lipid synthesis are known. This organism has also been reported to acquire oleaginous properties by no more than three genetic modifications(1).
In the yeast *S. cerevisiae*, activation of exogenous long-chain fatty acids to coenzyme A derivatives, prior to metabolic utilization, is mediated by the fatty acyl-CoA synthetases Faa1p and Faa4p. It has been shown that free fatty acids are secreted from a FAA1,4 double mutant (2). This modification will be combined with modifications of core fatty acid elongation, such as overexpression of acetyl-CoA synthetase (Acs1p), in an attempt to improve fatty acid production rate.
Essential for this work is to facilitate a biological platform for efficient fatty acid or lipid production.
In this work, the “delitto perfetto” method (3) was applied to delete these two fatty acyl-CoA synthetases generating genetically clean strains without markers or bacterial DNA. Results show that this technique can be used to generate multiple knockouts by recycling the marker gene.

Introduction

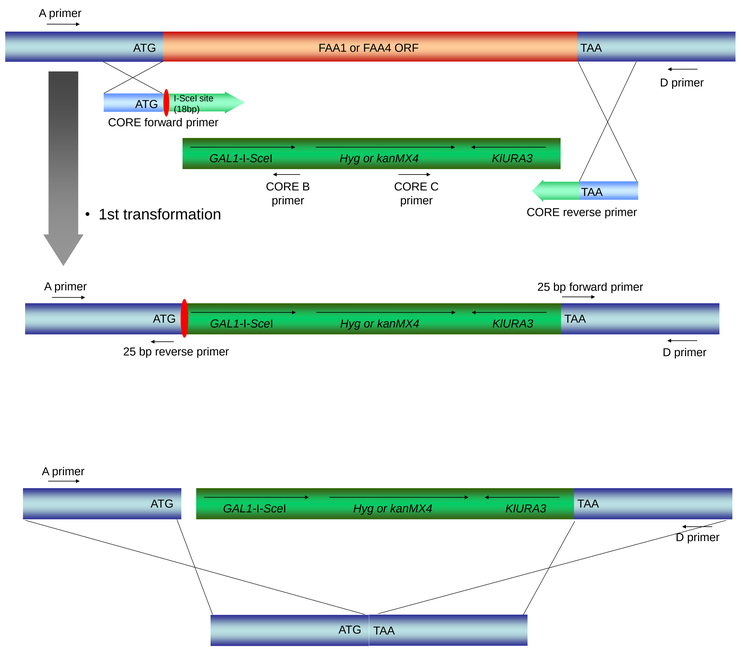


The pyruvate dehydrogenase bypass converts pyruvate into acetyl-CoA by the action of pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase. It was recently shown that engineering the pyruvate dehydrogenase bypass is effective for high level production of isoprenoids in yeast (Shiba *et* al. 2007).
Fatty acid biosynthesis, that begins with the carboxylation of acetyl-CoA, is essentially a reversal of fatty acid beta-oxidation but is carried out in the cytosol and is a demanding process in terms of reducing power in the form of NADPH. In *S. cerevisiae*, NADPH is mainly produced by oxidation of glucose to carbon dioxide in the oxidative pentose phosphate pathway.
ALD6 encodes a constitutively expressed, cytosolic, aldehyde dehydrogenase, activated by Mg^{2+}. It is required for the conversion of acetaldehyde to acetate and utilizes $NADP^{+}$ as the preferred coenzyme.
In *S. cerevisiae*, cytosolic acetyl-CoA is produced from acetate by acetyl-CoA synthetases Acs1p and Acs2p which catalyze the ligation of acetate and CoA.
In this work we engineered the pyruvate dehydrogenase bypass in order to enhance the supply of acetyl-CoA to the fatty acid biosynthesis pathway by overexpression of Acs1p and Ald6p, in a fatty acyl-CoA synthetase null mutant.

Methods

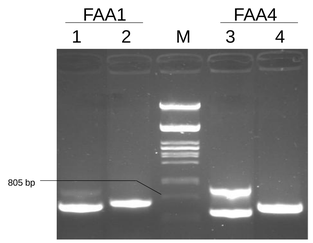
***Delitto perfetto* aproach to generate a *S. cerevisiae* FAA1,4 double mutant:**

The method used in this work is described in Storici F. and Resnick MA with the following modifications:
The CORE cassette is amplified using chimeric primers, consisting of 25 nucleotides homologous to the genomic target and an additional 20 nucleotides for the amplification of the CORE-I-SceI cassette.
25 bp oligonucleotides containing the sequence upstream immediately followed by the sequence downstream of the region to be knocked out were designed. One PCR was performed using primers A and D, and these 25 bp forward and reverse primers, producing 2 fragments that were then fused is a second PCR. The second step of the method is accomplished by transformation with the fusion product such that the CORE cassette is lost and the desired deleted strain is generated.



Results

CORE cassettes have been successfully inserted into the genome of CEN.PK 113-5D strain .
Integration of the CORE cassette in the correct chromosomal location was verified via PCR using primers A, CORE B, CORE C and D, designed to generate 600-1000 bp fragments (lanes 1 and 3).
Deletion was confirmed using primers A and D, generating fragments of 745 bp and 689 bp for the deletion of FAA1 and FAA4 respectively (lanes 2 and 4).
To overexpress genes, we constructed multicopy plasmids derived from p426TEF and p425GPD (Mumberg *et* al., 1995) where ALD6 and ACS1 were cloned.



Final remarks and future perspectives:

In this work, the “delitto perfetto” method was applied to delete these two fatty acyl-CoA synthetases generating genetically clean strains without markers or bacterial DNA.

Future plans include:

Physiological characterization of the modified strains.

Site-directed mutagenesis of *ALD6* to prevent possible inhibition by acetylation.

Further metabolic engineering is planned for the fatty acid elongation pathway.

Acknowledgments

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References

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