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Genome-scale reconstruction of *Salinispora tropica* CNB-440 metabolism to study strain-specific adaptation

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Abstract The first manually curated genome-scale metabolic model for *Salinispora tropica* strain CNB-440 was constructed. The reconstruction enables characterization of the metabolic capabilities for understanding and modeling the cellular physiology of this actinobacterium. The iCC908 model was based on physiological and biochemical information of primary and specialised metabolism pathways. The reconstructed stoichiometric matrix consists of 1169 biochemical conversions, 204 transport reactions and 1317 metabolites. A total of 908 structural open reading frames (ORFs) were included in the reconstructed network. The number of gene functions included in the reconstructed network corresponds to 20 % of all characterized ORFs in the *S. tropica* genome. The genome-scale metabolic model was used to study strain-specific capabilities in defined minimal media. iCC908 was used to analyze growth capabilities in 41 different minimal growth-supporting environments. These nutrient sources were evaluated experimentally to assess the accuracy of in silico growth simulations. The model predicted no auxotrophies for essential amino acids, which was corroborated experimentally. The strain is able to use 21 different carbon sources, 8 nitrogen sources and 4 sulfur sources from the nutrient sources tested. Experimental observation suggests that the cells may be able to store sulfur. False predictions provided opportunities to gain new insights into the physiology of this species, and to gap fill the missing knowledge. The incorporation of modifications led to increased accuracy in predicting the outcome of growth/no growth experiments from 76 to 93 %. iCC908 can thus be used to define the metabolic capabilities of *S. tropica* and guide and enhance the production of specialised metabolites.

Keywords *Salinispora tropica* · Metabolic capabilities · Strain adaptation · Genome-scale metabolic reconstruction

Introduction

At present, most pharmaceutical drugs available in the market are derived from nature (Cragg et al. 2009; Lam 2007). Bacteria within the order Actinomycetales are a well-known source of natural products or so called specialised (secondary) metabolites with potential biological activities such as antibiotics and anticancer agents, and the genus *Salinispora* is no exception. *Salinispora tropica* is a marine actinomycete that

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synthesizes diverse specialised metabolites, including many that possess pharmacological properties such as Salinosporamide A (NPI-0052, marizomib), a potent anticancer agent, and sporolides, candidates for antiviral compounds (Feling et al. 2003; Dineshkumar et al. 2014). In 1991, this aerobic Gram-positive bacteria was isolated in the Bahamas (Jensen et al. 1991), and later formally described as an obligate marine actinomycete (Mincer et al. 2002; Maldonado et al. 2005). To date many efforts have been made to elucidate the biosynthetic machinery associated with production of these specialised metabolites, and improvement of the fermentation process to optimize production (Udwary et al. 2007; Nett and Moore 2009; Tsueng and Lam 2008b). However, limited biological knowledge is available about the physiology of this organism and its metabolic capabilities. The strain has been examined for cultural and morphological features on different non-defined medias and a limited number of specific growth capabilities have been identified (Maldonado et al. 2005; Ahmed et al. 2013). These growth capabilities define the phenotypic properties that separate this strain from other *Salinispora* species. Furthermore, there is an increasing incentive to explore actinomycetes as a source of novel specialised metabolites as even well studied taxa have the potential to yield new metabolites (Chaudhary et al. 2013). The identification of unique nutrient sources can help with the development of fermentation methods to enhance the production of specific metabolites, previously cryptic or silent metabolic pathways and by selecting fermentation conditions, which could trigger the expression of dormant loci.

Genome-scale metabolic reconstructions enable determination of the metabolic capabilities of an organism. The reconstruction process itself allows a picture of the current knowledge about a specific organism to be built systematically using genome annotation, omics data and literature (Feist et al. 2009; Monk et al. 2014). Accurate annotations and identification of genes are critical to understand their effect on cellular functions and generate metabolic networks that link the annotations to observed phenotypes. Over the last decade, these metabolic models have been used successfully to predict cellular behavior under different physiological conditions, for the contextualization of high-throughput data, guidance of metabolic engineering, and helping hypothesis-driven discovery (Oberhardt et al. 2009; Edwards et al. 2001; Campodonico et al. 2014; Covert et al. 2004). Moreover, ever since the first genome-scale reconstruction was published in 1999, the number of reconstructions has been increasing steadily (Kim et al. 2011). However, few manually curated actinomycete reconstructions are available despite of their important contribution in drug discovery. These models have been used, for example, for detailed studies of metabolism, increasing specialised metabolite production, and integration of omic data-sets (Licona-Cassani et al. 2012; Borodina et al. 2005; Alam et al. 2010; Medema et al. 2011; Kjeldsen and Nielsen 2009).

In order to provide the best representation of the metabolic capabilities of *S. tropica*, we present the first manually curated genome-scale metabolic reconstruction for *S. tropica* strain CNB-440. The model was used to analyze growth capabilities in different minimal growth-supporting environments. To assess the predictive power of the model, predictions were compared to experimental observations. Comparison with the experimental results revealed a high level of accuracy with model predictions. All false predictions gave some insight about the missing context-specific information. In this study, all the growth experiments were performed in-house.

Materials and methods

Metabolic network reconstruction

The metabolic model generated by Alam et al. (2011) was selected as a template for the development of the model iCC908. The template was automatically generated using Model Seed (Henry et al. 2010) and partially curated focusing on core metabolic pathways (Alam et al. 2011). This draft reconstruction was manually inspected, curated and validated to include essential biomass components, specialised metabolic pathways, and to permit computation of steady-state properties at a broad range of growth conditions such as minimal media. Well-established reconstruction protocols and curation tutorials for models generated by Model Seed were followed through the process (Henry et al. 2010; Thiele and Palsson 2010). Literature, and organism-specific and unspecific databases (e.g. KEGG, PATRIC, ExPASy and NCBI) were used to obtain information for gene annotation, pathway

utilization and other physiological and phenotypical properties with particular focus on specialised metabolism. All gene-protein-reaction (GPR) associations were rewritten. Seed genes IDs were replaced by Locus Tags. Compartments, and molecular species with their identifiers and annotations were kept. A list of the reactions and metabolites included in iCC908 with corresponding references, associated genes, E.C. numbers and notes is available as Online Resource 1.

