A genome-scale metabolic reconstruction provides insight into the metabolism of the thermophilic bacterium Rhodothermus marinus

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Abstract

The thermophilic bacterium Rhodothermus marinus has mainly been studied for its thermostable enzymes. More recently, the potential of using the species as a cell factory and in biorefinery platforms has been explored, due to the elevated growth temperature, native production of compounds such as carotenoids and exopolysaccharides, the ability to grow on a wide range of carbon sources including polysaccharides, and available genetic tools. A comprehensive understanding of the metabolism of cell factories is important. Here, we report a genome-scale metabolic model of R. marinus DSM 4252T. Moreover, the genome of the genetically amenable R. marinus ISCaR-493 was sequenced and the analysis of the core genome indicated that the model could be used for both strains. Bioreactor growth data were obtained, used for constraining the model and the predicted and experimental growth rates were compared. The model correctly predicted the growth rates of both strains. During the reconstruction process, different aspects of the R. marinus metabolism were reviewed and subsequently, both cell densities and carotenoid production were investigated for strain ISCaR-493 under different growth conditions. Additionally, the dxs gene, which was not found in the R. marinus genomes, from Thermus thermophilus was cloned on a shuttle vector into strain ISCaR-493 resulting in a higher yield of carotenoids.

Keywords: Rhodothermus marinus; genome-scale metabolic model; carotenoids

Importance

A biorefinery converting biomass into fuels and value-added chemicals is a sustainable alternative to fossil fuel-based chemical synthesis. Rhodothermus marinus is a bacterium that is potentially well suited for biorefineries. It possesses various enzymes that degrade biomass, such as macroalgae and parts of plants (e.g. starch and xylan) and grows at high temperatures (55–77 °C) which is beneficial in biorefinery processes. In this study, we reviewed the metabolism of R. marinus and constructed a metabolic model. Such a model can be used to predict phenotypes, e.g. growth under different environmental and genetic conditions. We focused specifically on metabolic features that are of interest in biotechnology, including carotenoid pigments that are used in many different industries. We described cultivations of R. marinus and the resulting carotenoid production in different growth conditions, which aids in understanding how carotenoid yields can be increased in the bacterium.

Introduction

Rhodothermus marinus is an aerobic bacterium that belongs to the phylum Rhodothermaeota (Munoz et al. 2016). It grows optimally at 65°C on various proteinaceous and carbohydrate substrates and was first isolated from a submarine hot spring in Iceland (Alfredsson et al. 1988). The R. marinus genome encompasses several gene clusters encoding pathways for utilization and cellular import of diverse carbohydrates. Several enzymes from R. marinus have been characterized, and many have biotechnological potential, including a number of polysaccharide degrading enzymes, such as cellulase (Hálldórsdóttir et al. 1998), laminarinase (Krah et al. 1998) and xylanase (Nordberg Karlsson et al. 1997, 1998) (see Bjornsdottir et al. 2006 for a review). Rhodothermus marinus grows on a wide range of sugars from second and third generation biomass and can be cultivated to relatively high yields (Ron et al. 2019). It has anabolic pathways and precursor pools for production of various biotechnologically interesting primary and secondary compounds, such as polyamines (Hamana et al. 1992), exopolysaccharides (Sardari et al. 2017), carotenoids (Ron et al. 2018), compatible solutes (Nunes et al. 1995) and lipids (Nunes et al. 1992, Moreira et al. 1996).

Anaerobic fermentative organisms are generally preferred to produce low-value commodity chemicals, simple organic acids and alcohols that are typically catabolic waste products from incomplete oxidation of substrates. Conversely, heterotrophic
aeobes such as *R. marinus*, typically oxidize their carbon substrates completely under optimized conditions, and therefore utilize organic substrates more efficiently for both energy and carbon. Consequently, aeobes can carry a greater metabolic burden and are the preferred organisms for the anaerobic production of complex secondary metabolites.

Cultivation at high temperatures (60–70°C) may be beneficial in bioreactors as it reduces the cost of cooling, and higher temperatures protect the cultures from mesophilic spoilage bacteria. High temperature also increases the solubility of polysaccharides and leads to reduced viscosity of the fermentation broth. This may alleviate scale up problems of mixing and aeration to a significant extent and enable greater substrate loadings. Elevated temperatures may also enable cost-effective recovery of volatile products by distillation or gas stripping, reducing product inhibition and prolonging the production phase of the culture (López-Contreras et al. 2017).

*Rhodothermus marinus* has the potential to serve as a robust production organism in the emerging biorefinery industry and as a chassis species that can be metabolically engineered for the production of novel chemical compounds of industrial interest. For this purpose, comprehensive knowledge and understanding of its metabolism are needed. A well-curated genome-scale metabolic reconstruction contains a comprehensive overview of the metabolism of a given organism. A metabolic model can further be used to simulate phenotypic features, such as predict growth capabilities on different substrates and guide genetic engineering efforts (ÓBrien et al. 2015). Reconstructing the network of a poorly studied organism can result in gaps in the model. While this may skew predictions, it can nevertheless be useful for focusing future research efforts. Although *R. marinus* is not as well studied as common model organisms, it has been the subject of several studies, including the development and application of genetic tools (Bjornsdottir et al. 2007), particularly for the engineering of the carotenoid pathway (Kristjansdottir et al. 2020).

Carotenoids are pigments produced by many plants, fungi, algae and bacteria. Some non-photosynthetic bacteria produce carotenoids to stabilize cell membrane fluidity in response to extreme environments (high/low temperatures, pH, salinity, etc.) and to protect themselves against UV radiation and oxidative stress (Vereshnin 1999). Carotenoids are in demand for different applications, such as the food, feed and cosmetic industries. In a previous study, we engineered the carotenoid biosynthetic pathway in *R. marinus* to produce the industrially relevant carotenoid lycopene, instead of native ν-carotenoids (Kristjansdottir et al. 2020). In another, sequential batch cultivation resulted in higher carotenoid production than shake flask cultivation (Ron et al. 2019). Drawing upon the metabolic reconstruction of the current study, we further investigated the effects of culture conditions on carotenoid production and growth of *R. marinus*.

Here, we reconstructed the genome-scale metabolic network of the *R. marinus* type strain DSM 4252T, which has been the subject of most of the published studies so far and for which an annotated genome sequence is available (Nolan et al. 2009). However, the type strain is not amenable to genetic manipulation as it aggregates in liquid cultures and harbors a highly active DNA restriction enzyme with a 4 base recognition site (Rónká et al. 1991). Therefore, existing genetic tools were developed for another *R. marinus* strain, ISCar-493 (DSM 16675), which was selected after screening of numerous *R. marinus* strains for a restriction-deficient phenotype (Bjornsdottir et al. 2005). Here, the genome of strain *R. marinus* ISCar-493 was sequenced and the genomes of the two strains were compared. This analysis was used to find if any model genes from DSM 4252T could not be found in ISCar-493, and subsequently if the model could be extrapolated to strain ISCar-493. Growth curves and uptake and secretion rates of the main metabolites from bioreactor cultivations were obtained for both strains and the data used to validate the model. During the reconstruction process, several interesting features related to carotenoid production were identified and investigated further. This included heterologous expression of a gene from *Thermus thermophilus* encoding the terpenoid biosynthetic enzyme 1-deoxy D-xylulose 5-phosphate (DXP) synthase, which was not identified in the genomes of *R. marinus*.

**Methods**

**Strains, media and culture conditions**

Three *R. marinus* strains were used in this study, DSM 4252T, ISCar-493 (DSM 16675) and the mutant strain TK-4 (ISCar-493 derivative, ΔtrpBΔpurA:: trpBΔStr thermophilus, section “Genetic modification of *R. marinus*”). All cultivations were at 65°C and liquid cultures were set to shaking at 200 r/min. For each cultivation, *R. marinus* was first streaked on an agar plate containing rich medium, 10% medium 162 (Degryse et al. 1978), with modifications (2 mM MgSO4 and 0.2 mM CaCl2 in final volume) and addition of 1% NaCl, 0.03% K2HPO4, 0.1% yeast extract, 0.1% tryptone, 0.1% peptone, 0.05% glucose, 0.05% starch, 0.06% pyruvate and 0.018% Na2CO3. Utilization of different carbon sources (section “Reconstruction of a genome-scale metabolic model of *R. marinus* DSM 4252T”) was examined on defined medium agar plates containing 10% medium 162 (Degryse et al. 1978) with addition of 8 mM phosphate buffer (KH2PO4, Na2HPO4, pH = 7.2), 0.1% vitamin solution (Degryse et al. 1978) and 0.4% of each carbon source, except the amino acids which were 0.2%. *R. marinus* strain DSM 4252T from a rich medium agar plate (see above) was resuspended in a drop of 0.9% NaCl solution and subsequently streaked on agar plates containing the different carbon sources. The plates were incubated at 65°C and growth was examined after 1, 3, 5 and 7 days. Additionally, degradation of carboxymethyl-cellulose (CMC) was examined on rich medium agar plates (see above) supplemented with 0.4% CMC, by washing the agar plates with 0.1% Congo Red and then 1 M NaCl after 3 days of cultivation at 65°C. Halos formed around colonies where CMC had been degraded.

