Rewiring the Wax Ester Production Pathway of *Acinetobacter baylyi* ADP1

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S *Supporting Information*

ABSTRACT: Wax esters are industrially relevant high-value molecules. For sustainable production of wax esters, bacterial cell factories are suggested to replace the chemical processes exploiting expensive starting materials. However, it is well recognized that new sophisticated solutions employing synthetic biology toolbox are required to improve and tune the cellular production platform to meet the product requirements. For example, saturated wax esters with alkanol chain lengths C12 or C14 that are convenient for industrial uses are rare among bacteria. *Acinetobacter baylyi* ADP1, a natural producer of wax esters, is a convenient model organism for studying the potentiality and modifiability of wax esters in a natural host by means of synthetic biology. In order to establish a controllable production platform exploiting well-characterized biocomponents, and to modify the wax ester synthesis pathway of *A. baylyi* ADP1 in terms product quality, a fatty acid reductase complex LuxCDE with an inducible arabinose promoter was employed to replace the natural fatty acyl-CoA reductase *acr1* in ADP1. The engineered strain was able to produce wax esters by the introduced synthetic pathway. Moreover, the fatty alkanol chain length profile of wax esters was found to shift toward shorter and more saturated carbon chains, C16:0 accounting for most of the alkanols. The study demonstrates the potentiality of recircuiting a biosynthesis pathway in a natural producer, enabling a regulated production of a customized bioproduct. Furthermore, the LuxCDE complex can be potentially used as a well-characterized biopart in a variety of synthetic biology applications involving the production of long-chain hydrocarbons.

KEYWORDS: *wax ester, recircuiting, Acinetobacter baylyi ADP1, long chain aldehyde, fatty-acyl CoA reductase, luxCDE*
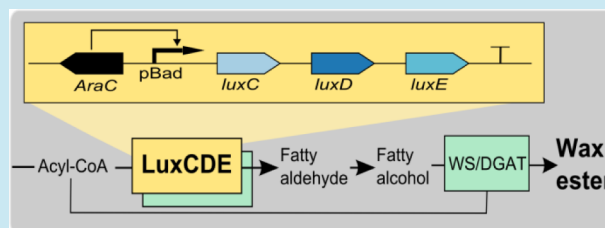
Wax esters (WE) are oxoesters of long-chain fatty acids esterified with long-chain alcohols. WEs are industrially valuable lipid compounds exploited in several purposes including cosmetics, printing inks, lubricants, surface coatings, and the food industry. At the moment, the supply of WE relies on biological plant-originated jojoba oil or expensive chemical synthesis.[1] Thus, bacteria provide an attractive alternative for producing WEs by biotechnological means. Among natural producers, the genus *Acinetobacter* is the most studied in terms of WE production, *Acinetobacter baylyi* ADP1 being the model representative.[2−4] As the wax ester production pathway is rather complicated and not yet fully characterized,[2] a natural producer serves as a convenient host for applications. Briefly, the biochemical pathway for wax ester synthesis in ADP1 involves three enzymatic steps; first, a fatty-acyl coenzyme A (acyl-CoA) is reduced to a corresponding long-chain aldehyde by a NADPH dependent fatty acyl-CoA reductase Acr1.[5] Fatty aldehyde is further reduced to a corresponding fatty alcohol by a yet uncharacterized aldehyde reductase. In the last step, the fatty alcohol is esterified with a fatty acyl-CoA by a well-characterized and highly unspecific bifunctional enzyme acyl-CoA:fatty alcohol acyltransferase (WE synthase, WS/DGAT),[3] resulting in the formation of a WE molecule.

Because of the broad range of applications, various chemical compositions of WE are needed. However, the natural alcohol moieties of WEs produced by ADP1 consistently comprise of unsaturated C16 and C18 chains,[6] whereas saturated carbon chains are often more appropriate for applications. Also, in order to broaden the application area of ADP1 based WEs, a wider spectrum of alkanol chain lengths is required. Even though WE composition can be potentially altered to some extent by using selected fossil substrates,[7] it is crucial to find more sustainable ways to produce WEs with desired qualities. Furthermore, the regulation of natural production is challenging, for which well-characterized bioparts with more straightforward tuning abilities are preferably exploited when conceivable. Thus, a natural target for modifying the wax ester pathway of ADP1 is the aldehyde producing step carried out by Acr1. A potential alternative for the production of long chain aldehyde within the pathway is the well-characterized but in the context of hydrocarbon production, to our knowledge, never applied[8] enzyme complex LuxCDE from *Photorhabdus luminescens*

consisting of three subunits: a transferase, a synthetase, and a reductase. In this study, a reconstructed WE pathway was introduced to *A. baylyi* ADP1 in order to demonstrate the usability of well-characterized biocomponents as a constituents of natural synthesis pathways, thus enabling a regulated production of modified bioproducts.

