Engineering the carotenoid biosynthetic pathway in \textit{Rhodothermus marinus} for lycopene production

Thordis Kristjansdottir\textsuperscript{a,b,1,*}, Emanuel Y.C. Ron\textsuperscript{c,1}, Daniel Molins-Delgado\textsuperscript{d}, Olafur H. Fridjonsson\textsuperscript{a}, Charlotta Turner\textsuperscript{d}, Snaedis H. Bjornsdottir\textsuperscript{e}, Steinn Gudmundsson\textsuperscript{b}, Ed W.J. van Niel\textsuperscript{1}, Eva Nordberg Karlsson\textsuperscript{c}, Gudmundur O. Hreggvidsson\textsuperscript{a,e}

\textsuperscript{a} Matis, Vinlandslei 12, 113 Reykjavik, Iceland
\textsuperscript{b} University of Iceland, Department of Computer Science, School of Engineering and Natural Sciences, Dunhagi 5, 107 Reykjavik, Iceland
\textsuperscript{c} Lund University, Department of Chemistry, Division of Biotechnology, Box 124, 221 00, Lund, Sweden
\textsuperscript{d} Lund University, Department of Chemistry, Division of Applied Microbiology, Box 124, 221 00, Lund, Sweden
\textsuperscript{e} University of Iceland, Department of Biology, School of Engineering and Natural Sciences, Starflugs 7, 102 Reykjavik, Iceland
\textsuperscript{1} Lund University, Department of Chemistry, Centre for Analysis and Synthesis, Box 124, 221 00, Lund, Sweden

* Corresponding author. Matis, Vinlandsleid 12, 113 Reykjavik, Iceland.
E-mail address: thordis@matis.is (T. Kristjansdottir).

\textsuperscript{1} These authors contributed equally to this work.

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\textbf{ABSTRACT}

\textit{Rhodothermus marinus} has the potential to be well suited for biorefineries, as an aerobic thermophile that produces thermostable enzymes and is able to utilize polysaccharides from different 2nd and 3rd generation biomass. The bacterium produces valuable chemicals such as carotenoids. However, the native carotenoids are not established for industrial production and \textit{R. marinus} needs to be genetically modified to produce higher value carotenoids. Here we genetically modified the carotenoid biosynthetic gene cluster resulting in three different mutants, most importantly the lycopene producing mutant TK-3 (\textit{ΔcrtBΔpurAΔcruFΔcrtBΔthermophilus}). The genetic modifications and subsequent structural analysis of carotenoids helped clarify the carotenoid biosynthetic pathway in \textit{R. marinus}. The nucleotide sequences encoding the enzymes phytoene synthase (CrtB) and the previously unidentified 1,2-hydration (CruF) were found fused together and encoded by a single gene in \textit{R. marinus}. Deleting only the \textit{cruF} part of the gene did not result in an active \textit{CrtB} enzyme. However, by deleting the entire gene and inserting the \textit{crtB} gene from \textit{Thermus thermophilus}, a mutant strain was obtained, producing lycopene as the sole carotenoid. The lycopene produced by TK-3 was quantified as 0.49 g/kg CDW (cell dry weight).

1. Introduction

\textit{Rhodothermus} is the type genus of the phylum \textit{Rhodothermaeota} (Munoz et al., 2016). The aerobic bacterium \textit{R. marinus} is thermophilic and moderately halophilic, growing optimally at 65 °C and 1–2% NaCl (Alfredsson et al., 1988). The bacterium has potential for biorefinery applications, as it possesses many features of importance for industrial bioconversion of recalcitrant 2nd and 3rd generation feedstock. This includes various biomass-degrading enzymes, as well as the capacity to produce interesting primary and secondary anaerobic compounds, such as carotenoids (Ron et al., 2018) and exopolysaccharides (EPS) (Sardari et al., 2017). In addition, as a robust extremophile, \textit{R. marinus} is adapted to growth at high temperatures which may be beneficial for biorefining of 2nd and 3rd generation feedstocks. High temperature increases the solubility of polysaccharides used as carbon sources and reduces the viscosity of fermentation broths. Consequently, it enables higher feedstock loads and facilitates enzymatic access to polysaccharides. In addition, growth at high temperatures in bioreactors mitigates scale-up problems of mixing and aeration, reduces costs of cooling, distillation and extraction and minimizes the danger of contamination of spoilage bacteria (López-Contreras et al., 2017).

To generate efficient biorefinery production strains, metabolic engineering is often required to modify the production profile of the microorganism. A few thermophiles have to date been the subject of metabolic engineering efforts, mainly anaerobic thermophiles for production of biofuels (and/or commodities), with encouraging results (Nordberg Karlsson et al., 2020). Tools for genetic engineering of the thermophilic aerobe \textit{R. marinus} have been developed, and genes have both been
heterologously expressed (Bjornsdottir et al., 2007) and deleted from the genome (Bjornsdottir et al., 2011).