The cultivations used to validate the model (section “Model validation for strains DSM 4252T and ISCar-493”) were carried out in bioreactors (Labfors 5, Infors HT, Bottmingen, Switzerland), where *pO2* was kept at 40% with stirrer speed at 200–500 r/min and airflow as needed, pH at 7.2 with addition of 16.5% NH4OH and the temperature at 65°C. Defined medium, which contained 10% modified medium 162 with the addition of 1% NaCl, 8 mM phosphate buffer (KH2PO4, Na2HPO4, pH = 7.2), 10 mM NH4Cl, 0.02% asparagine, 0.02% glutamine, 0.1% vitamin solution (Degryse et al. 1978) and the carbon sources 1% glucose and 0.09% pyruvate, was used. The cultures were inoculated with 10% of pre-culture and were performed in duplicates. Samples were taken for OD (620 nm) and high-performance liquid chromatography (HPLC) measurements every hour, until a stationary phase was reached (18–32 h).

The cultivations for examining cell density and carotenoid production under different conditions (section “Carotenoid production and growth of *R. marinus* ISCar-493”) were performed in shake flasks under light and dark conditions. All cultures were exposed to day light and additional light from a halogen lamp, except when grown under dark conditions where the flasks were covered from the light. A defined medium [same as in section “Model validation
for strains DSM 4252² and ISC:aR-493³, except Wolf's vitamin- and trace elements solution from Wolin et al. (1963) were used, was used for these cultivations, but with different carbon sources: 1% glucose, 1% pyruvate, 0.09% pyruvate, 0.1% alginate, 0.5% glucose and 0.25% pyruvate, 0.09% pyruvate, 0.18% pyruvate, and without a carbon source for a negative control. For cultivation of strain TK-4, adenine (0.0025%) was also supplemented. Two pre-cultures were prepared for each liquid culture to be monitored. First, cells were transferred from a fresh rich medium agar plate (see above) to defined liquid medium containing 0.5% glucose and 0.018% pyruvate and grown overnight (16 h). This culture was used to inoculate (10%) a fresh defined liquid medium containing 1% glucose and 0.09% pyruvate, which was also grown overnight (16 h). All the monitored cultures were inoculated (10%) with the second pre-culture. The cultures were stopped after 24 h and cell density was estimated by measuring OD at 620 nm in a spectrophotometer (Novaspec III+, Biochrom, Harvard Bioscience Inc., Holliston, Massachusetts, USA).

All media components were autoclaved, except for the vitamin solution and the trace element solution, which were filter sterilized. The alginates were not sterilized due to the brown coloration and degradation when autoclaved. The probability of contamination in the alginates was low, because of the high growth temperature and because alginates is not a trivial carbon source for most bacteria. The alginates were plated to verify that contamination had not occurred during growth. Individual colonies were obtained, which all had the characteristic red color, and were subsequently identified as R. marinus using MALDI-TOF MS (Microflex, Bruker, Billerica, Massachusetts, USA), according to the manufacturer's instructions.

**Genetic modification of R. marinus**

The dks gene from T. thermophilus was inserted into the shuttle vector pRM3000.0, between the constitutive groEL promoter sequence and the selective marker trpB. The genomic DNA from T. thermophilus HB8 was isolated using the MasterPure Complete DNA purification Kit (Lucigen, Middleton, Wisconsin, USA) and used as template for the amplification of the dks (1-deoxy D-xylulose 5-phosphate synthase) gene (TTHA0006) (1848 bp) by PCR. The amplification was performed using the Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, Massachusetts, USA), according to the manufacturer's instructions. The primers were designed to support HiFi DNA assembly (NEBuilder HiFi DNA Assembly Master Mix, New England BioLabs): dks_thermus_F (5’-AACCATGGGATTCGCGCATATGATCTTGGACGAAAGAGAC-3’) and dks_thermus_R (5’-AGCGTCTGCAGTCGTGACATTCAGGCGCCGTCTCATGCAAG-3’), with the underlined bases matching the dks gene and the rest matching a SalI (New England BioLabs) digested R. marinus shuttle vector pRM3000.0. The pRM3000.0 vector is the pRM3000 vector (Bjornsrdottir et al. 2007) with an ampicillin resistance gene added, for selection in Escherichia coli. The pRM3000.0-dks vector was introduced into chemically competent E. coli (NEB S-alpha) and plated on L-medium (Miller 1972) with 100 μg/ml ampicillin. After incubation overnight at 37°C, positive clones were identified by amplifying the dks gene, using the Taq DNA polymerase (New England BioLabs) according to the manufacturer's instructions. The primers dks_thermus_F and R were used. The pRM3000.0-dks vector was isolated from positive E. coli clones using the Monarch Plasmid Miniprep Kit (New England BioLabs).

For the carotenoid measurements, the cultures were diluted to OD = 1 at 620 nm. The cell pellets from 1 ml of the diluted cultures were mixed well with 1 ml of acetone and incubated in a sonication bath for 20 min. The samples were centrifuged at 16,000 g for 7 min, resulting in a colorless cell pellet and acetone supernatant containing the extracted carotenoids. The R. marinus carotenoids in acetone have maximum absorbance at ~480 nm (data not shown). The OD at 480 nm of the acetone extracts was measured in a 1 cm quartz cuvette in a spectrophotometer (Novaspec III+, Biochrom).

**Analytical methods**

The DNA content in R. marinus cells was estimated from exponential bioreactor cultures using the fluorometric Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, Waltham, Massachusetts, USA). A sample of freeze-dried biomass (approx. 10⁷ cells/ml) was dissolved in water and sonicated for 10 s to lyse the cells. A standard curve was obtained from PicoGreen measurements of λ-dsDNA and used to estimate DNA concentration in R. marinus cells.

Glucose, lactate and acetate concentrations in the samples taken during bioreactor cultivations were measured using HPLC. The samples were filtered through 0.2 μm filters (Phenomenex) and metabolites subsequently quantified using the Dionex 2000 HPLC system (Dionex, Idstein, Germany) with a Rezex ROA-Organic Acid H + (8%, Phenomenex, Aschaffenburg, Germany) and a RI-101 detector (Shodex, Munich, Germany). Chromatogram evaluation software version 6.80 (Dionex, Idstein, Germany) was used. Separation was obtained using 60°C column temperature with 0.2 mM sulfuric acid and at a flow rate of 600 μl/min for 30 min. External standards of HPLC grade (Merck, Darmstadt, Germany, Sigma-Aldrich, St. Louis, USA) were used. The pyruvate concentration was estimated using a pyruvic acid kit (Megazyme, Bray, Ireland). The two amino acids in the defined medium, asparagine and glutamine, were not measured. Their concentration in the medium was low and did not influence the model predictions to a significant extent. OD (620 nm) values from bioreactor cultivations were converted to cell dry weight (CDW, g/l) based on data obtained elsewhere (Dahlberg et al. 1993, Blücher et al. 2000) (CDW = 0.75 x OD).

**Genome sequencing and analysis**

Rhodothermus marinus strain ISC:aR-493 was cultured in rich liquid medium (see section “Strains, media and culture conditions”). For sequencing by short-read technology, genomic DNA was
extracted using the MasterPure Complete DNA purification Kit (Lucigen) and sequencing libraries made by both the Nextera XT (FC-131-1024) and Nextera Mate Pair (FC-132-1001) methods. The two resulting libraries were sequenced on the MiSeq sequencing platform using V3 2 × 300 bp and V2 2 × 250 bp chemistry, respectively. The sequence reads were quality assessed using FastQC (v0.11.7) (Andreas 2010) and trimmed using Trimmomatic (v0.39) (Bolger et al. 2014). A genome was assembled using SPAdes (v3.14.0) (Nurk et al. 2013) with flag for isolates. Genome polishing was done by mapping all shotgun short-reads (Nextera XT) to the largest contig from the novo assembly and generating a consensus sequence using the highest quality bases, using Genseq (v9.1.4). The genome was annotated using the PGAP pipeline from NCBI (Tatusova et al. 2016) and submitted to the database. Genome alignment of iSCaR-493 to the type strain DSM 4252\textsuperscript{T} was constructed using the software Diamond (Buchfink et al. 2015, 2021). Based on the Blast output parameters identity and coverage, core genes were identified as having identity equal or greater than 50\% across at least 50\% of the sequence. Genes were assigned functional categories in accordance with the cellular roles of the respective COGs by EggNog annotation (Huerta-Cepas et al. 2019).

### Metabolic reconstruction and simulations

A protocol for reconstructing genome-scale metabolic models (Thiele and Palsson 2010) was used to guide the reconstruction process. A draft reconstruction, based solely on the annotated genome sequence, was obtained using the model SEED which is a is a resource for the reconstruction, exploration, comparison, and analysis of metabolic models (Henry et al. 2010). Since no well-curated model of a closely related bacterium was available at the time of the reconstruction, the obtained draft was neither comprehensive nor functional. It was therefore used as a reference model, along with the well-curated model of E. coli (iJO1366) (Orth et al. 2011). The R. marinus model was manually reconstructed based on these two reference models and R. marinus experimental data, obtained here and from the literature. The BiGG (King et al. 2015), BRENDA (Jeske et al. 2018), KEGG (Kanehisa and Goto 2000) and MetaCyc (Caspi et al. 2017) databases and the BLAST tool (Altschul et al. 1990) were extensively used.