Metabolic modeling

Flux balance analysis (FBA) (Orth et al. 2010) from COBRA Tools (Becker et al. 2007) was used to predict growth rates and metabolite productions to match the experimental results reported in literature and experimental data generated in this work. The biochemical reactions of the reconstruction define a stoichiometric matrix, S, that allows testing of the capabilities of the system based on the structural knowledge of the metabolic reaction network and steady-state flux distributions. The matrix equation is formulated as:

$$S \cdot v = 0 \quad (1)$$

where S is the $m \times n$ stoichiometric matrix consisting of m metabolites and n net reactions, and $\boldsymbol{v}$ is the $n \times 1$ vector of net reaction rates. Constraint-based linear optimization was used to calculate the optimal flux distributions under the different testing conditions (Becker et al. 2007). This method utilizes linear optimization to estimate optimum value one can achieve given a particular objective function Z.

For simulation of salinosporamide and sporolide production by *S. tropica* wild type, growth rate was maximized and then used as an additional constraint to maximize the production of specialised metabolites since they are produced alongside biomass. For simulation of salinosporamide production by *S. tropica* mutants, in silico gene knockouts were added as constraints by setting the bounds of the corresponding reaction(s), as defined by the GPR association(s), to 0 mmol gDw^{-1} h^{-1}. The flux of non-growth associated ATP maintenance (ATPm) was fixed at 3 mmol gDw^{-1} h^{-1} as observed for *Streptomyces coelicolor* and *Saccharopolyspora erythraea* (Borodina et al. 2005; Licona-Cassani et al. 2012). This reaction simulates the consumption of ATP by non-growth associated processes such as maintenance of electrochemical gradients. Simulation conditions and constraints used in the validation process are available in Online Resource 2. Uptake constraints for non-defined media were set to match the experimental conditions as closely as possible. Aerobic condition was simulated by limiting the oxygen uptake rate to 20 mmol gDw^{-1} h^{-1}. All simulations were performed using the Matlab-based COBRA Toolbox. Gurobi™ Optimizer was employed as a linear programming solver. Paint4Net was used for the visualization of the reconstruction and analysis in MATLAB (Kostromins and Stalidzans 2012).

Biomass equation

The biomass equation defines all components required to synthesize a new cell of the organism of interest. This equation is fundamental to simulate growth rates using FBA, and it impacts every model prediction. Since it is a critical component of the reconstruction, the biomass reaction from the draft was replaced by a biomass equation developed based on the macromolecular composition of *S. tropica* (Maldonado et al. 2005; Ahmed et al. 2013) and related species. Essential components and their fractional contributions were adapted from Borodina et al. (2005). The biomass equation is described in detail in the Online Resource 3. The complete genome of *S. tropica* CNB-440 was released in 2007 (Udwary et al. 2007).

Strain and media compositions

S. tropica strain CNB-440 was kindly provided by Dr. Paul Jensen. The strain was maintained on medium M1 (Mincer et al. 2002) at 28.5 °C and 250 RPM and as glycerol suspensions (20 %, v/v) at −80 °C. M1 composition per liter of salt formulation: 10 g of starch (Sigma), 2 g of peptone (Sigma), and 4 g of yeast extract (Sigma). Medium M1 is also referred to as A1 (Tsueng and Lam 2008a, b; Lechner et al. 2011). In this case, salt formulation SF2 was used for the media. This salt formulation has been previously described by Tsueng and Lam (2010).

The strain was examined for its capacity to use a range of compounds as nutrients. To standardize experimental procedures, all the growth experiments to test the nutrient sources were performed in-house. For this purpose, we designed a defined minimum

medium (DMM) based on salt formulation SF2 (Tsueng and Lam 2010) and marine broth composition. DMM contains 20 mM of a carbon source supplemented with a salt formulation. Glucose was used as positive control. Salt formulation consists of the following ingredients per liter of deionized water: 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.01 g of KCl, 1 g of NH_4Cl, 3.4 g of $MgCl_2 \cdot 6H_2O$, 5.9 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.42 g of $NaH_2PO_4 \cdot H_2O$ supplemented with 23.5 mg of $CaCl_2 \cdot 2H_2O$, 20.2 g of NaCl, and trace elements. Trace elements consisted of: 0.1 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 2H_2O$, 1.9 mg of NaF, 2 mg of $NiCl_2 \cdot 6H_2O$, 2.5 mg of $MnCl_2 \cdot 2H_2O$, 3 mg of $Na_2MoO_4 \cdot 2H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 21.5 mg of H_3BO_3, and 85.9 mg of KBr. To identify unique growth supporting nutrients, DMM was modified to remove the carbon, nitrogen, phosphorous, and sulfur sources one at a time, and to add the selected nutrients to determine if they supported growth. Negative controls were done to discard toxicity problems. Details of the stock solutions, culture conditions and negative controls can be found in Online Resource 4. All experiments were done in duplicate.