RESULTS AND DISCUSSION

Reconstruction of the WE Production Pathway. For the reconstruction of the WE pathway, a knockout mutant strain of *A. baylyi* ADP1 known to be WE negative[2,9] was applied; the strain designated as ADP1*k* (ADP1Δ*acr1::Kan*[r]/*tdk*) lacks the fatty acyl-CoA reductase gene *acr1*, which is responsible for the conversion of fatty-acyl CoA to long chain aldehyde for the WE synthesis. Both *in vitro* and *in vivo* analyses indicate relatively narrow substrate range for Acr1, namely, strong preference for CoA-activated C16 and C18 fatty acids (FA).[5] In order to remold the WE production profile of ADP1, a fatty acid reductase complex genes *luxC*, *luxD*, and *luxE* from *P. luminescens* were exploited. The enzyme complex comprises of an acyl transferase (LuxD), which cleaves activated FAs for the acyl-protein synthetase (LuxE), which produces an acyl-protein thioester for FA reductase (LuxC) converting the thioester to corresponding fatty aldehyde.[10] For the transferase LuxD, there is a strong preference toward C14 acyl groups,[10] but hypothetically the substrate range for LuxCDE can be broader and not solely limited to tetradecanoate. Moreover, it has been recently suggested that the relevance of LuxD in the LuxCDE complex is more related to structural interactions between the proteins than to the catalytic transferase activity.[11] The use of LuxCDE complex is justified by the strong biochemical knowledge and the long history of its successful use in a number of applications, mainly related to biosensors.[12] Furthermore, it has been shown that some common thioesterases used in endogenous free FA synthesis, such as acyl−acyl carrier protein thioesterase from *Umbellularia californica*, might negatively affect the cell physiology through alterations of cell membrane composition.[13] The LuxCDE complex was cloned to a plasmid pBAV1K-T5[14] of which the kanamycin selection marker was replaced with a chloramphenicol resistance gene (C), and the promoter T5 was replaced with an inducible arabinose promoter (*ara*). The resulting plasmid pBAV1C-*ara-luxCDE* (Figure 1) was transformed to ADP1*k* by natural transformation and selected on LA plate containing 50 μg/mL of chloramphenicol.

The resulting strain ADP1*k* carrying the plasmid pBAV1C-ara-*luxCDE* was designated as ADP1*cde*. Figure 2 represents the hypothetical wax ester production pathway in ADP1 wild type (2A) and in the engineered strain ADP1*cde* (2B).

Studying the Functionality and Inducibility of Ara-LuxCDE Complex. For the detection of intracellular aldehyde production by LuxCDE and to verify the functionality of the arabinose promoter, a previously described real-time monitoring system, exploiting an integrative gene cassette, was applied;[2,15] ADP1*cde* was naturally transformed with the gene cassette i*luxAB_Cm*[r] containing the bacterial luciferase genes *luxAB* from *P. luminescens* that utilize long chain aldehydes as a substrate, producing a corresponding fatty acid molecule and a measurable luminescent signal. To verify the monitoring system, ADP1*k* strain was used as a negative control. The transformed strains were designated as ADP1*cde_ab* (genotype ADP1Δ*poxB::iluxAB_Cm*[r],Δ*acr1::Kan*[r]/*tdk* expressing pBAV1C-ara-*luxCDE*) and ADP1*k_ab* (genotype ADP1Δ*pox-*
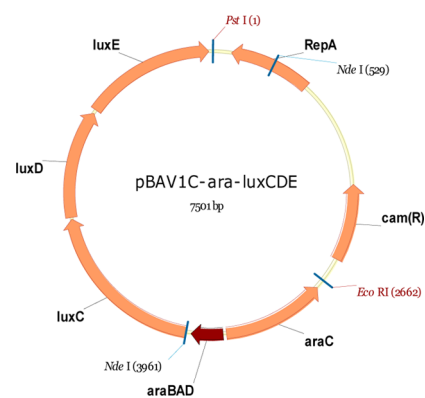

Figure 1. The expression plasmid pBAV1C-*ara-luxCDE* for *A. baylyi* ADP1. In the plasmid, the original[14] T5 promoter was replaced with an inducible arabinose promoter (*ara*), and a chloramphenicol marker (C) was used instead of kanamycin marker. The fatty acid reductase complex *luxCDE* was cloned into the plasmid for recircuited wax ester synthesis. In *luxE*, an internal *Nde*I recognition site and a possible transcription regulation element were disrupted.