Cobrapy (Ebrahim et al. 2013) was used in all model simulations, along with the GLPK solver. The corresponding code can be found as a Jupyter notebook on Github (https://github.com/steingrmarinus). For all simulations, flux balance analysis (FBA) was used (Fell and Small 1986, Savinell and Palsson 1992). Exchange reactions corresponding to metabolites taken up from the media (glucose and pyruvate) and secreted (lactate and acetate) during growth were constrained with experimentally obtained rates (Supplementary File 1). FBA was subsequently used to optimize for growth by maximizing flux through the biomass reaction.

For accurate growth rate predictions, the biomass reaction should ideally be based on data obtained for the target organism. Here, the biomass reaction was formulated based mostly on available data on R. marinus (Supplementary File 2). Separate biosynthetic reactions for each group of macromolecules (protein, lipid, DNA, etc.) were formulated, describing the ratio of the building blocks (amino acids, fatty acids, nucleotides, etc.) and the energy required. Sensitivity analysis, which shows how much variation in each macromolecule affects the predicted growth rate, was performed. This analysis helps to identify which biomass components most urgently need to be accurately measured.

### Results and discussion

#### Reconstruction of a genome-scale metabolic model of R. marinus DSM 4252\textsuperscript{T}

A genome-scale metabolic model of R. marinus DSM 4252\textsuperscript{T} was reconstructed, named Rmarinus_578 (https://github.com/steingrmarinus). The main features of the reconstruction are listed in Table 1. The Memote tool (Lieven et al. 2020) was used to help guide the reconstruction process, by verifying stoichiometric consistency, mass and charge balance and annotation quality (Supplementary File 3). Reactions and metabolites were usually abbreviated in accordance with the BiGG database and annotations with links to external databases are included. The genome sequence for strain DSM 4252\textsuperscript{T} was obtained from GenBank (accession nr: NC_013501). The genes in the reconstruction were identified with the locus tags from the GenBank file. They were annotated with the old gene locus tag from the GenBank file, the protein ID, protein annotation and protein sequence. Experimental data on R. marinus obtained in this study and the available literature was used to curate reactions, genes and gene-protein-reaction (GPR) rules. Several metabolic features were reviewed during the reconstruction process: in the following, we highlight a few, which are of interest for biotechnological application of R. marinus.

### Sugar metabolism

Rhodothermus marinus produces pyruvate from glucose through the Embden–Meyerhof–Parnas (EMP) pathway A 13C metabolic flux study of the central metabolism in R. marinus (Cordova et al. 2017) showed that the EMP pathway and the TCA cycle are both highly active while metabolizing glucose. The oxidative pentose phosphate pathway and the glyoxylate shunt had very low activity and the Entner–Doudoroff (ED) pathway, malic enzyme and phosphoenolpyruvate carboxykinase were inactive.

Growth of R. marinus strain DSM 4252\textsuperscript{T} was tested on many different carbon sources, both in vivo and in silico (Table 2). Growth has been shown on several mono-, di- and polysaccharides, which was also observed in silico. However, growth on cellulose was predicted in silico while not observed in vivo. Rhodothermus marinus does contain a gene encoding a GH12 endocellulase (EC 3.2.1.4) (Hreggvidsson et al. 1996, Halldorsson et al. 1998), which can degrade CMC and differently sized cellobioigosaccharides (the smallest hydrolyzable being cellobiose) to cellobiose and glucose (Crennell et al. 2002). Gene analysis has also shown presence of a signal peptide, indicating export, however, low specific activities were reported compared to benchmark enzymes. The strain does not contain genes encoding specific exocellulases (EC 3.2.1.91), which degrade glucans into β-celllobiose. However, it does

| Table 1. Main features of the genome-scale metabolic model of R. marinus DSM 4252\textsuperscript{T}, Rmarinus_578. |
|-----------------|-----------------|
| **Genome features** |                |
| Genome size      | 3.26 Mb         |
| Protein coding sequences | 2889         |
| **Model features** |                |
| Genes           | 578             |
| Metabolites (unique) | 871 (784)      |
| Reactions (with GPR) | 929 (771)    |
| Memote total score | 55%           |
Table 2. Growth of R. marinus strain DSM 4252\(^T\) on different carbon sources investigated in silico and in vivo. Green indicates growth, light green indicates weak growth, ‘w’ indicates white colonies (instead of the characteristic red), orange indicates no growth in silico except if a transport reaction was added to the model, red indicates no growth and no color indicates no data available.

| Metabolite            | In Silico | In Vivo |
|-----------------------|-----------|---------|
| Glucose\(^1\)         |           |         |
| Galactose\(^2\)       |           |         |
| Sucrose\(^3\)         |           |         |
| Lactose\(^4\)         |           |         |
| Raffinose\(^5\)       |           |         |
| Maltose\(^6\)         |           |         |
| Fructose\(^7\)        |           |         |
| Mannose\(^8\)         |           |         |
| Ribulose\(^9\)        |           |         |
| Xylose\(^10\)         |           |         |
| Pyruvate\(^11\)       | w         |         |
| Acetate\(^12\)        | w         |         |
| Lactate               |           |         |
| Formate               |           |         |
| Starch\(^13\)         | w         |         |
| Xylan\(^14\)          |           |         |
| Laminarin\(^15\)      |           |         |
| Alginate\(^16\)       |           |         |
| Cellulose (CMC)\(^17\)|           |         |
| Malate\(^18\)         |           |         |
| Fumarate\(^19\)       |           |         |
| Succinate\(^20\)      |           |         |
| Citrate\(^21\)        |           |         |
| Ornithine             |           |         |
| Oxaloacetate          |           |         |
| 2-oxoglutarate\(^22\) |           |         |

| Metabolite            | In Silico | In Vivo |
|-----------------------|-----------|---------|
| Glutamate\(^1\)      |           |         |
| Aspartate\(^2\)      |           |         |
| Glutamine\(^3\)      |           |         |
| Asparagine\(^2\)     |           |         |
| Arginine\(^3\)       |           |         |
| Leucine\(^2\)        |           |         |
| Phenylalanine\(^2\)  |           |         |
| Proline\(^2\)        |           |         |
| Serine\(^2\)         |           |         |
| Threonine\(^2\)      |           |         |
| Valine\(^2\)         |           |         |
| Alanine               |           |         |
| Histidine             |           |         |
| Glycine               |           |         |
| Lysine                |           |         |
| Tryptophan            |           |         |
| Tyrosine              |           |         |
| Cysteine              |           |         |
| Methionine            |           |         |
| Isoleucine            |           |         |

1 Data from this study  
2 Data from (Alfredsson et al. 1988).

Lactose and galactose are examples of the di- and monosaccharides that R. marinus can use for growth (Table 2). The gene encoding a β-galactosidase (EC 3.2.1.23), which hydrolyzes lactose into glucose and galactose, was found in the genome. Three steps are needed to convert galactose to glucose-6-phosphate, which then enters the glycolysis EMP pathway. The genes encoding galactokinase and galactose-1-phosphate uridylyltransferase, catalyzing the first two steps (galactose $\rightarrow$ galactose-1-phosphate $\rightarrow$ glucose-1-phosphate), were found in the genome. The third step, where glucose-1-phosphate is turned into glucose-6-phosphate, is usually performed by the enzyme phosphoglucomutase (EC 5.4.2.2). The gene for this enzyme was not found in the genome. However, a homology search showed similarity between known phosphoglucomutase genes from other bacteria and genes RMAR_RS01880 (E value 2e–37) and RMAR_RS08875 (E value 1e–25) which are annotated as phosphomannomutase (EC 5.4.2.8) and phosphoglucomutase (EC 5.4.2.10), respectively. The enzyme phosphoglucomutase in E. coli, which usually catalyzes the interconversion of glucosamine-6-phosphate and glucosamine-1-phosphate, was also shown to be able to catalyze the interconversion of glucose-6-phosphate and glucose-1-phosphate, at a lower rate (Jolly et al. 1999). The phosphorylation site of this enzyme...
Figure 1. The alginate degradation pathway of *R. marinus* and its connections to the EMP pathway, the partial ED pathway and the MEP terpenoid pathway. Names of substrates, products and energy molecules (ATP, NADH, NADPH) are shown, some abbreviated: 4-deoxy-1-erythro 5-hexoseulose uronic acid (DEH), 2-keto 3-deoxygluconate (KDG) and 2-keto 3-deoxygluconate 6-phosphate (KDGP). The molecular structure of a partial alginate molecule is shown: β-d-mannuronate (M) and α-l-guluronate (G). The missing reaction of the ED pathway in *R. marinus* is represented by a grey dotted arrow.

in E. coli is Ser102 and a mutational change of Ser100 to a threonine residue increased the phosphoglucomutase activity significantly. Gene RMAR_RS08875 from *R. marinus* was investigated and the serine residue responsible for the phosphorylation was found to be residue number 103. The corresponding residue to Ser100 in the *E. coli* enzyme was found to be a threonine, which indicates that this *R. marinus* enzyme may be responsible for the interconversion of glucose-6-phosphate and glucose-1-phosphate in *R. marinus*.