Growth measurements

Several methods have been used for growth analysis of *S. tropica* (Lechner et al. 2011; Tsueng and Lam 2008a, 2010). This spore-forming bacteria forms cell aggregates, which makes difficult to measure cell growth by standard spectrometric methods. A protein extraction protocol was selected to determine the growth of the culture over time (Lechner et al. 2011). Ultra-violet (UV) absorptions at 230 nm and 260 nm were measured using an Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech). Total protein concentration was calculated as follows:

$$\text{Total protein } (\text{ug/ml}) = (183 \times A_{230}) - (75.8 \times A_{260}).$$

In silico growth conditions

In silico simulations were performed to predict the strain's capacity to use a range of compounds as nutrients under minimal conditions. Details of DMM composition have been given above. DMM composition was simulated by allowing unlimited uptake of Ca^{2+}, Cl^-, Co^{2+}, Cu^{2+}, Fe^{2+}, H^+, H_2O, Mg^{2+}, Mn^{2+}, K^+, Na^+, Mo^{+7} and Zn^{2+}. The lower bounds of the respective exchange reactions were set to -100 mmol gDw^{-1} h^{-1}. The default carbon, nitrogen, phosphorous and sulfur sources were glucose, ammonium, phosphate, and sulphate. Default lower bounds were set to -20 mmol gDw^{-1} h^{-1}. To identify growth-supporting nutrients, the default carbon, nitrogen, phosphorous, and sulfur sources were removed one at a time by setting their lower bound to 0 mmol gDw^{-1} h^{-1}, and the selected nutrients were added to determine if they supported growth. Lower bound of the added nutrient was set to -20 mmol gDw^{-1} h^{-1}. FBA was used to determine growth rates for each condition one at the time. Aerobic condition was simulated by limiting the oxygen uptake rate to 20 mmol gDw^{-1} h^{-1} (Feist et al. 2010). Viability threshold was set to 10 % of the maximal growth rate under default conditions for computational identification of nutrients that support growth (Tepper and Shlomi 2010).

Results and discussion

Reconstruction of iCC908

A genome-scale reconstruction from Alam et al. (2011) served as a starting point for the reconstruction of iCC908. To assess the predictive power of the metabolic model, flux balance analysis (FBA) was used to compute growth at the reported minimum growth condition. Even when core metabolic pathways were well represented in the reconstruction of Alam et al. (2011), the model can only consider growth in complex medium, lacks secondary metabolism pathways, and the biomass reaction is not organism specific. Additionally, the model predicted a strain-specific auxotrophy for methionine. M4 and M5, low-nutrient media without supplementation of methionine, can support the growth of this strain (Mincer et al. 2002; Maldonado et al. 2005). For these reasons, the draft model was manually curated using literature, *S. tropica* pathway database in KEGG and other available databases to ensure that it captures the biochemical and physiological knowledge available at the time of the reconstruction. This allows generation of a model that can be used for detailed study of the organism's

metabolism. For this purpose, the biomass reaction and associated reactions were removed, all GPR associations were revised, 135 (9.8 %) reactions were removed, 376 (27 %) reactions underwent a change in directionality and 463 reactions were added to the model. A new organism specific biomass reaction was added to the reconstruction to assure that essential lipids, cell wall components, cofactors, protein, DNA, and RNA composition were included to represent this Gram-positive bacteria (Maldonado et al. 2005; Ahmed et al. 2013; Udwary et al. 2007). However, complete details of the cellular composition of *Salinispora* could not be found in the literature. Missing information of macromolecular biomass components was taken from bacteria of the family *Micromonosporaceae* (Hirsch and Valdés 2010; Kroppenstedt et al. 2005; Kawamoto 1992) and other manually curated actinomycete reconstructions (Licona-Cassani et al. 2012; Borodina et al. 2005). In addition, ATP consumption for growth is a unique property of each organism. To our knowledge experimental data about total energy production is not available for *S. tropica*. It was assumed that energy requirements must be similar to values reported for other actinomycetes. Growth associated maintenance requirements (GAM) was assumed to be 40 mmol ATP gDw^{-1} h^{-1}. An average of values observed for *S. coelicolor* and *S. erythraea* (Licona-Cassani et al. 2012; Borodina et al. 2005). As stated in protocols, this term was added as an ATP hydrolysis to the biomass reaction. Details about the biomass equation can be found in the Online resource 3.

Furthermore, oxidative phosphorylation is the major source of ATP in aerobic organisms. Recently, comparative genomics analysis revealed that any available Gram-positive marine bacterial genomes posses the sodium-dependent NADH dehydrogenase (Nqr) that is commonly found in gram-negative marine bacteria (Penn and Jensen 2012). *Salinispora* possesses NADH dehydrogenase components (NDH-1), a proton pump. Since no evidence from literature is available, it was assumed that ubiquinone is used as cofactor.

On the other hand, despite transporters being poorly characterized, different transport systems were added to allow interchange with the environment. Pathways for amino acid biosynthesis and metabolism were gap filled since no strain-specific auxotrophy has been identified. Four reactions were added for galactose utilization as carbon source (Maldonado et al. 2005). In addition, reactions were added to allow growth on fructose, mannitol, arabinose, mannose, sorbitol, melibiose, stachyose, and raffinose. The extensive manual curation and gapfilling process ensured that the reconstruction derived from the draft was able to produce all biomass precursors on aerobic minimal medium (Alam et al. 2011; Maldonado et al. 2005). The model includes metabolic pathways for primary and specialised metabolism. iCC908 describes 1373 reactions encoded by 908 genes, and 1317 metabolites. 673 of 864 genes accounted in the draft model are conserved in iCC908. Table 1 summarizes key properties of iCC908. Figure 1 shows the subsystem distribution in the model.

Table 1 Statistics of iCC908

Total reactions	1507
Unique reactions (no isoenzymes)	771
Transport reactions	204
Exchange reactions	134
Metabolic conversions	1169
Reactions with ORF assignments	1083
Reactions without ORF	424
% of reactions with ORF	72 %
Transport reactions with ORF assignments	60
% of transport reactions with ORF	29 %
Metabolic conversions with ORF assignments	1023
% of metabolic conversions with ORF	88 %
Number of ORFs	908
% ORF included in the model (from total ORFs)	20 %
% ORF included in the model (from ORF with assigned function)	30 %

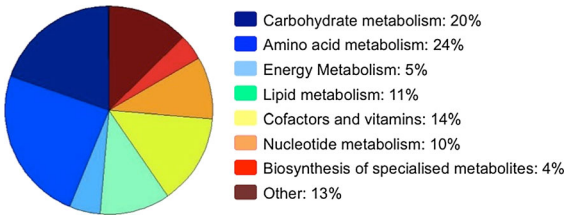


Fig. 1 Distribution of functional subsystems in *Salinispora tropica* iCC908

Specialised metabolite pathways

At least 19 biosynthetic gene clusters are harbored in the *S. tropica* genome (Nett and Moore 2009; Nett et al. 2009). Salinosporamides and sporolides are among the products of these pathways. These two metabolites were added to the model since these specialised metabolites have been characterized and their biosynthetic pathways have been reported with different grades of detail.