Selection of a chassis species for metabolic engineering of pathways for production of compounds of industrial interest should be based on the metabolic characteristics of the organism, including both the substrate utilization range required for a particular feedstock, and the synthetic capabilities or potential. Anaerobic fermentative species are generally preferred for production of lower value commodity chemicals, such as simple organic acids and alcohols, which are typically waste products from catabolic metabolism. However, aerobes can carry a greater metabolic burden and are the organisms of choice for the heterotrophic production of complex secondary metabolites. This includes carotenoids, which are pigments, naturally produced by many plants, algae and bacteria. Currently, over 1100 carotenoid structures from more than 600 organisms are known (Yabuzaki, 2017). They are widely used as colorants and additives in the food, feed and cosmetic industries. Numerous studies have shown that carotenoids have potential health benefits, both in preventing and treating various diseases (Mein et al., 2008; Sathasivam and Ki, 2018). However, the biological functions of carotenoids are complex and other studies have reported conflicting results (Young and Lowe, 2018). Nevertheless, the global market for carotenoids has reached USD 1.5 billion and is predicted to continue growing (Markets And Markets, 2020).

*R. marinus* natively produces y-carotenoids and their structures have been characterized (Lutnaes et al., 2004; Ron et al., 2018). Four variations of carotenoid glucoside esters were demonstrated, including that of salixinanthin. These native carotenoids are derived from y-carotene, while most of the carotenoids of industrial interest are b-carotenoids. Astaxanthin, b-carotene, canthaxanthin, zeaxanthin, lycopene and lutein are in highest demand (Markets And Markets, 2020).

We previously carried out a bioinformatic analysis of the carotenoid biosynthetic pathway in *R. marinus* (Ron et al., 2018). The feasibility of modifying the pathway has also been demonstrated in a prior study, where the genes encoding the phytoene desaturase (CrtI) and phytoene synthase (CrtB) enzymes of the pathway were deleted, resulting in the colorless *R. marinus* strain SB-71 (Bjornsdottir et al., 2011). The present study aims to clarify the function of the genes involved in the *R. marinus* carotenoid biosynthesis, and to engineer the pathway for lycopene production. Lycopene is one of the more demanded carotenoids on the market, used in cosmetics, pharmaceuticals and as a food coloring agent, and it is also the common precursor of y- and b-carotenoids. For this purpose and to illustrate the potential of *R. marinus* as a biorefinery strain, lycopene production is an ideal first target for metabolic engineering.

2. Methods and materials

2.1. Strains, plasmids, media and culture conditions

The strains and plasmids used and generated in this study are listed in Table 1. For genetic modifications of *R. marinus*, strain SB-62 ([ΔtrpBΔpurA](Bjornsdottir et al., 2011)) was used. Tryptophan selection was used in all cases, leaving the possibility of adenine complementation for further genetic modifications of the strains in the future. Genomic DNA from *R. marinus* ISCaR-493 and *Thermus thermophilus* HB8 as well as the plasmid vector pHCI9 (Yanisch-Perron et al., 1985) were used for generating recombinant molecules. NEB Stable *E. coli* cells (New England Biolabs) were used for molecular cloning. The pRM3000 plasmid (Bjornsdottir et al., 2007) was used as a control in the R. marinus transformation experiments. All *R. marinus* cells were cultured at 65 °C and the liquid cultures set to shaking at 200 rpm (New Brunswick Innova 4400 Incubator Shaker). Medium 162 (Degvre et al., 1978) was used, with modifications (2 mM MgSO4 and 0.2 mM CaCl2 in final volume) and additions of 1% NaCl and 0.053% NH4Cl. Two variations of the medium were used: A rich non-selective medium (R-medium) which contained 0.25% tryptone and 0.25% yeast extract and a selective agar medium (RS-medium), which contained 0.2% soluble starch, 0.2% casamino acids, vitamin solution (Degvre et al., 1978), 0.25% adenine and 2.5% agar. E. coli cells were cultured at 37 °C in LB-medium (Miller, 1972) with 100 μg/mL ampicillin.

For the analysis of carotenoids, *R. marinus* strains TK-1, TK-2, TK-3 and SB-71 were grown on agar plates with modified medium 162 supplemented with 1% NaCl and 0.025 g/L adenine. After 48 h at 65 °C, the colonies were transferred to 5 ml liquid LB-medium, supplemented with 0.025% adenine, and cultivated at 65 °C for 24 h in 50 ml falcon tubes. The cells were then cultivated in baffled shake flasks at 200 rpm increasing the cultivation volume from 50 mL to 100 mL sequential cultivations, maintaining 10% (v/v) inoculum volume. The harvested cells were then split and transferred to two 500 ml bioreactors (Multifors 2, Infors) for cultivation with the following parameters: 65 °C, pH 7, 1 VVM aeration, 200 rpm stirrer rate cascaded with dissolved oxygen tension (DOT) that was set to 40%. The cultivation was terminated at the onset of the stationary phase, indicated by a sudden increase of DOT, at which the cell culture was set to cool at 10 °C. The cells were harvested by centrifugation at 4 °C at 10,000 g for 10 min, after which the pellets were frozen at -80 °C and lyophilized until constant weights were reached.