B::iluxAB_Cm[r],Δ*acr1::Kan*[r]/*tdk*), respectively. The strains were incubated with different arabinose concentrations, and luminescence was measured (Figure 3). For ADP1*cde_ab*, luminescence could be detected with all arabinose concentrations, indicating the functionality of LuxCDE. Also, a correlation was observed between the measured luminescence signal and arabinose concentrations. For the control strain ADP1*k_ab*, no measurable luminescence was detected, neglecting any possibility for endogenous aldehyde and consequently WE formation.

In order to further confirm the compatibility of LuxCDE with the native WE production pathway of ADP1 the WE content of ADP1*cde* was determined. The cells were cultivated as four parallel samples at 30 °C and 300 rpm in MA/9 medium supplied with 5% glucose and different concentrations of arabinose. The cells were cultivated for 48 h, and the WE concentrations were measured for extracted lipids using nuclear magnetic resonance (NMR) spectroscopy (Table 1). It was found out that the strain ADP1*cde* was able to produce WEs with the reconstructed synthetic pathway in the presence of arabinose. It was observed that the biomass and total lipid content were similar in all samples regardless of the added arabinose concentration. However, the proportion of WE in total lipids varied between the samples, the highest WE concentration (0.65 mmol/g total lipids) being measured in the sample containing 0.5% (m/V) arabinose. Nevertheless, the proportion of WE in total lipids and consequently the WE titer produced by ADP1*cde* were found to be approximately 30−50% smaller than for the wild type strain ADP1 in the studied conditions. For control strain ADP1*k*, no WE production was detected.

Temperature Dependency of WE Production. A temperature range for a sufficient growth of *A. baylyi* ADP1 is relatively wide.[16] In order to study the effect of ADP1 growth temperature on the production of WE, and to potentially increase the amount of WE for qualitative alkanol determination, a temperature optimization was carried out using a temperature gradient incubator. The cells were cultivated for 48 h at seven different temperatures ranging from 20 to 37.5 °C. Biomasses were determined gravimetrically for cell dry weight (CDW), and for extracted lipids a thin layer chromatography (TLC) analysis was carried out. It was found out that the
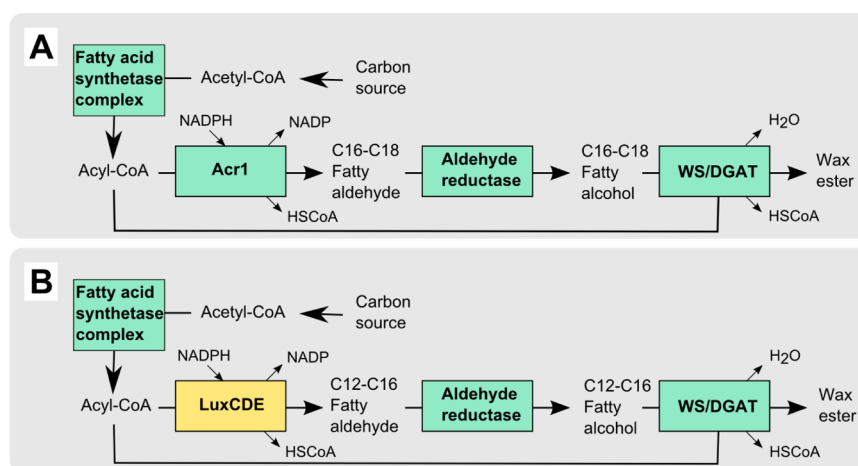
Figure 2. The hypothetical wax ester (WE) synthesis pathway in (A) *A. baylyi* ADP1 wild type and (B) ADP1*cde* (*A. baylyi* ADP1Δ*acr1::Kan^r/tdk* expressing pBAV1C-*ara-luxCDE*). In the engineered strain ADP1*cde* the natural fatty acyl-CoA reductase Acr1 (*acr1*, EC 1.2.1.n2) is replaced with a fatty acid reductase complex LuxCDE from *P. luminescens* for the production of modified WEs. The aldehyde reductase is unknown. The esterification of fatty alcohol and fatty acyl-CoA is carried out by bifunctional wax ester synthase/acyl CoA; diacylglycerol acyltransferase WS/DGAT (*wax-dgaT*, EC 2.3.1.20).
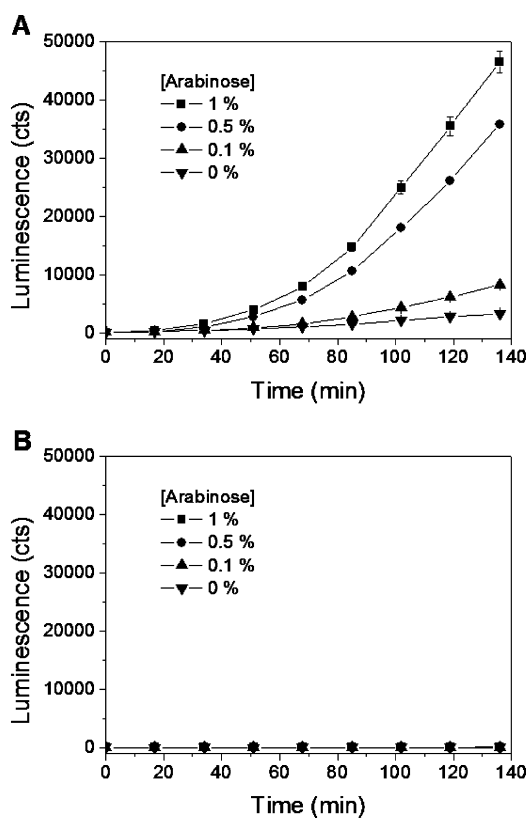