Salinosporamide A and B are assembled by a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS) system from three building blocks. Salinosporamide A is built from acetyl-CoA, chloroethylmalonyl-CoA and L-3-cyclohex-2′-enylalanine. Salinosporamide B is built form acetyl-CoA, ethylmalonyl-CoA and L-3-cyclohex-2′-enylalanine (Beer and Moore 2007; Eustáquio et al. 2009; Nett and Moore 2009). Both specialised metabolites incorporate an acetyl-CoA and the non-proteinogenic amino acid L-3-cyclohex-2′-enylalanine. However, only a part of the reaction stoichiometry could be identified with confidence, as a majority of the enzymes have not yet been characterized. Thus, the information on the uncharacterized enzymes was added to the model based on the predictions of bioinformatic analysis tools. BLAST and databases such as KEGG and BRENDA were used to find the most probable cofactors and reaction participants of the biosynthetic pathways. In addition, the pathway of the nonproteinogenic amino acid L-3-cyclohex-2′-enylalanine has not been fully identified up to this point (McGlinchey et al. 2008a; Nett and Moore 2009).

In the case of sporolides A and B, these metabolites are putatively derived from a 9-membered enediyne precursor on cyclization to a *p*-benzyne biradical and subsequent nucleophilic addition of a chloride anion (Nett and Moore 2009). A biosynthesis pathway of the sporolides A and B have been proposed (McGlinchey et al. 2008b). However, only the initial enzymatic reaction in the biosynthesis of the sporolides has been characterized. As stated above, the information on the uncharacterized steps was added to the model based on bioinformatic tools.

Fig. 2 Overview of biosynthetic pathways of: **a** salinosporamides A and B, and **b** sporolides A and B. *Aminoacids* L-isoleucine, L-leucine or L-valine as substrates ▶

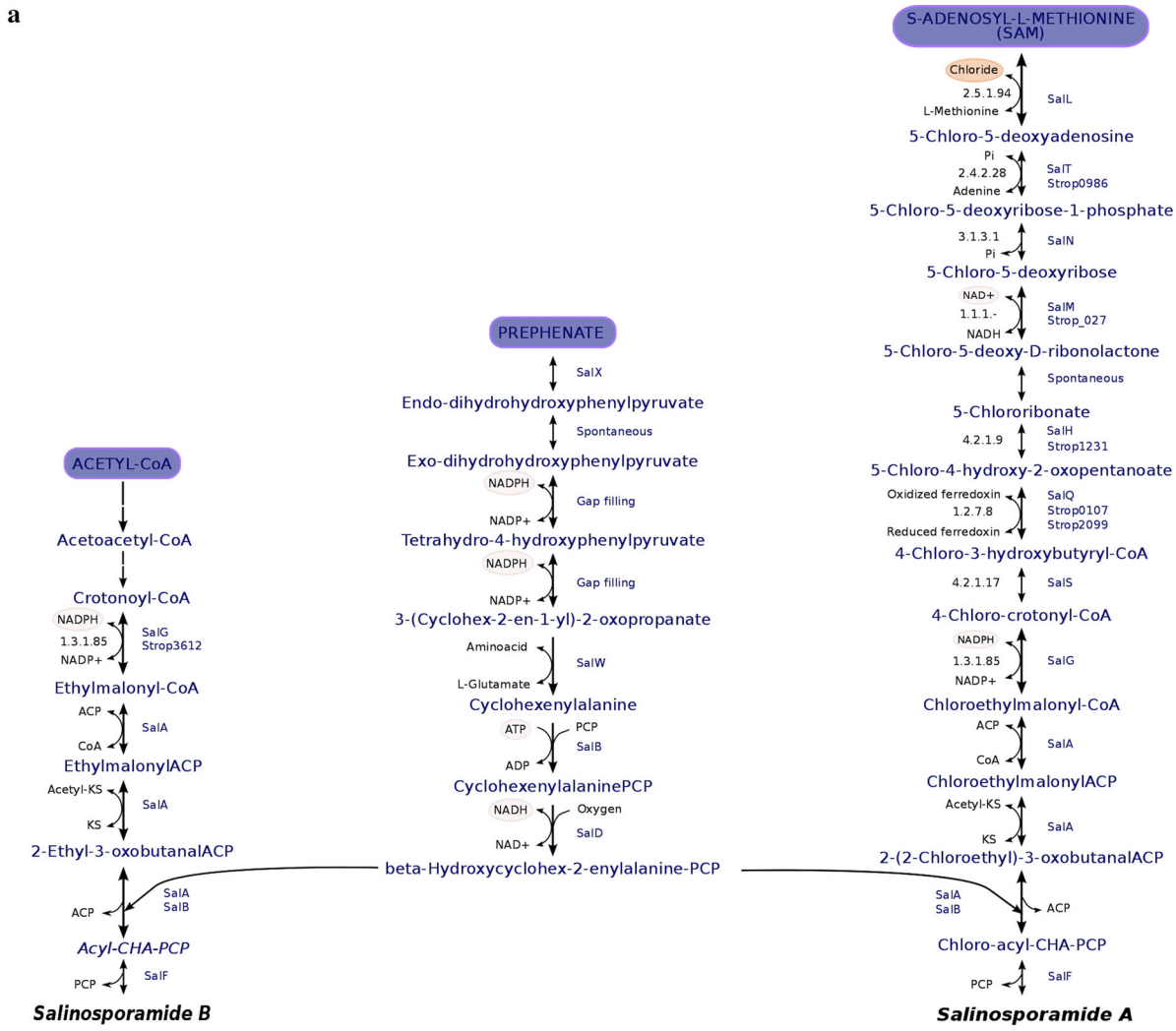
30 and 15 reactions were identified to represent the biosynthesis of salinosporamides and sporolides, respectively. The pathways were manually entered into the model. Figure 2 summarizes the information collected for the biosynthetic pathways of these specialised metabolites. A detail of the reactions can be found in Online Resource 1.