2.2. Design and generation of cloning molecules

Recombinant DNA molecules were designed using the plasmid vector pHCI9 and amplified regions of the genome sequences of *R. marinus* ISCaR-493 (Matís, unpublished) and *T. thermophilus* HB8 (NC_006461.1). DNA was isolated from the strains using the MasterPure Complete DNA purification Kit (Lucigen). Primers (Table S1) were designed for the amplification of ~1500 bp y’ and 3’ flank regions of the genes targeted for deletion as well as the trpB gene (selection marker) and the crtB gene from *T. thermophilus*. The primers were designed to support HIFI DNA assemblage (NEBuilder HiFi DNA Assembly Master Mix, New England Biolabs). The primers Gene5_5_F, Gene5_5_R (5’ flank region), Gene1_3_F, Gene1_3_R (3’ flank region), Gene5_Gene14_F and Gene5_Gene14_R (trpB gene) were used to amplify the fragments for the gene deletion
cassette used to obtain strain TK-1 (ΔmBΔpurAΔcrteYQ::trpB). The primers Gene9a_5_F, Gene9a_5_R (5' flanking region), Gene9a_3_F, Gene9a_3_R (3' flanking region), Gene9a_Gene14_F and Gene9a_Gene14_R (trpB gene) were used to amplify the fragments for the gene deletion cassette used to obtain strain TK-2 (ΔmBΔpurAΔcrteF::trpB). The primers Gene9_5_F, Gene9_5_R (5' flanking region), Gene9_3_F, Gene9_3_R (3' flanking region), Gene9_Gene13_F, Gene9_Gene13_R (crtB gene from T. thermophilus), Gene9_Gene14_F and Gene9_Gene14_R (trpB gene) were used to amplify the fragments for the gene deletion/insertion cassette used to obtain strain TK-3 (ΔmBΔpurAΔcrteF::trpHocBtrt::trpB::crtF::trpB::crtB::trpB::crtB::trpB::crtB::trpB::crtB::trpB::crtB::trpB::crtB::trpB::crtB::trpB::<br> thermophilus). All following enzymes, kits and cells in this section were obtained from New England BioLabs. Q5 High-Fidelity DNA polymerase was used in all amplifications according to the manufacturer’s instructions. The correct sizes of the resulting fragments were verified by electrophoresis and they were subsequently purified from gel using the Monarch DNA Gel Extraction Kit. The fragments were assembled into Smal restricted pUC19 vector and introduced into competent E. coli cells (NEB stable) by chemical transformation. Positive clones were confirmed by verifying the presence of the trpB gene by PCR, using Taq DNA polymerase and the primers Gene14.Verify_F and Gene14.Verify_R (Table S1). Vectors were isolated from positive clones using the Monarch Plasmid Miniprep Kit. Linear inserts for R. marinus transformation for the construction of strains TK-1 and TK-2 were obtained by PCR, using the Q5 High-Fidelity DNA polymerase and the primers Gene5_5_F and Gene1_3_R, and TK2.Total_F and TK2.Total_R (Table S1), respectively. A linear insert for R. marinus transformation for the construction of strain TK-3 was obtained by digesting the plasmid with XhoI and KpnI-HF.

2.3. Transformation of R. marinus

R. marinus cells were prepared for electroporation as described elsewhere (Bjornsdottir et al., 2005). The electroporation protocol was followed, using the GenePulser Xcell electroporation system (Bio-Rad) with 20 kV/cm pulses delivered at 20 kV/cm 1 μg of DNA was used per transformation, in ≤5 μL and mixed carefully with 40 μL of washed cells. Negative (sterile MilliQ water) and positive (pRM3000) controls were included. Transformed cells were grown on selective agar plates (without tryptophan) for 3–5 days.

2.4. Verification of genotypes using PCR and sequencing

Positive R. marinus clones were verified by PCR, using Taq DNA polymerase. Modifications of strain TK-1 were verified using primers Gene7,-Verify_F, Gene7.Verify_R, Gene5.Verify_F, Gene5.Verify_R, Gene4.Verify_F, Gene4.Verify_R, Gene14.Verify_F and Gene14.Verify_R (Table S1). Modifications in strains TK-2 and TK-3 were verified using the primers Gene9a.Verify_F, Gene9a.Verify_R, Gene9b.Verify_F, Gene9b.Verify_R, Gene13.Verify_F, Gene13.Verify_R, Gene14.Verify_F and Gene14.Verify_R (Table S1). Additionally, the modifications were confirmed by sequencing. Several PCR reactions were performed for strains SB-62 and TK-1 using the primers Gene1_seq_F, Gene1_seq_R, Gene4_seq_F, Gene4_seq_R, Gene5_seq_F and Gene5_seq_R (Table S1) and Q5 DNA polymerase. The resulting amplicons were sequenced using the ABI3730 system (Applied Biosystems, Thermo Fisher Scientific). For strain TK-2, Q5 DNA polymerase and primers Gene9_seq_F and Gene9_seq_R (Table S1) were used to amplify a 5188 bp region. For strain TK-3, Q5 DNA polymerase and primers Gene9_seq_F and Gene9_seq_R (Table S1) were used to amplify a 4686 bp region. Sequencing libraries were made from the amplicons using the Nextera DNA (TK-2) and Nextera Flex (TK-3) methods (Illumina) and libraries were sequenced on the Illumina MiSeq sequencing platform using the V3 2 × 300 cycle chemistry. Obtained fragments were assembled using Geneious.