*Rhodothermus marinus* possesses several genes encoding polysaccharide degrading enzymes. As a marine bacterium, seaweed is common in its natural environment. Alginate and laminarin are major polysaccharides of brown algae, which *R. marinus* can break down and use as sole carbon sources for growth (Allahgholi et al. 2020). Alginate is a structural component of brown algae and can comprise up to 40% of its dry matter (Qin 2008). It is a polyeuronate that consists of β-d-mannuronate (M) and α-l-guluronate (G) units forming (1 → 4) linked G-, M- and mixed blocks in the polysaccharide chain. The *R. marinus* genome has four genes encoding alginate lyases (Hreggvidsson et al. 2016, Hreggvidsson and Fridjonsson 2020) that, together, depolymerize alginate into the same unsaturated mono-uronate derivative of the M and G units. The *R. marinus* genome also possesses the genes coding for the remaining enzymes of the alginate catabolic pathway enabling its utilization. The unsaturated monouronate is converted to 4-deoxy 1-erythro 5-hexoseulose uronic acid (DEH) by a spontaneous reaction and further catalyzed to 2-keto 3-deoxygluconate (KDG) by an aldose reductase (Preiss and Ashwell 1962). KDG enters the partial ED pathway in *R. marinus*, where it is catalyzed to 2-keto 3-deoxygluconate 6-phosphate (KDGP) by 2-keto 3-deoxygluconokinase (EC 2.7.1.45) and then by 2-dehydro-3-deoxyphosphogluconate aldolase (EC 4.1.2.14), to pyruvate and glyceraldehyde 3-phosphate which enter central metabolism (Preiss and Ashwell 1962, Takase et al. 2010) (Fig. 1). *Rhodothermus marinus* does not possess a key enzyme in the ED pathway, phosphogluconate dehydrogenase (EC 4.2.1.12), which is needed to metabolize glucose. This explains why the ED pathway is not active when *R. marinus* is grown on glucose (Cordova et al. 2017), while the partial pathway is essential for utilization of alginate (Fig. 1).

**Terpenoid and carotenoid metabolism**

The carotenoids produced by several *R. marinus* strains have been characterized (Lutnaes et al. 2004, Ron et al. 2018). A carotenoid pathway was proposed in (Ron et al. 2018) using structural and bioinformatic data, and later refined (Kristjansdottir et al. 2020). This pathway is included in the reconstruction along with candidate genes. Terpenoids, which serve as precursors to carotenoids, can be produced through two different pathways, the mevalonate (Katsu & Bloch 1967, Lynen 1967) and the non-mevalonate (MEP) (Rohmer et al. 1993) pathways. In the latter, which is used...
by most bacteria including *R. marinus*, geranylgeranyl diphosphate (GGDP) is produced from pyruvate and glyceraldehyde 3-phosphate in multiple steps. *Rhodothermus marinus* possesses genes coding for all the enzymes in the MEP pathway, except for 1-deoxy D-xylulose 5-phosphate (DXP) synthase (DXS, EC 2.2.1.7.), which catalyzes the first step in the pathway. Studies of the MEP pathway in other bacteria have shown that DXS is not strictly necessary for the synthesis of DXP. Examples include a mutated form of pyruvate dehydrogenase that is known to rescue *E. coli* cells defective in DXS (Sauter-Güeto et al. 2006) and a mutated RibB protein and a YajO protein that synthesized DXP from ribulose 5-phosphate, also in *E. coli* (Kirby et al. 2015). Another possibility to bypass DXS is via the MTA-isoprenoid shunt, as has been shown in *Rhodospirillum rubrum* (Erb et al. 2013). Here, the dead-end metabolite of polyamine biosynthesis, 5-methylthioadenosine (MTA) is metabolized by an alternative methionine salvage pathway, which produces DXP as a side-product. Phylogenetic analysis showed that the genes in this pathway are partially present in the *R. marinus* genome. At present, it is not known how DXP is produced in *R. marinus* and without further evidence of alternative pathways, the DXP synthase reaction is present in the reconstruction without any gene candidates assigned. The absence of DXS in *R. marinus* directly suggests heterologous expression of a thermostable DXS as means to increase flux through the terpenoid pathway.

Light-inducible carotenoid production has been observed in many organisms, including non-photosynthetic bacteria, and the regulatory mechanisms have been studied in some of them, including *Myxococcus xanthus* (Pérez-Marín et al. 2008), *Thermus thermophilus* (Takano et al. 2011), *Streptomyces colorcol* (Takano et al. 2005) and *Bacillus megaterium* (Takano et al. 2015). The MerR family transcriptional regulator, LitR, acts as a repressor in the carotenoid gene cluster. Its activity is dependent on the binding with adenosyl B12 and the LitR-AdoB12 complex becomes inactivated when illuminated. This causes cell cultures to become colorless under dark conditions while producing carotenoids in light. A homologue for the *T. thermophilus* litR gene was previously identified in the carotenoid gene cluster in *R. marinus* (Kristjansdottir et al. 2020), located upstream of the carotenoid gene *crb* (phytoene synthase). This suggests that light might help to increase carotenoid yields in *R. marinus*.

Other pathways

The respiratory chain in *R. marinus* has been extensively studied. The first two complexes, NADH dehydrogenase and succinate dehydrogenase, have been characterized (Fernandes et al. 2001, 2002) and found to be similar to those of other bacteria. The third complex, cytochrome dehydrogenase, is not the typical bc1, but an alternative complex (Pereira et al. 2007). It has an entirely different structure but carries out the same function, oxidizing reduced menaquinones-7 (Tindall 1991) and reducing high potential iron-sulfur protein (HiPIP) and cytochrome c. Finally, three different types of the fourth complex have been characterized in *R. marinus*, cb3 (Pereira et al. 2000), cu3 (Pereira et al. 1999) and ba3 (Verissimo et al. 2007). These reactions and associated genes are included in the reconstruction.

Polyamines are alkaline organic compounds with at least two primary amino groups. They can be found in most forms of life and have diverse functions. Some polyamines are biotechnologically interesting because they can be used to produce plastics and are used as curing agents in polymer applications (Schneider and Wendisch 2011). In thermophilic bacteria unusual, long and branched polyamines have been observed (Fukuda et al. 2015). They are believed to have protective effects on nucleic acids and proteins under high-temperature conditions. Seven different polyamines have been characterized in *R. marinus* to date, putrescine, spermidine, cadaverine, spermine, thermopentamine, N4-aminopropylspermidine and N4-bis(aminopropyl)spermidine (Hamana et al. 1992), and their biosynthesis is included in the reconstruction. *Rhodothermus marinus* produces compatible solutes to protect the cell against sudden osmotic changes. They include amino acids, monosaccharides such as trehalose, small peptides and, most abundantly, mannosylglycerate (Silva et al. 1999). Mannosylglycerate in *R. marinus* is synthesized via two pathways (Borges et al. 2004), which have been studied in detail, along with the corresponding enzymes and genes (Nunes et al. 1992, Borges et al. 2004, Nielsen et al. 2011). These pathways are present in the metabolic reconstruction, but as their products are only produced in response to stress, they are not included in the biomass reaction. These pathways are therefore not active during growth simulations when the biomass reaction is maximized.

Genomic information indicates that *R. marinus* can synthesize all the amino acids needed for protein synthesis. This is supported by growth experiments which show that *R. marinus* can grow in defined medium, without any addition of amino acids (Mukti et al. 2021). Biosynthetic pathways for all the 20 amino acids are included in the reconstruction. The fatty acid and lipid composition of *R. marinus* have been characterized (Nunes et al. 1992, Moreira et al. 1996). The dominating fatty acids are iso- and anteiso-C15 and iso- and anteiso-C17, with iso-C16 and iso-C18 as minor components. The major polar lipids are phosphatidylethanolamine, diphosphatidylglycerol and one unidentified lipid and phosphatidylglycerol was identified as a minor lipid (Tindall 1991, Nunes et al. 1992). The biosynthesis of the fatty acids and lipids are also included in the reconstruction.

The biomass objective function

In genome-scale metabolic models, a biomass objective function (BOF) is used to simulate growth. The BOF describes the ratio between the macromolecules (protein, DNA, RNA, lipids, etc.), the composition of each macromolecule (amino acids, nucleotides, fatty acids, etc.) and the energy that the cell requires to grow and maintain itself. We collected data on several studies that describe different components of the biomass in *R. marinus*. Protein, RNA, lipid and glycogen content and amino acid ratio in the biomass were based on Cordova et al. (2017), carotenoid composition on Lutnaes et al. (2004), exopolysaccharide (EPS) on Sardari et al. (2017), lipids on Nunes et al. (1992) and Moreira et al. (1996) and polyamines on Hamana et al. (1992). Quantification of DNA in the biomass was measured in this study (Supplementary File 2). Nucleotide compositions of DNA and RNA were derived from the DSM 4252T genome. The RNA estimate assumes equal transcription of all genes and is therefore expected to be somewhat inaccurate. Growth associated maintenance (GAM) was estimated using experimental data obtained here (Supplementary File 1). The remainder of the biomass components was adopted from *E. coli* (Feist et al. 2007). A detailed overview of the biomass, sources of information and calculations can be found in Supplementary File 2.