Validation

As stated above, the genome-scale metabolic reconstruction of *S. tropica*, iCC908, is capable of predicting growth on aerobic minimal medium. In this case, the conditions described by Alam et al. (2011) and Maldonado et al. (2005) were tested. Furthermore, the model was validated and interrogated by contrasting model predictions in terms of growth/no growth in comparison to published results of growth studies using several carbon sources (Maldonado et al. 2005; Ahmed et al. 2013; Tsueng and Lam 2010, 2008b; Tsueng et al. 2008). The media conditions included in these studies were aerobic growth on glucose minimal media and on rich media with different carbon sources and salt formulations. For the validation of the specialised metabolite pathways, gene deletions, and chemical complementation studies were used to compare the predictions of the model (Eustáquio et al. 2009; McGlinchey et al. 2008b; Tsueng et al. 2007). These allowed evaluation of the structure of the pathways, precursors and production ratios. Only scenarios with strong evidence were evaluated in the validation process.

Since most of the data reported in the literature gives qualitative results, the quantitative results from literature and simulations were simplified to growth/no growth and production/no production values to facilitate the overall comparison between experiment data and in silico simulations.

Growth simulations and in silico gene deletions were performed by using flux balance analysis (FBA) as


a
S-ADENOSYL-L-METHIONINE (SAM)
Chloride
2.5.1.94
L-Methionine
SalL
5-Chloro-5-deoxyadenosine
Pi
2.4.2.28
Adenine
SalT
Strop0986
5-Chloro-5-deoxyribose-1-phosphate
3.1.3.1
Pi
SalN
5-Chloro-5-deoxyribose
NAD+
1.1.1.-
NADH
SalM
Strop_027
5-Chloro-5-deoxy-D-ribonolactone
Spontaneous
5-Chlororibonate
4.2.1.9
SalH
Strop1231
5-Chloro-4-hydroxy-2-oxopentanoate
Oxidized ferredoxin
1.2.7.8
Reduced ferredoxin
SalQ
Strop0107
Strop2099
4-Chloro-3-hydroxybutyryl-CoA
4.2.1.17
SalS
4-Chloro-crotonyl-CoA
NADPH
1.3.1.85
NADP+
SalG
Chloroethylmalonyl-CoA
ACP
CoA
SalA
ChloroethylmalonylACP
Acetyl-KS
KS
SalA
2-(2-Chloroethyl)-3-oxobutanalACP
SalA
SalB
ACP
Chloro-acyl-CHA-PCP
PCP
SalF
Salinosporamide A
PREPHENATE
SalX
Endo-dihydrohydroxyphenylpyruvate
Spontaneous
Exo-dihydrohydroxyphenylpyruvate
NADPH
NADP+
Gap filling
Tetrahydro-4-hydroxyphenylpyruvate
NADPH
NADP+
Gap filling
3-(Cyclohex-2-en-1-yl)-2-oxopropanate
Aminoacid
L-Glutamate
SalW
Cyclohexenylalanine
ATP
ADP
PCP
SalB
CyclohexenylalaninePCP
NADH
NAD+
Oxygen
SalD
beta-Hydroxycyclohex-2-enylalanine-PCP
ACETYL-CoA
Acetoacetyl-CoA
Crotonoyl-CoA
NADPH
1.3.1.85
NADP+
SalG
Strop3612
Ethylmalonyl-CoA
ACP
CoA
SalA
EthylmalonylACP
Acetyl-KS
KS
SalA
2-Ethyl-3-oxobutanalACP
ACP
SalA
SalB
Acyl-CHA-PCP
PCP
SalF
Salinosporamide B
b
L-TYROSINE
2-Oxoglutarate
Strop_3195
Strop_4252
L-Glutamate
2.6.1.9
3-(4-Hydroxyphenyl)pyruvate
Oxygen
SpoT1
CO2
1.13.11.46
(S)-4-Hydroxymandelate
ATP
SpoT2
PPi
6.-.-.-
4-HMA-AMP
SpoT2
6.-.-.-
SpoT2-SH
AMP
S-Adenosyl-L-homocysteine
S-Adenosyl-L-methionine
6-M-5HQG-T2
SpoT6
2.1.1.-
5-HQG-T2
NADH
SpoT5
NAD+
1.1.1.-
2,4,5-tHMA-T2
H2O2
SpoT4
Oxygen
1.-.-.-
3,4-dHMA-T2
FAD
SpoT3
FADH2
1.14.14.9
4-HMA-T2
Oxygen
S-Adenosyl-L-methionine
2.1.1.-
SpoT7
S-Adenosyl-L-homocysteine
6-M-5HQ-MG-T2
NADH
SpoT8
SpoT9
NAD+
5.1.-.-
Oxygen
Cyclohexenone epoxide
SpoT10
SpoT2-SH
Preprosporolide
H2O
Spontaneous
Presporolide
Spontaneous
Chloride
SPOROLIDE A
Spontaneous
Chloride
SPOROLIDE B
ACETYL-CoA
SpoE
SpoE2-E11
CoA
2.-.-.-
CO2
Precore
SpoF
3.3.2.-
Enediyne core
MALONYL-CoA

described in the "Materials and methods" section. The media conditions for the simulations were set to match the experimental conditions as closely as possible. From the simulations, it was verified that all predictions were consistent with the published reports. Table 2 summarized the literature sources, criteria and conditions used for validation of the *S. tropica* model. Overall, 42 different conditions from published data were tested during the validation process. The predicted growth rates and specialised metabolite productions for each condition can be found in Online Resource 2.