2.5. Carotenoid extraction

Carotenoids were extracted from R. marinus strains TK-1, TK-2, TK-3, SB-71 and ISCaR-493 using two methods. In the first method, aqueous cell suspensions were sonicated in an ice bath for 5 × 2 min, with 1-min rests in-between in order to keep cold sample conditions. After sonication the samples were mixed with ethyl acetate (1:1). In the second method, lyophilized cells were powdered with a glass rod before mixing with dichloromethane (25 mL solvent per g freeze-dried cells). All organic phase extracts were vacuum filtered before being dried by rotary evaporation (Heidolph instruments) at 80 rpm at 40 °C. The extracts were reconstituted in 3 mL of dichloromethane, filtered through a 0.2 μm PTFE syringe filter, flushed with N2 gas and stored at -80 °C until mass spectrometry analysis.

Carotenoids were additionally extracted from strains TK-1, TK-2, TK-3, SB-71 and ISCaR-493, for absorbance spectra analysis, by mixing full loops of cells from agar plates with hexane:acetone (1:1) and sonicating in a bath for 20 min. This was done in triplicates.

2.6. Carotenoid analysis

The carotenoid extracts were analyzed by means of supercritical fluid chromatography – mass spectrometry (MS) in an Ultra Performance Convergence Chromatography system (UPC²) coupled to a quadrupole–orthogonal acceleration time-of-flight tandem mass spectrometer (XEVO-G2 Q-TOP) with an electrospray ion source, both instruments from Waters (Milford, MA, USA). Both systems were controlled, and all data was analyzed, with Masslynx™ (v. 4.1, SCN 77; Waters). The chromatographic method used was based on a modification of the method described in (Jumaah et al., 2016). Briefly, the chromatographic separation was achieved using Acquity UPC² Torus 1-Aminoanthracene (100 mm × 3 mm, 1.7 μm) column from Waters. The mobile phase consisted of (A) CO2 and (B) methanol in a gradient elution analysis programmed as follows: 0–0.5 min, 5% (B); 0.5–2.5 min, 5–15% (B); 2.5–8 min, 15% (B); 8–9 min, 15-5% (B); and 9–10 min, 5% (B). The flow rate was 1.5 mL min⁻¹, column temperature was kept at 56 °C and backpressure at 160 bar. Ammonium formate (10 mM) in methanol was used as a make-up solvent at a flowrate of 0.5 mL min⁻¹. The total run-time of the program was 10 min.

UV-Vis spectra were recorded in the range of 200–500 nm by the diode array detector (DAD). The electrospray ionization (ESI) ion source was operated in positive mode and full-scan MS spectra were obtained by scanning the range m/z 50–1000. The mass spectrometer was calibrated with sodium formate. Centroid mode data was collected after mass correction during acquisition using an external reference comprising of 10 μL/min solution of leucine-enkephalin (2 ng/μL). The capillary and cone voltage were set at 3 kV and 40 V, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (800 L/h). The source and desolvation temperature were set at 150 and 300 °C, respectively. Simultaneous acquisition of exact mass at high and low collision energy, MS² (where E represents collision energy), was used to obtain full scan accurate mass fragment, precursor ion, and neutral loss information. The collision energy in function 1 (low energy) was off while in function 2 (high energy) and the collision energy ranged between 15 and 60 V. MS/MS analysis of the carotenoids was performed with the quadrupole set at m/z 536 in MS² mode.

The absorbance spectra (350–600 nm) of the carotenoids of strains TK-1, TK-2, TK-3, SB-71 and ISCaR-493, extracted with hexane:acetone (1:1), were analyzed in a benchtop spectrophotometer (MULTISKAN Sky, Thermo Scientific), using 1 cm cuvette. This was done to investigate the profile of the spectra and ΔAmax Values of obtained peaks.

Lyophilized TK-3 cells from shake flask cultivations were weighed before organic solvent extraction and reconstitution in chloroform. The extracts were then analyzed by a spectrophotometer at 485 nm. The lycopene concentration was calculated using Beer’s law with the molar attenuation coefficient of 150855 L mol⁻¹ cm⁻¹ for lycopene in chloroform (Naviglio et al., 2008). Lycopene, α-, β- and γ-carotene standards were of analytical grade quality (Supelco).