Figure 3. The effect of arabinose concentration on the intracellular aldehyde formation in ADP1*cde_ab*. A previously described real-time monitoring system[2] based on luminescence produced by bacterial luciferase LuxAB was applied in studying the fatty aldehyde formation in ADP1*cde_ab* (ADP1Δ*poxB::iluxAB_Cm^r,Δacr1::Kan^r/tdk* expressing pBAV1C-ara-*luxCDE*) (A). LuxAB utilizes fatty aldehydes as a substrate producing measurable luminescent signal. A knockout mutant strain ADP1*k_ab* (ADP1Δ*poxB::iluxAB_Cm^r,Δacr1::Kan^r/tdk*) lacking the enzyme for fatty aldehyde synthesis was used as a control (B). The mean and standard deviation of two independent cultures are shown. cts: counts.

Table 1. Total Lipid and Wax Ester (WE) Contents of the Engineered Strain ADP1*cde* (*A. baylyi* ADP1Δ*acr1::Kan^r/tdk* Expressing pBAV1C-*ara-luxCDE*), *A. baylyi* ADP1 Wild Type (ADP1 wt), and the Control Strain ADP1*k* (*A. baylyi* ADP1Δ*acr1::Kan^r/tdk*) Determined by NMR Spectroscopy

strain	[arabinose] (%)	CDW (g/L)	total lipids (%)	WE (mmol/g of lipids)	WE (mmol/L)
ADP1*cde*	0	6.7	4.8	0	0
	0.1	6.2	4.6	0.37	0.11
	0.5	6.2	4.4	0.65	0.18
	1.0	6.2	4.3	0.34	0.09
ADP1 wt	–	9.0	4.3	0.90	0.32
ADP1*k*	–	11	2.7	0	0

highest CDW is obtained at 29.5 °C, whereas the most optimal temperature for WE production within the studied range is 20 °C (Figure 4). This suggests that the WE production is strongly dependent on temperature. This can be partly explained by the shift in activity of WS/DGAT in relation to temperature between the synthesis of triacylglycerol (TAG) and WE.[17] For
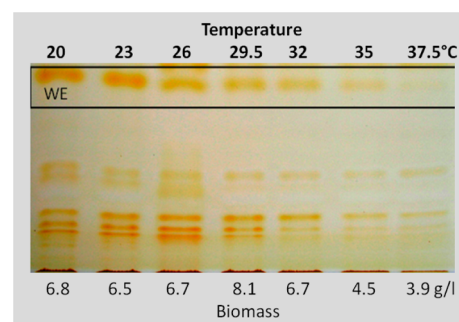

Figure 4. Temperature dependency of wax ester synthesis and biomass production. Thin layer chromatography (TLC) analysis was carried out to demonstrate the temperature dependency of wax ester production in *A. baylyi* ADP1. For biomass determination, cell dry weight was measured.