Phenotypic properties

In order to refine the model, a wider range of experimental conditions was needed. Minimal media with only glucose as carbon source or rich media composition make it difficult to identify structural problems of the network, incapabilities, or missing metabolic functions in the reconstruction. The strain has been examined for its ability to degrade a range of organic compounds and for the ability to grow on a range of sole carbon and nitrogen sources by using established methods (Pridham and Gottlieb 1948; Stevenson 1967; Williams et al. 1983). However, all the growth screening experiments reported in these studies are qualitative and not quantitative results, experimental data sets are not available, different basal mineral salts formulations were used in each study, and either liquid culture or agar plates were used. Furthermore, salt formulations used do not take into account the growth requirements for medium ionic strength, and discrepancies have been found in the literature about the definition of minimal growth conditions (Alam et al. 2011; Maldonado et al. 2005). It is now known that *S. tropica* strains will only grow if

Table 2 Experimental data used for validation of the *Salinispora tropica* model

References	Criteria	Gene KOs	Media conditions
Alam et al. (2011)	Growth	–	Minimal media
Maldonado et al. (2005)	Growth	–	Minimal media
Tsueng et al. (2008)	Growth	–	Glucose complex media
Tsueng and Lam (2010)	Growth	–	A1 growth media
Tsueng et al. (2008b)	Growth	–	A1 growth media
Ahmed et al. (2013)	Growth	–	ISP2,5,4,3,1,6,7 medias
DSMZ (Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)	Growth	–	Marine broth
This study	Growth	–	Glucose complex media
Lechner et al. (2011)	Sal. production, growth	–	A1 growth and production media
Tsueng and Lam (2009)	Sal. production	–	Defined salt formulation I
Tsueng et al. (2007)*	Sal. Production	–	Production media
Eustáquio et al. (2009)	Sal. production	salL, salA, salT, salM, salH, salQ, salG, Strop_3612	A1 production media with/without chemical complementation
McGlinchey et al. (2008b)	Spo. productions	–	A1 production media; supplementation with tyrosine

Sal. salinosporamides, *Spo.* sporolides

* Experiment with *S. tropica* NPS21184

the growth medium has a high ionic strength (Tsueng and Lam 2010). For these reasons, all growth/no growth experiments were performed in-house to standardize experimental procedures.

The ability of the strain to use sole growth supporting sources was examined on minimal saline media. The nutrients tested were divided into four categories: carbon; nitrogen; sulfur and phosphorous sources. In total, 42 growth conditions were evaluated. Additionally, negative controls were performed to discard toxicity problems. The default carbon, nitrogen, sulfur, and phosphorous sources used in the defined minimum medium (DMM) are required to support *S. tropica* growth. Experimental evidence shows that the cells may be able to store sulfur. No growth was detected in the controls except for the sulfur case. The sulfur negative control was able to grow for over 20 days. Figure 3 shows growth curves for sulfur sources including the respective negative control. Growth of the negative control was lower compared to the sulfur sources tested. A subculture of this culture was done and no growth was detected as seen in Fig. 3b, which implies that sulfur must be supplemented to the media to support growth after the depletion of the accumulated sulfur. To our knowledge, this is the first report showing this behavior in *S. tropica*. Certain bacteria are able to store sulfur globules both extracellularly and intracellularly. In the literature, some actinomycete strains have been mentioned to possess genes with similar sequences to the sulfur globule protein (SgpA) from *Allochromatium vinosum* (Maki 2013). This protein is related to intracellular storage of sulfur. A BLASTP search of the amino acid sequence of the sulfur globule proteins (SgpA (YP_003443861), SgpB (YP_003442351), and SgpC (YP_003443294)) from *A. vinosum* against *S. tropica* genome revealed low identities (less than 40 %) at the amino acid level (data not shown). Therefore, *S. tropica* does not have any Sgp ortholog. Genes involved in sulfur accumulation in *S. tropica* could have a low degree of sequence homology to any other organism since there is not a universal mechanism for sulfur accumulation (Dahl and Prange 2006). A detailed study of the sulfur metabolism is required to gain new insights, which is not in the scope of the present study.

Table 3 includes the characterization of all growth screening experiments. Although different shades of orange pigments (dark orange, orange or light orange)

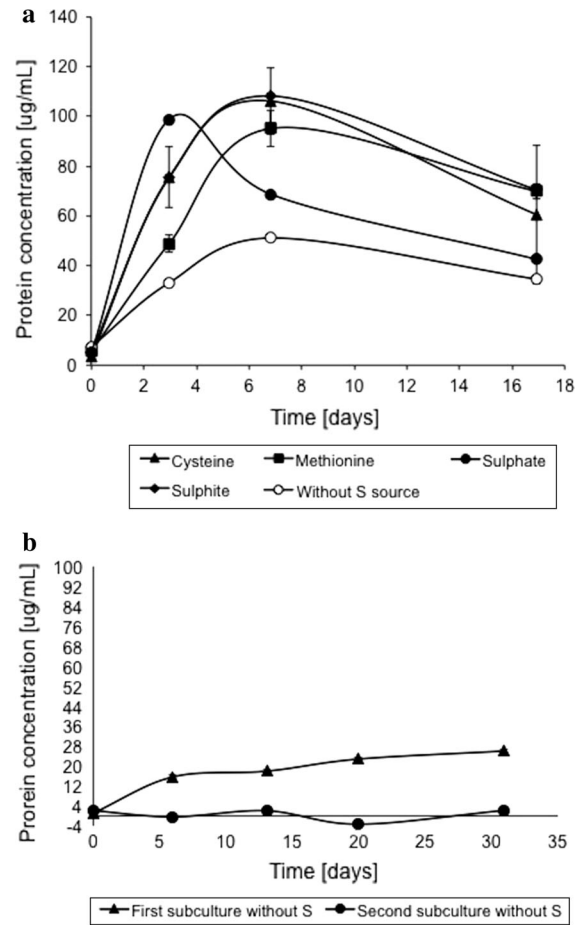


Fig. 3 Growth curves of *Salinispora tropica* CNB-440 under different sulfur sources. **a** Growth capabilities under sulfur sources; **b** negative controls: first culture and subculture. Nutrients tested and negative controls are indicated in the graphs. All experiments were done in duplicate. *Error bars* represent standard deviation

were produced, a simplified color classification was used to keep objectivity. At six growth conditions, the strain was poorly pigmented. Experiments confirm that this strain has biosynthetic pathways for all essential amino acids. Furthermore, *S. tropica* has the ability to adapt its metabolism to a large range of substrates. Cells have the ability to assimilate 21, 8, and 4 diverse carbon, nitrogen, and sulfur compounds as sole sources for growth, respectively. From the table, it is observed that the strain's enzymatic setup very likely is arranged according to the growth conditions to enable a fast or slow adaptation to use the tested compounds. Thereby, the lag phase varies depending on the nutrient source and some slight