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3. Results

3.1. Bioinformatic analysis of genes encoding carotenoid biosynthetic enzymes

Several strains of the thermophilic bacterium *R. marinus* produce carotenoids derived from γ-carotenoid. Previous gene homology searches for known carotenoid biosynthetic genes using the genomic data of the type strain *R. marinus* DSM 4252T, resulted in the discovery of genes encoding homologues to enzymes in the carotenoid biosynthesis pathways from other species. This allowed the identification of a gene cluster, with two adjacent operons, one small and one larger (Bjornsdottir et al., 2011; Ron et al., 2018). In this work, the bioinformatic analysis of genomic sequence data was expanded, enabling further identification of the enzymes in the biosynthetic pathway for carotenoid production in *R. marinus* (Fig. 1).

Here, we report the identification of the *cruF* gene in the gene cluster (Fig. 2), which likely acts as a 1′,2′-hydratase (Sun et al., 2009). This gene was not identified in the previous work because it is fused together with the phytoene synthase (*crtB*) gene (Fig. 2A, gene 9) and together they were annotated as a phytoene synthase. These genes are usually separate, while *cruF* in *R. marinus* is without a stop codon and fused with the downstream *crtB* in one open reading frame (ORF). The fused genes, along with the carotenoid genes acyltransferase (*cruD*) and phytoene desaturase (*crtI*) (Fig. 2A, genes 9, 11 and 12, respectively) are located in the smaller operon in the gene cluster. Two additional genes are also present in the smaller operon, encoding products showing homology to hypothetical proteins (gene 10) and a MerR family transcriptional regulator from *T. thermophilus* (31% similarity) (gene 8).

Two genes in the larger operon were also identified based on sequence homology, with high sequence similarity to the known carotenoid genes encoding lycopene cyclase (*crtY*) (50%) (Fig. 2A, gene 2) and carotene ketolase (*crtO*) (60%) (Fig. 2A, gene 3). Five other genes (Fig. 2A, genes 1, 4–7) were found in this larger operon, but since they lack sequence homology to known genes in carotenoid biosynthetic pathways, their role in the pathway remains unknown. They were annotated as hypothetical protein (gene 1), NAD dependent epimerase (gene 4), FAD dependent oxidoreductase (gene 5), which showed slight

![Fig. 1. Proposed carotenoid biosynthetic pathway in *R. marinus*, based on (Ron et al., 2018), with added information obtained in this study. The molecular structures and abbreviated enzyme names are shown, phytoene synthase (*CrtB*), 1′,2′-hydratase (*CruF*), phytoene desaturase (*CrtI*), glycosyltransferase (*CruC*), acyltransferase (*CruD*), lycopene cyclase (*CrtY*) and carotene ketolase (*CrtO*).](image-url)
Homology (36% similarity) to cfr, deoxyribodipyrimidine photo-lyase (gene 6) and short-chain dehydrogenase/reductase (gene 7). Several known carotenoid biosynthetic genes belong to the cfr gene family, including cfrO (gene 3, above) and also 3',4'-desaturase, an enzyme for which the corresponding gene is not identified in R. marinus. The native structures of the carotenoids in R. marinus suggest that a 3',4'-desaturase is a part of the biosynthetic pathway, but since the cfr homology of gene 5 is low and there is no other evidence so far that it encodes a 3',4'-desaturase, this step in the pathway is still labeled as unknown (Fig. 1).

3.2. Genetic modifications to alter the carotenoid production

Three different genetic modifications were performed in the carotenoid gene cluster of R. marinus strain SB-62 (ΔtrpBΔpurA) (Fig. 2). The target genes were deleted and replaced with the selective marker trpB (encoding the tryptophan synthase beta chain), using double crossover homologous recombination using linear insertion cassettes. In the first modification, a 5890 bp region was deleted from the larger operon, resulting in the R. marinus mutant TK-1 (ΔtrpBΔpurAΔcrtYO::trpB) (Fig. 2B). This region includes the genes cfrY (gene 2) and cfrO (gene 3) which encode the enzymes responsible for the 4-keto β-ionone ring modifications displayed on the left side of the carotenoid backbone (Fig. 1), as well as gene 5, where the deduced amino acid sequence is showing slight homology to cfr (see section 3.1). The other two modifications involved knocking out the cfrF gene (1',2'-hydratase), which encodes one of the enzymes that modify the right side of the carotene backbone (Fig. 1). In the first cfrF modification, only the cfrF part of the gene was deleted, leaving the cfrB part intact, resulting in the R. marinus mutant TK-2 (ΔtrpBΔpurAΔcfrF::trpB) (Fig. 2C). The cfrB part of the gene includes a start codon, which raises the question if it could encode a functional enzyme without the cfrF part. In the second cfrF modification, both the cfrF and cfrB parts of the gene were deleted and the cfrB gene from Thermus thermophilus strain HB8 was inserted, resulting in the R. marinus mutant TK-3 (ΔtrpBΔpurAΔcrtB::trpBcrtBT.thermophilus) (D). Genes involved in the carotenoid biosynthesis are colored grey. Gene deletions and insertions were verified using PCR (1–14) and the sequencing of PCR products (a–g). Pictures showing gel electrophoresis following PCR are shown to the right side of the gene clusters. Lane M1: 100 bp DNA ladder. Lane M2: 1 kb DNA ladder. Unedited electrophoresis pictures and sequencing results are shown in the supplementary file (Figs. S1–S11).