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other lipid groups, there is no significant temperature dependency observed apart from the biomass production.

Qualitative Analysis of Lipids Produced by the Synthetic Pathway. For studying the effect of the recircuited pathway on WE quality of ADP1, the strains ADP1cde, ADP1k, and ADP1 wild type were cultivated at 20 °C for 70 h in standard MA/9 medium supplied with glucose and 0.5% arabinose. Total lipids were analyzed by GC-FID and GC−MS, and the alcohol moieties measured in total lipids are presented in Figure 5. It was demonstrated in the arabinose induction
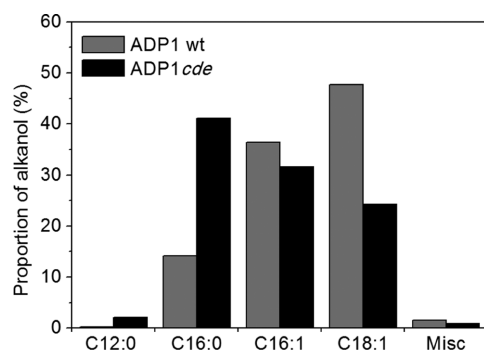

Figure 5. Qualitative analysis of fatty alcohols in *A. baylyi* ADP1 wild type (ADP1 wt) and *A. baylyi* ADP1Δacr1::Kan^r/tdk expressing pBAV1C-ara-luxCDE (ADP1cde). The alkanol moieties of wax esters were analyzed using GC−MS. Results are presented as proportions of alcohols in samples.

tests exploiting luminescence determination and NMR analysis, and also our previous studies[2] that the long chain alcohols produced through aldehyde synthesis route in the ADP1 cells are constituents of WEs. In the ADP1 wild type strain, C18:1-OH accounts for most of the alcohols (48%), whereas in ADP1cde C16:0-OH represent the major alcohol fraction (41%). Furthermore, C12:0-OH peak was detected for ADP1cde (2.1%). For ADP1 wild type strain, only traces of dodecanol were found (0.2%).

Surprisingly, C14:0-OH groups are not found in the ADP1cde samples in spite of the strong preference of the acyl transferase LuxD toward C14 FAs. Yet in our previous studies, we demonstrated the effect of luxD deletion from the lux operon luxCDABE: the luminescence measurements showed a major drop in the signal in the absence of LuxD; however, luminescence signal could be still detected (Supporting Information). In that sense, the catalytic role of LuxD regarding the WE synthesis in the engineered strain is somewhat unclear even though the functionality of LuxD in ADP1cde could be verified in the arabinose induction tests. Thus, it can be proposed that LuxD is important for the reductase complex to provide structural support, but the catalytic activity is relatively weak. In addition, endogenous acyl transferases of ADP1 can be potentially involved in the aldehyde synthesis, providing C12−C18 acyl groups for the synthesis complex. It is also obvious that the yet uncharacterized endogenous aldehyde reductase(s) of ADP1 plays a major role in determining the final product content and carbon chain length since the preference and specificity of the enzyme is not known. Therefore, it can be speculated that even though tetradecanal can be potentially produced in the cells the aldehyde product is directed to catabolic reaction or components other than WEs.

Further, it was observed that the deletion of *acr1* and the expression of *luxCDE* affects the proportions of different

acylglyceride fractions of ADP1cde and ADP1k in comparison to ADP1 wild type (Table 2) even though acylglycerides are

Table 2. Distribution (%) of Lipid Fractions in *A. baylyi* ADP1 Wild Type (ADP1 wt), *A. baylyi* ADP1Δacr1::Kan^r/tdk (ADP1k), and *A. baylyi* ADP1Δacr1::Kan^r/tdk Expressing pBAV1C-ara-luxCDE (ADP1cde) Analyzed with HPLC-GPC

	ADP1 wt	ADP1k	ADP1cde
mono[a]	0.80	1.3	4.1
di[a]	55	16	37
tri[a]	37	68	17
oligomers	0.70	3.8	4.4
fatty acids	7.2	11	38

[a] Acylglycerine fractions.