Table 3 Growth and cultural characteristics of *Salinispora tropica* strain CNB440 under minimal growth conditions in DMM

	Growth	Lag phase [d]	Characteristics
Carbon sources			
D-Glucose	+	0 ± 0	Orange
D-Mannose	+	4 ± 0	Poor pigmentation
D-Xylose	+	2 ± 0	Orange
Glycerol	+	5 ± 1	Poor pigmentation
Lactose	−		
Sucrose	+	3 ± 2	Orange
D-Fructose	+	1 ± 1	Orange
Mannitol	+	0 ± 0	Orange
Maltose	+	5 ± 3	Orange
Acetate	+	1 ± 1	Poor pigmentation
D-Sorbitol	+	1 ± 1	Orange
L-Arabinose	+	2 ± 0	Orange
Alginate	+	30 ± 0	Orange
Citrate	+	3 ± 0	Orange
L-Glutamate	+	14 ± 0	Orange
Glycine	+	3 ± 1	Orange
Cellobiose	+	3 ± 3	Orange
Raffinose	+	3 ± 3	Orange
Uracil	−		
Uridine	+	19 ± 2	Poor pigmentation
D-Galactose	+	5 ± 0	Orange
Lactate	−		
Adenosine	+	5 ± 0	Orange
Trehalose	+	6 ± 0	Poor pigmentation
Inosine	−		
L-Glutamine	−		
Nitrogen sources			
Glycine	+	2 ± 0	Orange
Ammonium	+	0 ± 0	Orange
Nitrate	+	8 ± 2	Orange
Urea	+	0 ± 0	Orange
L-Glutamate	+	0 ± 0	Orange
Nitrite	−		
Inosine	+	5 ± 4	Orange
L-Glutamine	+	4 ± 2	Orange
Adenosine	+	4 ± 0	Poor pigmentation
Sulfur sources			
Sulfate	+	0 ± 0	Orange
Sulfite	+	1 ± 1	Orange
L-Cysteine	+	0 ± 0	Orange
L-Methionine	+	0 ± 0	Orange
Phosphorous sources			
Pyrophosphate	−		
Orthophosphate	+	0 ± 0	Orange
β-Glycerophosphate	−		

+, Growth; −, no growth; values are average of at least two independent experiments ± SD

differences were observed in the replicates. According to the results, the strain could be classified as having a fast or slow response to changes in the availability of the substitutable nutrient source. Long or short-term adaptation to growth depends on the transport systems and the ability to metabolize the different compounds. Adaptation to growth on certain substrates is likely to be due to the induction of key enzymes or transport permeases. In some cases, such as glucose and mannitol cells are able to enter the exponential phase rapidly. On the contrary, other sources such as alginate implied a long adaptive phase. Alginate is the major polysaccharide constituent of brown macroalgae, and many efforts are being made to utilize alginate as a carbon source (Enquist-Newman et al. 2014; Contador et al. 2015). A preliminary BLASTp search was done and low identities (less than 40 %) at the amino acid level to genes that code for alginate lyases were found (data not shown). Several alginate lyases have been described for marine bacteria (Wong et al. 2000) and further studies are necessary to characterize these enzymes and transport systems in *S. tropica*.

Inability to assimilate certain compounds as sole carbon sources for growth have been mentioned in previous publications (Maldonado et al. 2005; Ahmed et al. 2013). These compounds included fructose, mannose, mannitol and xylose. In contrast, our study shows that the strain is able to use these nutrient sources. In addition, disagreement in lactose utilization was observed. Growth on lactose previously described by Ahmed et al. (2013) cannot be verified in this study. The discrepancies between published results and the current study could be due to a number of reasons including different thresholds for growth, different incubation time, different inoculum size or physiological state of the inoculum, nutrient concentrations, or even different ionic strengths of the media. This last factor may be critical to determine the nutrient and growth conditions of this strain. DMM has a conductivity of 33.34 mS/cm, which is higher than other reported media used to test the phenotypic properties of *S. tropica* (Maldonado et al. 2005; Ahmed et al. 2013).

Analyzing the metabolic capabilities of the network

The genome-scale metabolic reconstruction for *S. tropica* strain CNB-440 was converted into a computational model to allow the simulation of phenotypes. The computational model was used to predict carbon, nitrogen, sulfur and phosphorus sources that could support growth in minimal medium using a constraint-based approach (Becker et al. 2007). Alginate, whose transport and metabolism is not understood, was not included in the analysis or the reconstruction.

Overall, there were 20 carbon, 6 nitrogen, 2 phosphorous and 3 sulfur sources that were predicted to support growth using flux balance analysis (FBA). Predictions were contrasted with the experimental data to assess the accuracy of the model. Figure 4 details the comparison between the computational and experimental predictions. Comparison with the experimental results revealed a high level of accuracy with 76 % of model predictions agreeing with experiments. In most cases the model correctly predicted growth/no growth capabilities on various carbon, nitrogen, phosphorus and sulfur sources for *S. tropica*.