For strain SB-62 all reactions resulted in the expected sized amplicons, except for the cfrB gene from T. thermophilus in TK-2 would be non- or low functioning after the deletion of the cfrB part. Successful genetic modifications were verified by amplifying target regions from the genomes of putative mutants, and sequencing. PCR reactions amplifying several genes in the cluster (genes 1, 4, 5, 7, 9a (cfrA), 9b (cfrB), 13 and 14 in Fig. 2), using primers designed to bind inside each gene, were performed for all three mutants and strain SB-62. For strain SB-62 all reactions resulted in the expected sized amplicons, except for the cfrB gene from T. thermophilus and the trpB gene (Fig. 2A). The presence of an amplicon using primers for amplifying the cfrB gene from T. thermophilus in R. marinus was not expected. However, in all R. marinus strains the same sized unknown amplicon (~300 bp) was obtained and was subsequently sequenced. This showed that the amplification occurred from a location on the chromosome distant from the carotenoid gene cluster (Fig. S9). This gene did not show homology to cfrB from T. thermophilus. The primers used to amplify the cfrB gene from T. thermophilus did however show homology to this region, which explains the unexpected amplification. The trpB gene was not amplified in strain SB-62 which was to be expected since it has the trpB gene deleted, enabling us to use it as a selective marker.

For strain TK-1 (ΔtrpBΔpurAΔcfrYO::trpB), the PCR results showed two main differences compared to strain SB-62 (Fig. 2B). First, genes 1, 4 and 5 were not amplified, which indicated the successful deletion of the
region. Second, the reaction for the trpB gene (gene 14) did result in the expected sized amplicon, indicating the insertion of the selection marker. To further verify this, additional PCR reactions (a – f in Fig. 2) were performed for strains SB-62 and TK-1 and the products were sequenced (Figs. S5 and S6).

For strains TK-2 (ΔtrpBΔpurAΔcruF::trpB) and TK-3 (ΔtrpBΔpurAΔcruFcrB::trpBcrB::thermophilus), the cruF part of gene 9 was not amplified, while gene 14 was, indicating the expected substitution of cruF for trpB. Additionally, an amplicon of the expected size for gene 13 (crtB from T. thermophilus) was observed in strain TK-3. An identical amplicon was observed from T. thermophilus (Fig. S1). The native crtB gene (second half of gene 9) was deleted from strain TK-3 and the amplicon seen in strain SB-62 (~600 bp) was not amplified in strain TK-3. However, a smaller unknown amplicon (~400 bp) was obtained and was subsequently sequenced. The results showed that the amplification did not occur from the carotenoid gene cluster (Fig. S9), but in a gene on the chromosome distant from the cluster. This gene did not show homology to the crtB gene, but the primers used to amplify crtB in R. marinus did show homology to this region, which explains the unexpected amplification. To further verify the successful modifications in strains TK-2 and TK-3, additional PCR reactions were performed (g in Fig. 2) and the resulting products were sequenced (Figs. S10 and S11).

3.3. Analysis of carotenoids by UHPSFC-DAD-QTOF/MS and spectrophotometry

Absorbance spectra of carotenoids from strains TK-1 (ΔtrpBΔpurAΔcruF::trpB), TK-3 (ΔtrpBΔpurAΔcruFcrB::trpBcrB::thermophilus) and ISCar-493 extracted with hexane:acetone (1:1), showed A\text{max} for strain ISCar-493 at 478 nm, for TK-1 at 488 nm and for TK-3 at 472 nm, with an additional peak at 502 nm (Fig. 3and Figs. S17-S19). The absorption spectra of the carotenoids for strain ISCaR-493 at 478 nm, for TK-1 at 488 nm and for TK-3 at 472 nm, showed A\text{max} values of obtained peaks.

To further verify this, additional PCR reactions (a – f in Fig. 2) were performed for strains SB-62 and TK-1 and the products were sequenced (Figs. S5 and S6). The middle row shows absorbance spectra from 350 to 600 nm of carotenoids isolated with acetone:hexane (1:1) and A\text{max} values of obtained peaks. Graphs with both axes are shown in Figs. S17-S19. The bottom row shows the chemical structures of identified carotenoids: Native carotenoids (4-keto 2′-hydroxy β-ψ-carotene acyl glycoside), modified lycopene (2′-hydroxy ψ-ψ-carotene acyl glycoside) and lycopene. For more information on R₁ and R₂, refer to Fig. 1.