Sensitivity analysis was performed to investigate how sensitive the predicted growth rate was to changes in biomass and energy components. The growth rate was predicted while varying each component by 50%, for multiple glucose uptake rates. The components tested were protein, DNA, RNA, lipid, exopolysaccharide, lipopolysaccharide, glycogen, peptidoglycan, GAM and non-growth associated maintenance (NGAM) (Supplementary File 2). The predicted growth rate was most sensitive to changes in the protein component, followed by the lipid component.
Adapting model to strain ISCaR-493

The metabolic model was reconstructed based on genomic information from *R. marinus* DSM 4252T. This strain is, however, not amenable to genetic manipulation so it aggregates in liquid cultures and shows high DNA degrading activity due to the presence of a cut restriction enzyme, Rmal (type II restriction endonuclease) (Ronka et al. 1991). Therefore, genetic tools were developed for a derivative of the strain ISCaR-493 (Bjornsdottir et al. 2005). The genome of strain ISCaR-493 was sequenced (GenBank accession nr: CP172416) and compared to the genome of strain DSM 4252T.

A total of 578 genes were used in the metabolic network modelling of *R. marinus* 4252T. According to genome comparison, only seven model genes were not found in the genome of ISCaR-493 and only three of them did not have isozymes in the genome that showed high similarity to DSM 4252T genes (Table 3). Strain DSM 4252T contains two genes encoding xylanases while strain ISCaR-493 contains only one homologue. Both strains grow on xylan as the sole carbon source (Table 2 for DSM 4252T, data not shown for ISCaR-493). Strain DSM 4252T contains four genes encoding alginate lyases and one of them is missing in ISCaR-493. The latter strain can grow in a medium with alginate as the sole carbon source (Allahgholi et al. 2020), suggesting that the three alginate lyases are sufficient to degrade alginate for utilization.

Many enzymes take part in EPS biosynthesis and assembly, and their corresponding genes were all found in the core genome. A putative o-antigen polymerase (wzy) found in the accessory genome of strain ISCaR-493 showed a low similarity to a functionally corresponding protein in DSM 4252T (E-value 0.004). However, the encoded gene showed high similarity to genes in more distantly related bacteria annotated as o-antigen ligase and O-antigen polymerase. This enzyme activity is essential for EPS synthesis and must be present in ISCaR-493 as EPS is produced (Sardari et al. 2017). Taken together, this suggests that the Rmarinus_578 model can be used for both strains DSM 4252T and ISCaR-493.

### Table 3. The model genes that are not present in *R. marinus* strain ISCaR-493. The model gene IDs from DSM 4252T are listed, with corresponding annotations and the gene IDs of the isozymes, when applicable.

| DSM 4252T gene ID | Annotation                      | Isozyme present          |
|-------------------|---------------------------------|---------------------------|
| RMAR_RS02620      | Dihydropterin triphosphate pyrophosphatase | Yes (RMAR_RS06180)        |
| RMAR_RS14780      | O-antigen polymerase (wzy)      | No                        |
| RMAR_RS05305      | Xylanase                        | No                        |
| RMAR_RS134585     | Alginate lyase                  |                           |
| RMAR_RS11910      | Asparagine synthase (glutamine-hydrolyzing) | Yes (RMAR_RS10310)        |
| RMAR_RS12230      | Beta-ketoacyl-ACP synthase      | Yes (RMAR_RS14045)        |
| RMAR_RS12885      | N-acetylated glutamate synthase  | Yes (RMAR_RS13420)        |

Model validation for strains DSM 4252T and ISCaR-493

Experimental growth data was obtained for strains DSM 4252T and ISCaR-493 in bioreactors, along with measured uptake of glucose and pyruvate and secretion of acetate and lactate (Supplementary File 1). The average rates of two replicates (Supplementary File 1) was used to constrain the model to validate the accuracy of growth predictions (Fig. 2A). The experimental growth data showed that the growth of *R. marinus* DSM 4252T did not follow the typical batch growth curve of bacteria. A true exponential phase was not observed throughout the growth phase. Instead, the apparent specific growth rate decreased over time until stationary phase was reached (Fig. 2B). The specific growth rate in strain ISCaR-493 was closer to being constant, with exponential growth during a longer period (Fig. 2B). Data from time points 3–6 for strain DSM 4252T and 1–5 for strain ISCaR-493 were used here. This analysis showed that the model accurately predicts growth for both strains (Fig. 2A).

For both strains, but more so for DSM 4252T, secretion rates of lactate and acetate increased during the growth phase (Supplementary File 1). A decrease in growth rate during batch cultivations has been observed in other bacteria, such as *E. coli* (Berney et al. 2006) where the main reason was oxygen limitation that could also lead to an increase in organic acid secretion. The cultivations here were carried out with high aeration as oxygen levels were kept fixed at 40% pO2. A plausible explanation for why the cells would experience oxygen limitation in a medium with excess oxygen levels is local limitation due to cell aggregation (Wessel et al. 2014). Aggregation of several *R. marinus* strains has been reported previously (Bjornsdottir et al. 2005), especially in DSM 4252T and *R. marinus* is also shown to produce exopolysaccharides (Sardari et al. 2017), which can cause cells to aggregate (Nwodo et al. 2012).

When the model was optimized for growth, without oxygen limitation and free secretion of acids, it did not predict any acid production and the predicted growth rate was slightly higher than observed in vivo. When oxygen was limited in the model, the predicted growth rate decreased, and the model predicted lactate secretion (data not shown). Experimental data showed that lactate was first secreted, followed by acetate (Supplementary File 1). The model predicted slightly higher growth rate when lactate was the sole acid produced, opposed to when it was forced to also produce acetate.

Carotenoid production and growth of *R. marinus* ISCaR-493

While constructing the metabolic model, several factors were identified that could potentially impact the production of carotenoids. To better understand this, a cultivation experiment comparing different conditions was performed. Besides obtaining high yields of carotenoids per cell, high cell density is important for achieving high yields of carotenoids. Therefore, both extracted carotenoids (from 1 ml of cells diluted to OD620 nm = 1) and cell densities were measured from cultivations after 24 h (Fig. 3). The ISCaR-493 strain was used in this experiment, as it can be genetically modified and thus likely to be used for future cell factory designs.

Glucose and pyruvate

*Rhodothermus marinus* can grow on several monosaccharides, as predicted by the model. However, we have often observed better growth on oligo- and polysaccharides (data not shown). Growth
of strain ISCaR-493 in defined medium with glucose (1%) as the sole carbon source resulted neither in high cell density nor high carotenoid production (Fig. 3). *Rhodothermus marinus* can utilize pyruvate as the sole carbon source (Table 2). Pyruvate is used in several pathways essential for growth and is the substrate, together with glyceraldehyde 3-phosphate, in the first step of the MEP terpenoid pathway (Fig. 1). To increase both cell density and carotenoid production, pyruvate (0.09%) was added to the glucose-based medium. This resulted in increased carotenoid production and highly increased cell density (Fig. 3). Visually, these cultures exhibited much stronger red color than the glucose cultures, which can be explained by both increased carotenoids yields and higher cell densities.

**Impact of light**

During the model reconstruction, a homolog for a light dependent regulatory gene was found in the carotenoid gene cluster in *R. marinus*. This indicates that carotenoid production in *R. marinus* is light induced (section "Reconstruction of a genome-scale metabolic model of *R. marinus* DSM 4252 T"). This was investigated here by cultivating ISCaR-493 in glucose (1%) and pyruvate (0.09%) medium in the dark and compared to the corresponding cultures grown in the light. The former cultures were colorless and the lack of carotenoids was confirmed by measurements (Fig. 3).

**Alginate**

*Rhodothermus marinus* can grow on many different polysaccharides (Table 2), making it an interesting candidate for processing second or third generation biomass, such as seaweed. Alginate is one of the major polysaccharides of brown algae. The products from alginate degradation are pyruvate and glyceraldehyde 3-phosphate (Fig. 1), which are the same metabolites as used in the first step of the MEP terpenoid pathway. This raised the question whether *R. marinus* produces more carotenoids when grown on alginate, since it produces the two metabolites needed for the biosynthesis concurrently and in equal amounts. Cultivations in defined medium with alginate (1%) as the sole carbon source showed less cell density compared to glucose and pyruvate, but highly increased carotenoid production (Fig. 3).

**Glucose and pyruvate in equal quantities**

To further examine if the availability of glyceraldehyde 3-phosphate and pyruvate in equal amounts results in higher carotenoid production, cultivation in defined medium with glucose (0.5%) and pyruvate (0.25%) was investigated. These cultivations showed lower cell density and higher carotenoid production compared to growth on glucose (1%) and pyruvate (0.09%) (Fig. 3). The increased carotenoid production could be due to the equal availability of the two metabolites. Another possibility is that increased concentration of pyruvate alone in the medium caused higher carotenoid production.