not directly linked to the WE synthesis pathway: the diacylglycerol fraction mostly required for cell growth is significantly lower in ADP1k and ADP1cde compared to the wild type strain. According to the results obtained by HPLC-GPC analysis and our previous studies,[9] in ADP1k the diacylglycerols are substantially directed to triacylglycerol synthesis, whereas in ADP1cde the most significant differences are observed for the FA fraction containing polar lipid groups. Qualitative analysis for the FA fractions was carried out with GC−MS (Figure 6). It was found out that considering the
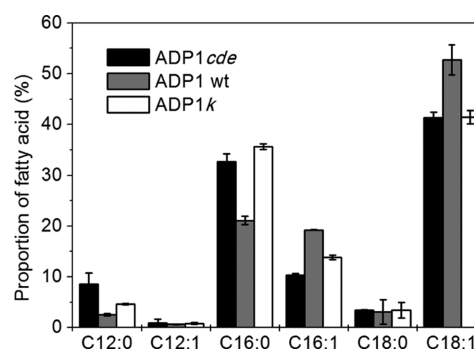

Figure 6. Distribution (%) of fatty acid (FA) carbon chain lengths in *A. baylyi* ADP1 wild type (ADP1 wt), *A. baylyi* ADP1Δacr1::Kan^r/tdk (ADP1k), and *A. baylyi* ADP1Δacr1::Kan^r/tdk expressing pBAV1C-ara-luxCDE (ADP1cde) analyzed with GC−MS. The mean and standard deviation of at least two independent cultures are shown. In addition to FAs presented in the figure, traces (<0.6%) of C8:0, C10:0, C13:0, C17:1, and C18:2 fatty acids were detected. Fatty acids C6:0, C11:0, C14:0, C14:1, C15:0, and C17:0 were not detected.

carbon chain lengths, the distribution of FAs in total lipids varied between the strains; most interestingly, the proportion of C12:0 FAs was found to be approximately 2-fold greater in ADP1cde compared to the ADP1 wild type strain and ADP1k. This indicates slightly increased acyltransferase activity toward C12 acyl-CoA potentially afforded by LuxD.

Even though the lipid profile was not as broadly diversified through the engineering as could have been expected, with regards to the substrate preference of LuxCDE, it can be concluded that the reconstruction of the WE pathway has an impact on the overall cellular lipid distribution and composition.

WE production in *A. baylyi* ADP1 is a multistep process involving strict regulation and several enzymes of which are

Table 3. Primers Used in the Study

name	description	oligo sequence (5′ → 3′)
ab98	pBAV1, sense, *Nhe*I	TAATAGCTAGCTATTTAAAGATACCCCAAGAAGCTAATTATAAC
ab99	pBAV1, antisense, *Apa*LI	TAATAGTGCACTCGCTTGGACTCCTGTTGATAG
ab100	*cat*, sense, *Nhe*I	TAATAGCTAGCCTGTAGAAAAGAGGAAGGAAATAATAAATGGAGAAAAAAATCACTGGATATAC
ab101	*cat*, antisense, *Apa*LI	TAATAGTGCACTTACGCCCCGCCCTGCCAC
ab102	*ara*, sense, *Eco*RI	GTTTCTTCGAATTCGCGGCCGCTTCTAGAGCAATTCCGATAAAAGCGGATTC
VS10_10	*ara*, antisense, *Nde*I	ATCCCATATGTAATTCCTCCTGTTAG
ab61	*luxCD*, sense, *Nde*I	AATACCTAGGAAGAAGGAGATATACATATGACTAAAAAAATTTCATTCATTATTAAC
ab104	*luxCD*, antisense, *Bam*HI	CAATGGATCCTTAAGACAGAGAAATTGCTTGATTTTC
ab105	*luxE*, sense, *Bam*HI	CAATGGATCCTTATCTTGAGGAGTAAAACAGGTATGACTAGTTATGTTGATAAACAAGAAATTACAG
ab106	*luxE*, antisense, *Pst*I	GTTTCTTCCTGCAGCGGCCGCTACTAGTATTAACTATCAAACGCTTCGGTTAAGC

only partly characterized. Therefore it is quite evident that the WE concentration obtained with the reconstructed synthetic pathway at this stage is low, approximately half the amount obtained from the wild type strain in studied conditions. In order to significantly accelerate the WE production it is crucial to optimize the complete set of enzymes and conditions related to the WE synthesis. In addition, the localization and compartmentalization of the pathway related enzymes should be considered; especially the membrane-bound enzymes may possess physical proximity and interaction between the enzymes.[18]

Long-chain aldehydes are important precursors for several high-value hydrocarbons such as alkanes, fatty alcohols and wax esters.[19,20] However, in many natural long-chain hydrocarbon-producing microbes the production pathway is still poorly understood, including the conversion of fatty acids to fatty aldehydes.[21] As demonstrated in this study, the LuxCDE complex provides a potential alternative for complementation of hydrocarbon production pathway involving fatty aldehyde synthesis.