![Absorbance spectra and chemical structures](image-url)
extracted from ISCaR-493 and TK-1 (Fig. 3) show a clear shift towards higher wavelengths for the TK-1 carotenoids, suggesting the presence of a ψ-end and therefore the deletion of the crtY gene.

The extract of \textit{R. marinus} strain TK-3 \((\Delta \text{tcrB} \text{purA} \Delta \text{cruF} \text{crte} B::\text{trpB} \text{crte} B_{\text{thermophilus}})\) showed a sole peak \((t_q 2.75 \text{ min})\) by DAD \((\lambda_{250} \text{ nm})\). This peak had a mass \((m/z 536.428 \pm 0.010)\) corresponding to \(\text{C}_{48} \text{H}_{83}\) carotenoids, such as \(\alpha\)-caroteone, \(\beta\)-caroteone, \(\gamma\)-caroteone and lycopene. By comparing the \(t_q\) of the peak of TK-3 to these carotenoid standards, TK-3 had the same \(t_q\) as lycopene (Figs. S12 and S13). In addition, MS/MS fragmentation spectra were analyzed. The caroteones \(\gamma\)-caroteone and lycopene have acidic ψ-ends, which can be determined by removal of an isoprene unit, resulting in a \([M-69]^+\) fragment of \(m/z 467.368\) \((\text{van Breemen et al., 2012})\). This fragment could be detected in the standards \(\gamma\)-caroteone and lycopene but also in the TK-3 MS/MS spectrum at \(t_q 2.75\) min. Moreover, absorption spectra of the TK-3 extract (Fig. 3) shows an identical wavelength profile as established spectra for lycopene \((\text{Britton et al., 2004})\). From these results it can be concluded that lycopene is produced by TK-3 as the sole product from the carotenoid biosynthetic pathway. Lycopene was quantified spectrophotometrically using Beer’s law in TK-3 extracts in a separate shake flask experiment. The result was a yield of \(0.49 \pm 0.01\) g/kg cell dry weight (CDW), which corresponded to \(0.14 \pm 0.004\) mg/L cultivation volume. In conclusion, the results suggest that the \text{cruF}-\text{crte}B gene was successfully deleted from the genome of \textit{R. marinus} and that the heterologous \text{crte}B gene from \textit{T. thermophilus} encodes an active enzyme in strain TK-3. Additionally, and somewhat surprisingly, the only active carotenoid enzymes in TK-3 seem to be \text{CrtB} and \text{CrtI}, leaving the other enzymes, such as \text{CrtY}, non-functional.

4. Discussion

Three successful modifications were performed in the carotenoid gene cluster of \textit{R. marinus}, resulting in two carotenoid producing mutant strains. Based on the results obtained here, we propose the carotenoid biosynthesis pathway in \textit{R. marinus}, which enzymes are involved and in what order they act. The mutant TK-1 \((\Delta \text{tcrB} \text{purA} \Delta \text{cruF} \text{crte}Y::\text{trpB})\) produces a lycopene backbone with all native modifications (displayed on the right side in Fig. 1) still present, but without the 4-keto \(\beta\)-ionone ring. The carotenoid genes \text{crtY} and \text{crtO}, which encode the enzymes that catalyze the keto ionone ring formation, were a part of the 5890 bp region from the larger operon that was deleted in TK-1. The remaining genes of the region apparently do not play a role in the biosynthetic pathway. The enzymes responsible for the modifications displayed on the right side of the backbone are mostly encoded by genes located in the smaller operon of the gene cluster (Fig. 2). They are active without the keto ionone ring according to the structure analysis of the carotenoids obtained from TK-1. This means they are not dependent on the activity of \text{CrtY} and \text{CrtO}.