**Pyruvate**

To examine if pyruvate alone affects the carotenoid production, two additional cultivations were set up, with pyruvate (0.09% and 0.18%) as the sole carbon source. The cell density in these cultures was low, only increased slightly after inoculation. This indicated that ISCaR-493 struggles to grow in liquid defined medium.
Figure 3. Cell density (OD620nm) and carotenoid (OD480nm) production following growth of \textit{R. marinus} strain ISCaR-493 for 24 h on glucose; mixture of glucose and pyruvate under light and dark conditions; alginate; pyruvate and without any carbon sources. Additionally, the modified strain TK-4 (\textit{Atrp8\Delta purA:trp8\Delta dxs.Thermophilus}) was grown on a mixture of glucose and pyruvate. The carotenoids were always extracted from a fixed number of cells (1 ml of OD620nm = 1). The total amount of carotenoids was calculated by multiplying the cell density by the measured carotenoids. The average cell density of each culture condition is represented by a blue bar (top graph), the average measured carotenoid value as a red bar (middle graph) and the average total carotenoids by an orange bar (bottom graph). Dots represent individual replicates.
with pyruvate as the sole carbon source. The carotenoids per fixed cell density in the pyruvate cultures (both 0.09% and 0.18%) were much higher compared to cultures on a mixture of glucose (1%) and pyruvate (0.09%). Additionally, increased pyruvate concentration resulted in increased carotenoid production (Fig. 3). This suggests that the pyruvate is used for carotenoid production. Producing glyceraldehyde 3-phosphate from pyruvate costs energy (glyconeogenesis) and it cannot be determined from this data if this is the case for the observed growth. However, glycogen is an alternative source of glyceraldehyde 3-phosphate. The amount of glycogen in the biomass of R. marinus has been estimated as 14% (Cordova et al. 2017) and is relatively high compared to other bacteria. Inclusion that could possibly contain glycogen can be discerned on electron micrographs of R. marinus (Bjornsdottr et al. 2006). Considering the natural habitat of R. marinus in coastal hot springs, it is not unreasonable to assume that it accumulates high levels of glycogen. Due to tides, the availability of nutrients in the surroundings of R. marinus varies widely and it is likely that the bacterium stores carbohydrates when they are in abundance in the environment. Since little or no growth was observed on pyruvate in liquid cultures it is likely that the cells experienced starvation and therefore started the breakdown of glycogen and carotenoid production. This was also seen for the negative control cultures without a carbon source (Fig 3). The cell density did not increase from inoculation, while the carotenoid production did.

**Addition of the dxs gene from T. thermophilus**

During the manual curation of the reconstruction process, we realized that the dxs gene encoding 1-deoxy-d-xylulose-5-phosphate synthase (DXS), which catalyzes the first step in the MEP terpenoid pathway (section “Reconstruction of a genome-scale metabolic model of R. marinus DSM 4252T”), could not be identified in the genomes of R. marinus. In an effort to increase carotenoid yields, the dxs gene from T. thermophilus was cloned on a shuttle vector into R. marinus strain SB-62 (ISCaR-493 derivative, ΔtrpBΔpurA), resulting in the mutant strain TK-4 (ΔtrpBΔpurA::trpBdxs::thermophilus) (Supplementary File 4). Compared to ISCaR-493, cultivation of TK-4 resulted in lower cell density but highly increased carotenoid production. Presumably the added dxs gene resulted in a higher flux of carbons through the terpenoid and carotenoid pathways. However, it is also possible that this strain struggles to grow and responds by producing carotenoids. The dramatically lower cell density compared to ISCaR-493 can most likely be explained by the metabolic burden caused by the replication of the shuttle vector and the expression of its genes. Inserting the dxs gene into the chromosome could reduce such effects.

In summary, these experiments showed that the highest cell density was obtained in glucose medium supplemented with pyruvate, while higher carotenoid production was observed during growth on alginate, with pyruvate added to a glucose-based medium and in the presence of light. It also showed that the carotenoid production per cell increased during stress, e.g. decreased pH and/or starvation. Increased carotenoid production has been observed during stationary phase of R. marinus growth before (Ron et al. 2019), where the carbon sources and the pH levels have decreased. This indicates that yields can potentially be increased by either allowing the culture to reach and stay in stationary phase or transfer the cells after growth to new medium with limited or no carbon source. The motivation for the latter is that after the growth phase, the medium might not be optimal, e.g. due to accumulation of by-products that alter the pH, and the cells might stay alive and produce carotenoids longer in fresh medium. However, the lowered pH might also contribute to the increased carotenoid production. Finally, cloning the dxs gene from T. thermophilus in R. marinus resulted in the highest yields of carotenoids, but much lower cell density than the wild type strain ISCaR-493.

**Conclusions**

A manually curated genome-scale metabolic model of R. marinus DSM 4252T was reconstructed and made publicly available (https://github.com/steining/marinus). Experimental data from the literature and from this study was used to curate and validate the model. This includes growth data on various carbon sources, bioreactor cultivations and HPLC measurements of main metabolites, used for model validation, multiple studies on different metabolic pathways, components, genes and enzymes, and data on biomass components, which was used to formulate a species-specific BOF.

The model was also evaluated for use with R. marinus ISCaR-493, from which the genetically modified SB-62 (ΔtrpBΔpurA) was derived. The genome of strain ISCaR-493 was sequenced and the resulting draft genome was compared to that of strain DSM 4252T. This analysis showed that only seven model genes were absent in strain ISCaR-493 and four of them were replaced by genes encoding isozymes that exhibited high similarity to the DSM 4252T enzymes. The remaining three genes are involved in EPS formation and xylan- and alginate degradation. EPSs of both strains have been previously studied (Sardari et al. 2017) and shown to be of similar structures. It was also observed that strain ISCaR-493 grows well in defined medium with xylan and alginate as the sole carbon sources. In conclusion, this analysis suggests that the model is applicable for both strains DSM 4252T and ISCaR-493. Both strains should be considered when any future changes or additions to the model reconstruction are made. Data on growth and metabolites were used to constrain the model and compare the experimental and simulated growth rates. This revealed that the model predicts correct growth rates for both strains.

Different aspects of the metabolism of R. marinus were reviewed during the reconstruction process. Here, an emphasis was on those with a potential biotechnological aspect, carotenoids in particular. Cell density and carotenoid production of strain ISCaR-493 grown at different conditions were investigated. Addition of pyruvate to a glucose-based medium resulted in highly increased cell density. Carotenoid production varied considerably under different growth conditions. Higher carotenoid yields were observed when pyruvate was present in the growth medium, alginate was used as the sole carbon source, cultivating the cells in light conditions and the cells experienced starvation. Additionally, we cloned the dxs gene from T. thermophilus on a shuttle vector into R. marinus and cultivation of the resulting mutant showed low cell density compared to ISCaR-493, but higher carotenoid production. With its thermostable enzymes, wide range of potential carbon sources for growth and marketable products, R. marinus application potential is highly relevant in biotechnology and bioenergy. A genome-scale metabolic model helps us to understand its metabolism and should be useful in future strain designs.

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Supplementary data
Supplementary data is available at FEMSEC Journal online.