Conclusions. Synthetic biology provides tools for novel solutions *via* reconstructed pathways, expanding the usability of natural producers in more economical, diverse, and sustainable manner. In this study, the reconstructed wax ester synthesis pathway in *A. baylyi* ADP1 enabled the establishment of a controlled production platform for modified wax esters using well-characterized bioparts. The used fatty acid reductase complex LuxCDE shows potential for a variety of applications regarding long-chain hydrocarbon production.

◼ METHODS

Strains. The wild type strain *A. baylyi* ADP1 (DSM 24193) and a single gene knockout strain of ADP1 (gene deletion *acr1*, ACIAD3383, referred here as ADP1*k*), kindly provided by Veronique de Berardinis (Genoscope, France), were used in the study. In the single gene knockout mutant strain, the gene in question is replaced with a gene cassette containing a kanamycin resistance gene (*Kan*^{r}).[22] The knockout strain was transformed with the construct pBAV1C-*ara*-*luxCDE* described in the Plasmid Construction and Transformation section. The resulting strain is referred as ADP1*cde*.

Plasmid Construction and Transformation. For digestions and ligations, the enzymes and buffers were provided by Fermentas (Lithuania) and used according to manufacturer's instructions. PCR reagents were provided by Finnzymes (Finland) (DNA polymerase Phusion and buffer) and Fermentas (nucleotides). Primers were from ThermoFisher Scientific (USA) with appropriate restriction sites. The primer sequences are presented in Table 3.

For chloramphenicol resistance (C) plasmid, the vector pBAV1K[14] was partially amplified with PCR using primers ab98 and ab99 and ligated to *cat* gene from pAK400c[23] (primers ab100 and ab101 with restriction sites *Nhe*I and *Apa*LI). The arabinose promoter and AraC repressor designated as *ara* was amplified from the plasmid SAKcB (kind gift from Urpo Lamminmäki, unpublished) by primers ab102 (*Eco*RI) and VS10_10 (*Nde*I). The genes *luxC* and *luxD* (ab61 *Nde*I, ab104 *Bam*HI), and *luxE* (ab105 *Bam*HI, ab106 *Pst*I) were amplified from the plasmid pCGLS1,[24] ligated together, amplified again using primers ab61 and ab106, and ligated to *ara*. The fragment *ara*-*luxCDE* was amplified using primers ab102 and ab106 and inserted to the plasmid pBAV1C using restriction sites *Eco*RI and *Pst*I. An internal *Nde*I site within the gene *luxE* were disrupted by appropriate primer design. Purifications of the PCR products were carried out using PCR purification kit (Fermentas) or gel extraction kit (Fermentas) for agarose gel.

The plasmid constructed was transformed to ADP1Δ*acr1*::-*Kan*^{r}/*tdk* (ADP1*k*) by natural transformation as described previously.[9] The colonies were selected on LA plates containing chloramphenicol (50 μg/mL) and glucose. The correct construct was verified by restriction analysis.

Medium Composition. The minimal salts medium MA/9 described previously[9] was used for the cultivations. Cas amino acids (Difco, USA) were added at concentration (0.2 wt %). Glucose (5%) was used as a carbon and energy source. L(+)Arabinose was used for induction when appropriate.

Functional Verification of the Construct. For testing the induction abilities of the construct, the strains ADP1*cde* and ADP1*k* were cultivated at 30 °C, 300 rpm until an early exponential growth phase was reached. A plasmid carrying a gene cassette with bacterial luciferase genes *luxAB* was added to two parallel cultivations (500 ng of plasmid DNA/2 mL of culture) and incubated for 1 h at 30 °C 300 rpm. The details of the gene cassette are described elsewhere;[2] briefly, the cassette contains *luxAB* genes under T5 promoter and flanking regions for the genome integration. For all cultivations, 200 μL of cell cultures (transformed strains and controls strains without cassette addition) were added to the microtiter plate wells as three parallel samples, and different concentrations of arabinose (1.0, 0.5, 0.1, and 0%) were added to the wells to induce the *luxCDE* expression. The bioluminescence was monitored at 15 min intervals by Chameleon multilabel counter (Hidex, Finland).