The results indicate that the mutant strain TK-3 \((\Delta \text{tcrB} \text{purA} \Delta \text{cruF} \text{crte} B::\text{trpB} \text{crte} B_{\text{thermophilus}})\) produces lycopene as the sole carotenoid. This mutant has the \text{cruF}-\text{crte}B gene (fused \text{cruF} and \text{crte}B genes) deleted from its genome and the \text{crte}B gene from \textit{T. thermophilus} inserted. Deleting only the \text{cruF} part of the gene did not result in a carotenoid producing strain (TK-2). Apparently, the \text{crte}B part by itself does not result in the corresponding enzyme activity that is high enough to produce carotenoids at detectable levels. Fused carotenoid genes have been observed in other species, such as the \text{crt}B gene in \textit{Xanthophyllomyces dendrorhous}. Deletions in the lycopene cyclase domain of this gene did not result in an active phytoene synthase either (Xie et al., 2015). TK-3, however, produces lycopene, implying that the \text{crtB} gene from \textit{T. thermophilus} encodes an active enzyme in \textit{R. marinus} and also that without \text{CruF} activity, most of the remaining enzymes downstream in the pathway cannot act on the carotenone backbone. The \text{CrtB} enzyme is clearly still active, as it is essential for producing lycopene. This means that \text{Crt}I is not dependent on the modification done by \text{CruF} and that the two enzymes might act simultaneously in the native pathway. Since \text{cruF} and \text{crte}B are fused in one gene, it can be argued that their corresponding enzymes likely act together or successively. Whether the enzymes are fused into one polypeptide chain or not cannot be concluded from the data presented here. However, our results do show that \text{CrtB} is inactive without \text{CruF}, which suggests that the two enzymes might be acting as one entity. Also, coupled \text{CruF} and \text{CrtB} activities could explain how the organism is able to produce asymmetric carotenoids. While we can only speculate on the order of which these enzymes act, further studies on substrate specificity of the enzymes could elucidate their respective functions. The \text{CruF} enzyme is likely a 1’,2’-hydratase. Without its activity, it was to be expected that the \text{CruC} and \text{CruF} enzymes, which add a glycosyl and an acyl group to the C-1’ hydroxyl group, would not modify the backbone. Our results confirmed this. Two additional native modifications on the right side of the backbone were absent in the carotenoid from TK-3, which are catalyzed by unknown enzymes in \textit{R. marinus}. They add the C-2’ hydroxyl group and the C-3’,4’ double bond. \text{Crt}I is a desaturase and has previously been reported to produce 3,4-dihydrolycopene in \textit{Neurospora crassa} (Hausmann and Sandmann, 2006). It is therefore possible that the \text{Crt}I in \textit{R. marinus} is responsible for the 3’,4’-desaturation. However, since the C-3’,4’ double bond is not present in TK-3, this putative activity of \text{Crt}I must be dependent on the hydration of the C-1’2’ double bond. While we do not have stronger evidence of \text{Crt}I catalyzing the 3’,4’-desaturation in \textit{R. marinus}, this step in the pathway remains unknown (Fig. 1). On the left side of the native carotenone backbone is a 4-keto \(\beta\)-ionone ring, which is not present in the carotenoid produced in TK-3. The \text{crt}Y gene, which encodes a lycopene cyclase, was not disrupted during the genetic modifications, which means that the corresponding enzyme simply cannot act on the backbone without the modifications on the right side of it. Based on this, the enzymes responsible for the modifications on the right side are proposed to modify the backbone before \text{Crt}Y and \text{Crt}O (Fig. 1). To confirm this hypothesis, substrate specificity of the enzymes would have to be tested.

The objective of this work was to engineer the carotenoid biosynthetic pathway in \textit{R. marinus} to produce lycopene instead of its native carotenoids. We successfully obtained the \textit{R. marinus} mutant strain TK-3 \((\Delta \text{tcrB} \text{purA} \Delta \text{cruF} \text{crte} B::\text{trpB} \text{crte} B_{\text{thermophilus}})\), that produced 0.49 g/kg CDW of lycopene. This can be compared to optimized commercial lycopene producing microorganisms, such as \textit{Escherichia coli}, \textit{Saccharomyces cerevisiae} and \textit{Blakeslea trispora}, which have been reported to produce 43.7 (Zhu et al., 2015), 55.6 (Chen et al., 2016) and 15 (Hu et al., 2013; Tereshina et al., 2002) g/kg CDW of lycopene, respectively. Currently, \textit{R. marinus} produces 1-2 orders of magnitude less lycopene than these strains. However, there is considerable room for improvement. Further optimization of the carotenoid production through genetic engineering and culture conditions is likely to yield significantly. For instance, in previous work on cultivation of \textit{R. marinus}, a 28-fold increase of 450 nm absorption was observed in native carotenoid extracts from sequential batch cultivation with cell recycling, as compared to that of shake flask cultivations (Ron et al., 2019). Taking the higher cell densities into consideration, carotenoid absorption per cell density was still 11-fold higher than shake flask cultivations.

\textit{R. marinus} is a robust versatile organism and in many aspects a pre-adapted production organism for utilization of recalciarant polysaccharides in 2nd and 3rd generation biomass, an important task of biorefinery development. In the present study we have demonstrated that \textit{R. marinus} is amenable to metabolic engineering, comprising both gene deletions and insertions, leading to efficient production of a metabolite of industrial interest. The work also revealed the potential of the methodology to help unravel complex pathways.

Declaration of competing interest

None.

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CRediT authorship contribution statement

Thordis Kristjansdottir: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization. Emanuel Y.C. Ron: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. Daniel Molins-Delgado: Methodology, Writing - review & editing. Olafur H. Fridjonsson: Conceptualization, Writing - review & editing. Charlotta Turner: Methodology, Writing - review & editing. Snaedis H. Bjornsdottir: Methodology, Writing - review & editing. Steinn Gudmundsson: Conceptualization, Writing - review & editing. Ed W.J. van Niel: Writing - review & editing. Eva Nordberg Karlsson: Conceptualization, Project administration, Funding acquisition, Supervision, Writing - review & editing. Gudmundur O. Hreggvidsson: Conceptualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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