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References
Alfredsson GA, Kristjansdottir JK, Hjuleifsdottir S et al. Rhodothermus marinus, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. J Gen Microbiol 1988;134:299-306.
Allagholi I, Sardari RRR, Hakvág S et al. Composition analysis and minimal treatments to solubilize polysaccharides from the brown seaweed Laminaria digitata for microbial growth of thermophiles. J Appl Phycol 2020;32:1933-47. https://doi.org/10.1007/s10811-020-20103-6.
Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. J Mol Biol 1990;215:403–10. https://doi.org/10.1016/S0022-2836(05)80360-2.
Andrews S. FastQC, Babraham Bioinformatics. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (1 June 2020, date last accessed).
Ara KZG, Mänberger A, Gabriško M et al. Characterization and diversity of the complete set of GH family 3 enzymes from Rhodothermus marinus DSM 4253. Sci Rep 2020;10:1329. https://doi.org/10.1038/s41598-020-58015-5.
Berney M, Weilenmann H-U, Ihansen J et al. Specific growth rate determines the sensitivity of Escherichia coli to thermal, UVA, and solar disinfection. Appl Environ Microbiol 2006;72:2586-93. https://doi.org/10.1128/AEM.72.4.2586-2593.2006.
Bjornsdottir SH, Blondal T, Hreggvidsson GO et al. Rhodothermus marinus: physiology and molecular biology. Extremophiles 2006;10:1–16. https://doi.org/10.1007/s00792-005-0466-z.
Bjornsdottir SH, Fridjonsson OH, Hreggvidsson GO et al. Generation of targeted deletions in the genome of Rhodothermus marinus. Appl Environ Microbiol 2011;77:5505–12. https://doi.org/10.1128/AEM.02070-10.
Bjornsdottir SH, Fridjonsson OH, Kristjansson JK et al. Cloning and expression of heterologous genes in Rhodothermus marinus. Extremophiles 2007;11:283–93. https://doi.org/10.1007/s00792-006-0373-y.
Bjornsdottir SH, Thorbjarnardottir SH, Eggertsson G. Establishment of a gene transfer system for Rhodothermus marinus. Appl Microbiol Biotechnol 2005;66:675–82. https://doi.org/10.1007/s00253-004-1730-3.
Blücher A, Karlsson EN, Holst O. Substrate-dependent production and some properties of a thermostable, α-galactosidase from Rhodothermus marinus. Biotechnol Lett 2000;22:663–9. https://doi.org/10.1023/A:1005627501609.
Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–20. https://doi.org/10.1093/bioinformatics/btu170.
Borges N, Marugg JD, Empadinhas N et al. Specialized roles of the two pathways for the synthesis of mannosylglycerate in osmoadaptation and thermoadaptation of Rhodothermus marinus. J Biol Chem 2004;279:9892–8. https://doi.org/10.1074/jbc.M312186200.
Buchfink B, Reuter K, Drost H-G. Sensitive protein alignments at tree-of-life scale using DIAMOND. Nat Methods 2021;18:366–8. https://doi.org/10.1038/s41592-021-01101-x.
Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods 2015;12:59–60. https://doi.org/10.1038/nmeth.3176.
Caspi R, Billington R, Fulcher CA et al. The MetaCyc database of metabolic pathways and enzymes. Nucleic Acids Res 2018;46:D633–9. https://doi.org/10.1093/nar/gkx935.
Cordova LT, Cipolla RM, Swarup A et al. 13C metabolic flux analysis of three divergent extremely thermophilic bacteria: Geobacillus sp. LC300, Thermus thermophilus HB8, and Rhodothermus marinus DSM 4252. Metab Eng 2017;44:182–90. https://doi.org/10.1016/j.ymben.2017.10.007.
Crennell SJ, Hreggvidsson GO, Nordberg Karlsson E. The structure of Rhodothermus marinus Cel12A, a highly thermostable family 2 endoglucanase, at 1.8 Å resolution. J Mol Biol 2002;320:883–97. https://doi.org/10.1006/jmbi.2002-2836(02)00446-1.
Dahlberg L, Holst O, Kristjansson JK. Thermostable xylanolytic enzymes from Rhodothermus marinus grown on xylan. Appl Microbiol Biotechnol 1993;40:63–8. https://doi.org/10.1007/BF01704340.
Degryse E, Glenadorff N, Pièrard A. A comparative analysis of extreme thermophilic bacteria belonging to the genus Thermus. Arch Microbiol 1978;117:189–96. https://doi.org/10.1007/BF00402307.
Ebrahim A, Lerman JA, Palisson BO et al. COBRApy: cO Nóstrants-Based Reconstruction and Analysis for Python. BMC Syst Biol 2013;7:74. https://doi.org/10.1186/1752-0509-7-74.
Erb TJ et al. A RubisCO like protein links SAM metabolism with iso-prenoid biosynthesis. Nat Chem Biol 2013;9:926–32. https://doi.org/10.1038/nchembio.1087.A
Feist AM, Henry CS, Reed JL et al. A genome-scale metabolic reconstruction for Escherichia coli K-12 MG1655 that accounts for 1260 ORFs and thermodynamic information. Mol Syst Biol 2007;3:121. https://doi.org/10.1038/msb4100155.
Fell DA, Small JR. Fat synthesis in adipose tissue. An examination of stoichiometric constraints. Biochem J 1986;238:781–6. https://doi.org/10.1042/bj2380781.
Fernandes AS, Pereira MM, Teixeira M. The succinate dehydrogenase from the thermohalophilic bacterium Rhodothermus marinus: redox-Bohr effect on heme b1.1. J Bioenerg Biomembr 2001;33:343–52. https://doi.org/10.1023/A:100663424846.
Fernandes AS, Pereira MM, Teixeira M. Purification and characterization of the complex I from the respiratory chain of Rhodothermus marinus. J Bioenerg Biomembr 2002;34:413–21. https://doi.org/10.1023/A:1022509907553.

Fukuda W, Hidese R, Fujiwara S. Long-chain and branched polyamines in thermophilic microbes. In: Kusano T, Suzuki H (eds.), Polyamines: a Universal Molecular Nexus for Growth, Survival, and Specialized Metabolism. Tokyo: Springer Japan, 2015, 15–25. https://doi.org/10.1007/978-4-431-55212-3_2.

Guzmán GI, Sandberg TE, Lacroix RA et al. Enzyme promiscuity shapes adaptation to novel growth substrates. Mol Syst Biol 2019;15:e8462. https://doi.org/10.15252/msb.20188462.

Hallórdsdóttir S, Thórólfsdóttir ET, Spillaert R et al. Cloning, sequencing and overexpression of a Rhodothermus marinus gene encoding a thermostable cellulase of glycosyl hydrolase family 12. Appl Microbiol Biotechnol 1998;49:277–84. https://doi.org/10.1007/s002530011569.

Hamana K, Hamana H, Niitsu M et al. Distribution of unusual long and branched polyamines in thermophelic eubacteria belonging to “Rhodothermus,” Thermus and Thermonema. J Gen Appl Microbiol 1992;38:575–84. https://doi.org/10.2322/jgam.38.575.

Henry CS, Dejongh M, Best AA et al. High-throughput generation, optimization and analysis of genome-scale metabolic models. Nat Biotechnol 2010;28:977–82. https://doi.org/10.1038/nbt.1672.

Hrengviddson G, Kaiste E, Holst O et al. An extremely thermostable cellulase from the thermophilic eubacterium Rhodothermus marinus. Aem 1996;62:3047–9. https://doi.org/10.1128/62.8.3047-3049.1996.

Hrengviddson GO, Fridjonsson OH. International publication number WO 2020/044379 A1. 2020. https://patents.google.com/patent/WO2020044379A1/en (1 April 2021, date last accessed).

Hrengviddson GO, Jonsson Wheat JO, Bjornsdottir B et al. International publication number EP 3092247 A1. 2016. https://patents.google.com/patent/EP3092247A1/en (1 April 2021, date last accessed).

Huerta-Cepas J, Szklarczyk D, Heller D et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res 2019;47:D309–14. https://doi.org/10.1093/nar/gky1085.

Jeske L, Placzek S, Schomburg I et al. BRENDAP in 2019: a European ELIXIR core data resource. Nucleic Acids Res 2019;47:D542–9. https://doi.org/10.1093/nar/gky1048.

Jolly L, Ferrari F, Blanot D et al. Reaction mechanism of phosphoglucomamine mutase from Escherichia coli. Eur J Biochem 1999;262:202–10. https://doi.org/10.1111/j.1432-1327.1999.00373.x.

Kanehisa M. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28:27–30. https://doi.org/10.1093/nar/28.1.27.

Katsuki H, Bloch K. Studies on the biosynthesis of ergosterol in yeast. Formation of methylated intermediates. J Biol Chem 1967;242:222–7.

King ZA, Lu J, Dräger A et al. BiGG models: a platform for integrating, standardizing and sharing genome-scale models. Nucleic Acids Res 2016;44:DS15–22. https://doi.org/10.1093/nar/gkv1049.

Kirby J, Nishimoto M, Chow RWN et al. Enhancing terpene yield from sugars via novel routes to 1-deoxy-d-xylulose 5-phosphate. Appl Environ Microbiol 2015;81:130 LP–138. https://doi.org/10.1128/AEM.0.02920-14.

Krah M, Misselwitz R, Politz O et al. The lamarinase from thermophilic eubacterium Rhodothermus marinus. Eur J Biochem 1998;257:101–11. https://doi.org/10.1046/j.1432-1327.1998.2570101.x.

Kristjansdottir T, Ron EYC, Molins-Delgado D et al. Engineering the carotenoid biosynthetic pathway in Rhodothermus marinus for ly-copene production. Metab Eng Commun 2020;11:e00140. https://doi.org/10.1016/j.mec.2020.e00140.

Lieveen C, Beber ME, Olivier BG et al. MEMOTE for standardized genome-scale metabolic model testing. Nat Biotechnol 2020;38:272–6. https://doi.org/10.1038/s41580-020-0446-y.

López-Contreras A, Harmsen P, Hou X et al. Biorefinery approach to the use of macroalgae as feedstock for biofuels. In: Pereira L (ed.) Algal Biofuels. Boca Raton: CRC Press, 2017. https://doi.org/10.1201/9781315152547-5.

Lutnaes BF, Strand Å, Pétursdóttir SK et al. Carotenoids of thermal bacteria—Rhodothermus marinus from submarine Icelandic hot springs. Biochem Syst Ecol 2004;32:455–68. https://doi.org/10.1016/j.bse.2003.09.005.

Lynen F. Biosynthetic pathways from acetate to natural products. Pure Appl Chem 1967;14:137–68. https://doi.org/10.1557/pac19671401014.

Miller JH. Experiments in Molecular Genetics (Bacterial Genetics—E. coli). Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory, 1972.

Moreira L, Fernanda Nobre M, Sa-Correia I et al. Genomic typing and fatty acid composition of Rhodothermus marinus. Syst Appl Microbiol 1996;19:83–90. https://doi.org/10.1016/S0723-2020(96)80014-2.

Mukti JJ et al. Growth and Production of Carotenoids and Exopolysaccharides by the Extremophile Rhodothermus marinus DSM16675 in Defined Media. 2021.