Cultivations. For confirming the WE formation in ADP1*cde,* the strain was cultivated as four parallel cultures in 50 mL of medium at 30 °C and 300 rpm for 48 h. The cultures were supplemented with arabinose at concentrations 0, 0.1, 0.5, and 1.0%.

For determining the optimal temperature for wax ester production, ADP1 was precultivated in 5 mL of medium at 30 °C and 300 rpm overnight. For seven growth tubes, 9.5 mL of fresh medium were inoculated with 500 μL of the preculture and incubated in Terratec temperature gradient incubator (Tasmania) at temperatures 20, 23, 26, 29.5, 32, 35, and 37.5 °C at oscillation speed 120 rpm for 48 h. Optical density was measured, and 2 mL of TLC samples were taken after 24 and 48 h. At the end-point, 5 mL of cells were collected and freeze-dried for determination of CDW.

For qualitative GC−MS analysis of WEs, the strains ADP1 wild type, ADP1k, and ADP1cde were cultured as two to four individual batch cultures in 50 mL of medium at 20 °C and 200 rpm for 70 h. The cultures for strains ADP1k and ADP1cde were supplemented with kanamycin 30 μg/mL and for ADP1cde also with chloramphenicol 25 μg/mL. Cultures contained also 0.5% (m/V) arabinose.

For all cultivations, the cells were collected by centrifugation, and the cell pellets were freeze-dried in ALPHA 1−4 LD plus freeze-dryer (Martin Christ, Germany).

Lipid Extraction and Analysis. For TLC and NMR analysis the lipids were extracted from the freeze-dried biomass samples as described in Santala et al.[2,9] The amount of total lipids was determined gravimetrically. For GC−MS analysis, the lipids were extracted with the following procedure prior to the analysis. Sample (30−100 mg) was weighed in Eppendorf tube, steel bead (5 mm) was added, and sample was homogenized for 2 min at 50 Hz. Methylnonadecanoate was added as an internal standard. 400 μL of 0.5 M NaOH solution in methanol (2.0 g of NaOH per 100 mL of methanol) was added to the tube, and sample was treated 3 min at 40 Hz in Qiagen TissueLyser. Thereafter, samples were shaken (650 rpm) at 60 °C for 20 min. After this, 500 μL of BF3 in methanol (15% BF3 in methanol) was added to the tubes, and shaking was continued for additional 3 min. Heating was stopped, and 250 μL of iso-octane was added. After this, 700 μL of NaOH solution (NaOH saturated in water) was added, and samples were mixed. Samples were centrifuged at 13 000 rpm for 10 min, and 50−100 μL of upper phase containing iso-octane was transferred to the GC vial. Fatty acids and fatty alcohols were quantified with GC-FID according to ISO15304 method and identified with GC−MS. Glycerolipid distribution was analyzed from biomass samples according to EN ISO 15304:2002M. Samples were extracted with direct saponification and glycerolipid distribution was analyzed by HPLC-GPC (EN ISO 15304:2002).

In order to visualize the temperature dependency of WE production, TLC analyses were carried out for extracted lipids as described previously by Santala et al.[2] Mobile phase used was n-hexane:diethyl ether:acetic acid 90:15:1. Palmitoyl-palmitate (Sigma) was used as a standard. Of extracted lipids, 30 μL of each sample was applied on the TLC plate, and iodine was used for visualization.

For quantitative determination of the construct functionality and the effect of arabinose induction on ADP1cde the WE content of the cells was determined by ^{1}H NMR (Varian Mercury spectrometer 300 MHz) as described by Santala et al.[2] Spectra were recorded in chloroform-d_3 with trifluortoluene as an internal standard (1.47 mg/0.7 mL of chloroform-d_3). Chemical shifts were quoted as parts per million relative to tetramethylsilane ($\delta = 0$), and spectra were processed using ACD NMR processor program. Phase correction and baseline correction were applied to all the spectra.

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Author Contributions

S.S. and V.S. designed the study. S.S. performed the molecular work, microbiological work, TLC analyses, and wrote the manuscript. E.E. carried out the NMR lipid analyses. P.K. was responsible for the GC−MS and HPLC-GCP analysis of lipids. V.S. and M.K. supervised and coordinated the study. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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