Munoz R, Rosselló-Móra R, Amann R. Revised phylogeny of bacteroidetes and proposal of sixteen new taxa and two new combinations including Rhodothermaeota phyl. nov. Syst Appl Microbiol 2016;39:281–96. https://doi.org/10.1016/j.syapm.2016.04.004.

Nielsen MM, Suits MDL, Yang M et al. Substrate and metal ion promiscuity in mnnosylglycerate synthase. J Biol Chem 2011;286:15155–64. https://doi.org/10.1074/jbc.M110.199844.

Nolan M, Tindall BJ, Pomrenke H et al. Complete genome sequence of Rhodothermus marinus type strain (R-10 T). Stand Genomic Sci 2009;1:283–90. https://doi.org/10.4056/sigs.46736.

Nordberg Karlsson E, Bartonek-Roxå E, Holst O. Cloning and sequence of a thermostable multidomain xylanase from the bacterium Rhodothermus marinus. Biochim Biophys Acta 1997;1353:118–24. https://doi.org/10.1016/s0007-4717(97)00059-6.

Nordberg Karlsson E, Bartonek-Roxå E, Holst O. Evidence for substrate binding of a recombinant thermostable xylanase originating from Rhodothermus marinus. FEMS Microbiol Lett 1998;168:1–7. https://doi.org/10.1111/j.1574-6968.1998.tb13247.x.

Nunes OC, Donato MM, Mancia CM et al. The polar lipid and fatty acid composition of rhodothermus strains. Syst Appl Microbiol 1992;15:59–62. https://doi.org/10.1016/S1433-7909(00)80139-6.

Nunes OC, Mancia CM, Da Costa MS et al. Complementary solutes in the thermophilic bacteria rhodothermus marinus and “thermus thermophilus”. Appl Environ Microbiol 1995;61:2351–7. Available at: https://pubmed.ncbi.nlm.nih.gov/16530503.

Nurk S, Bankevich A, Antipov D et al. Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng Met al. (eds.), Research in Computational Molecular Biology, Berlin, Heidelberg: Springer Berlin Heidelberg, 2013,158–70.

Nwodo UIU, Green E, Okoh AI. Bacterial exopolysaccharides: functionality and prospects. Int J Mol Sci 2012;13:14002–15. https://doi.org/10.3390/ijms131114002.

O’Brien EJ, Monk JM, Palsson BO. Using genome-scale models to predict biological capabilities. Cell 2015;161:971–87. https://doi.org/10.1016/j.cell.2015.05.019.
Orth JD, Conrad TM, Na J et al. A comprehensive genome-scale reconstruction of Escherichia coli metabolism-2011. Mol Syst Biol 2011;7:1–9. https://doi.org/10.1038/msb.2011.65.

Pereira MM, Carita JN, Anglin R et al. Heme centers of Rhodothermus marinus respiratory chain. Characterization of its cbb3 oxidase. J Bioenerg Biomembr 2000;32:143–52. https://doi.org/10.1023/A:10055829301.

Pereira MM, Refoyo PN, Hreggvidsson GO et al. The alternative complex III from Rhodothermus marinus—a prototype of a new family of quinol-electron acceptor oxidoreductases. FEBS Lett 2007;581:4831–5. https://doi.org/10.1016/j.febslet.2007.09.008.

Pereira MM, Santana M, Soares CM et al. The ca3 terminal oxidase of the thermophilic bacterium Rhodothermus marinus: a HiPIP oxygen oxidoreductase lacking the key glutamate of the D-channel. Biochim Biophys Acta 1999;1413:1–13. https://doi.org/10.1016/S0005-2728(99)00073-0.

Pérez-Marin MC, Padmanabhan S, Palanco MC et al. Vitamin B12 partners the CarH repressor to downregulate a photoinducible promoter in Myxococcus xanthus. Mol Microbiol 2008,67:804–19. https://doi.org/10.1111/j.1365-2958.2007.06086.x.

Preiss J, Ashwell G. Alginic acid metabolism in bacteria. The enzymatic reduction of 4-deoxy-L-erythro-5-hexoseulose uronic acid to 2-keto-3-deoxy-D-guconic acid. J Biol Chem 1962,237:317–21.

Qin Y. Alginate fibres: an overview of the production processes and applications in wound management. Polym Int 2008,57:171–80. https://doi.org/10.1002/pi.2296.

Rohner M, Knani M, Simonin P et al. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diposphate. Biochem J 1993,295:517–24. https://doi.org/10.1042/bj950517.

Ron EYC, Plaza M, Kristjansdottir T et al. Characterization of carotenoids in Rhodothermus marinus. MicrobiologyOpen 2018,7:e536. https://doi.org/10.1002/mbo3.536.

Ron EYC, Sardari RRR, Anthony R et al. Cultivation technology development of Rhodothermus marinus DSM 16675. Extremophiles 2019,23:735–45. https://doi.org/10.1007/s00792-019-01129-0.

Ronka J, Hjorleifsdottir S, Tenkanen T et al. Rmal, a type II restriction endonuclease from Rhodothermus marinus which recognizes 5′-CTAG 3′. Nucleic Acids Res 1991,19:2789. https://doi.org/10.1093/nar/19.10.2789.

Sardari RRR, Kulczynska E, Ron EYC et al. Evaluation of the production of exopolysaccharides by two strains of the thermophilic bacterium Rhodothermus marinus. Carbohydr Polym 2017,156:1–8. https://doi.org/10.1016/j.carbpol.2016.08.062.

Sauret-Güeto S, Urós EM, Ibáñez E et al. A mutant pyruvate dehydrogenase E1 subunit allows survival of Escherichia coli strains defective in 1-deoxy-d-xylulose 5-phosphate synthase. FEBS Lett 2006,580:736–40. https://doi.org/10.1016/j.febslet.2005.12.092.

Savinell JM, Palsson BO. Network analysis of intermediary metabolism using linear optimization. I. Development of mathematical formalism. J Theor Biol 1992,154:421–54.

Schneider J, Wendisch VF. Biotechnological production of polyamines by bacteria: recent achievements and future perspectives. Appl Microbiol Biotechnol 2011,91:17–30. https://doi.org/10.1007/s00253-011-3252-0.

Silva Z, Borges N, Martins LO et al. Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by Rhodothermus marinus and Rhodothermus obamensis. Extremophiles 1999,3:163–72. https://doi.org/10.1007/s007920050112.

Takano H, Kondo M, Usui N et al. Involvement of CarA/LitR and CRP/FNR family transcriptional regulators in light-induced carotenoid production in Thermus thermophilus. J Bacteriol 2011,193:2451–9. https://doi.org/10.1128/JB.01125-10.

Takano H, Mise K, Hagiwara K et al. Role and function of LitR, an adenosyl B12-bound light-sensitive regulator of Bacillus megaterium QM B1551, in regulation of carotenoid production. J Bacteriol 2015,197:2301–15. https://doi.org/10.1128/JB.02528-14.

Takano H, Obitsu S, Beppu T et al. Light-induced carotenogenesis in Streptomyces coelicolor A3(2): identification of an extracytoplasmic function sigma factor that directs photodependent transcription of the carotenoid biosynthesis gene cluster. J Bacteriol 2005,187:1825–32. https://doi.org/10.1128/JB.187.5.1825–1832.2005.

Tkase R, Ochiai A, Mikami B et al. Molecular identification of unsaturated uronate reductase prerequisite for alginate metabolism in Sphingomonas sp. A1. Biochim Biophys Acta 2010,1804:1925–36. https://doi.org/10.1016/j.bbapap.2010.05.010.

Tatusova T, Dicuccio M, Badreddin A et al. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res 2016,44:6614–24. https://doi.org/10.1093/nar/gkw569.

Thiele I, Palsson BØ. A protocol for generating a high-quality genome-scale metabolic reconstruction. Nat Protoc 2010,5:93–121. https://doi.org/10.1038/nprot.2009.203.

Tindall BJ. Lipid composition of Rhodothermus marinus. FEMS Microbiol Lett 1991,80:65–8.

Verássimo AF, Pereira MM, Melo AMP et al. a ba3 oxygen reductase from the thermohalophilic bacterium Rhodothermus marinus. FEMS Microbiol Lett 2007,269:41–7. https://doi.org/10.1111/j.1574-6968.2006.00598.x.

Vershinin A. Biological functions of carotenoids—diversity and evolution. Biofactors 1999,10:99–104. https://doi.org/10.1002/biof.5520100203.

Wessel AK, Arshad TA, Fitzpatrick M et al. Oxygen limitation within a bacterial aggregate. mBio 2014,5:e00992. https://doi.org/10.1128/mBio.00992-14.

Wicher KB, Abou-Hachem M, Hallárdóttir S et al. Deletion of a cytotoxic, N-terminal putative signal peptide results in a significant increase in production yields in Escherichia coli and improved specific activity of Cel12A from Rhodothermus marinus. Appl Microbiol Biotechnol 2001,55:578–84. https://doi.org/10.1007/s002530000599.

Wolin EA, Wolin MJ, Wolfe RS. Formation of methane by bacterial extracts. J Biol Chem 1963,238:2882–6.