To further improve and validate the reconstruction, the disagreements with the experimental results were analyzed. Discrepancies with experimental data were categorized in false positive and false negative cases. The first case indicates potential errors in the reconstruction or missing regulatory constraints. False negative cases define areas where additional knowledge is necessary to represent *S. tropica* metabolism. Instances where experimental growth was reported and no growth is predicted by the model was observed in usage of: acetate, and trehalose as sole carbon sources; nitrate, urea, and inosine as sole nitrogen sources, and sulfite as sole sulfur source. The disagreement of acetate was resolved by relaxation of directionality constraints of existing irreversible reactions in the model. Sulfite exchange and transport reactions were added to the reconstruction to allow the use of this compound as sole sulfur source. These reactions were not present in the draft model or added during the curation process. The rest of the inconsistencies were resolved by adding reactions from the KEGG database to gap fill the missing knowledge. A BLASTp search of amino acid sequences of the genes coding for enzymes that catalyze these reactions against the *S. tropica* genome was done to evaluate the proposed corrections. In some cases, these revealed significant sequence similarities (BLASTp expectation value of less than 10^{-10}). However, low sequence similarity enzymes could carry out the activity, or

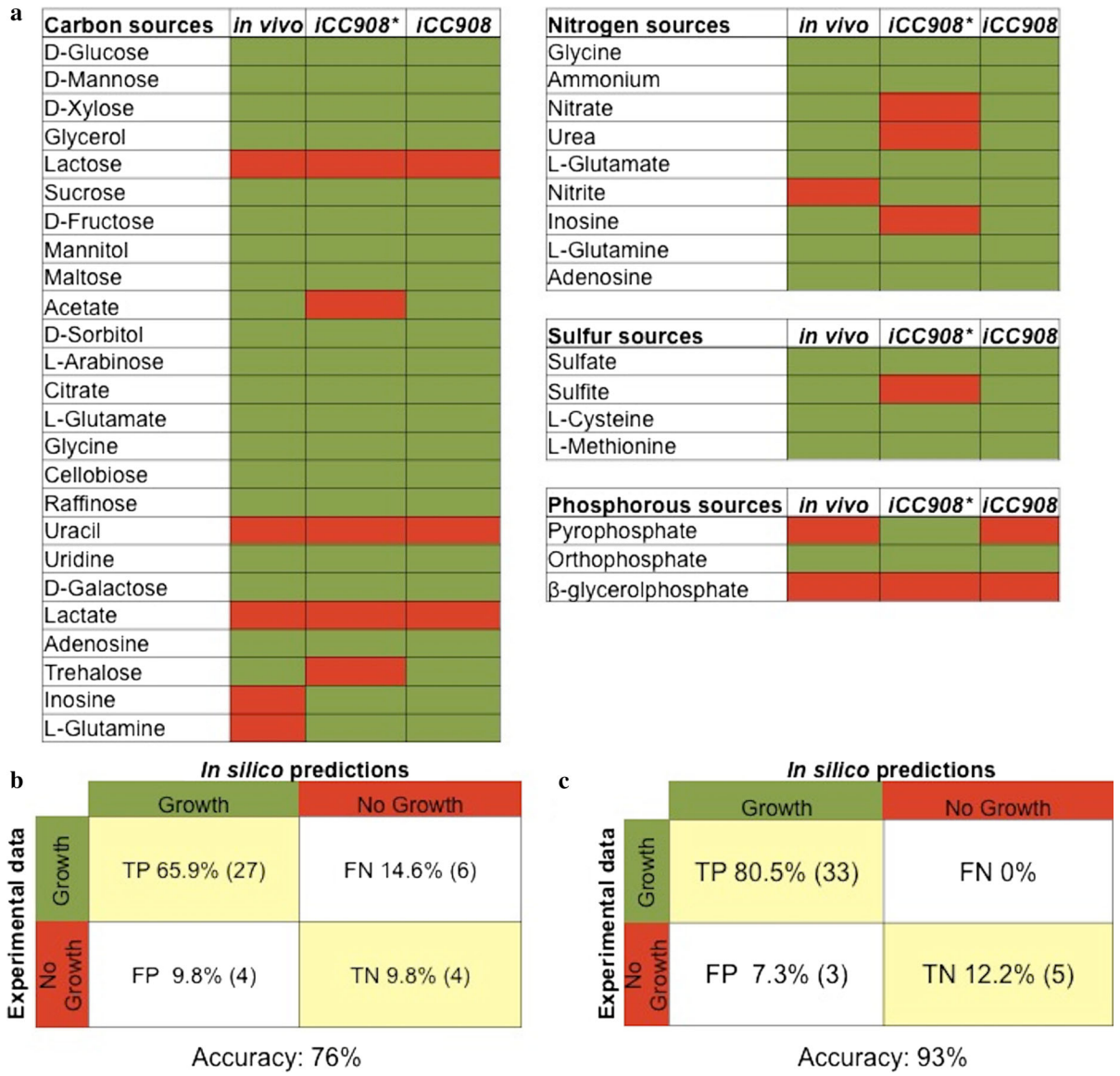


Fig. 4 **a** Comparison of model predictions to experimental data. Accuracy of iCC908 was calculated before (**b**) and after (**c**) modifications using sole growth supporting sources on minimum saline media. The number of false and correct predictions is indicated in each case. *iCC908** indicates model before modifications, *green* growth, *red* no growth

unknown promiscuous enzymatic activities or even alternate pathways for the metabolism of these compounds. After the modifications, the growth inconsistencies were resolved. It was verified that all the proposed modifications do not invalidate any of the correct model predictions.

Cases in which growth is predicted by the model and has not been observed experimentally was seen in usage of inosine, and glutamine as sole carbon sources; nitrite as sole nitrogen source, and pyrophosphate as sole phosphorous source. Inosine and glutamine can be used as sole nitrogen sources, but not as carbon sources by *S. tropica*. Inosine can presumably be converted to alpha-D-ribose 1-phosphate, which can be converted to ribose 5-phosphate and then catabolized to 5-phospho-alpha-D-ribose 1-diphosphate to be used in the synthesis of nucleotides. There is evidence that nucleoside catabolism can differ greatly between different bacteria (Schuch et al. 1999). Regulatory factors or a slow nucleoside

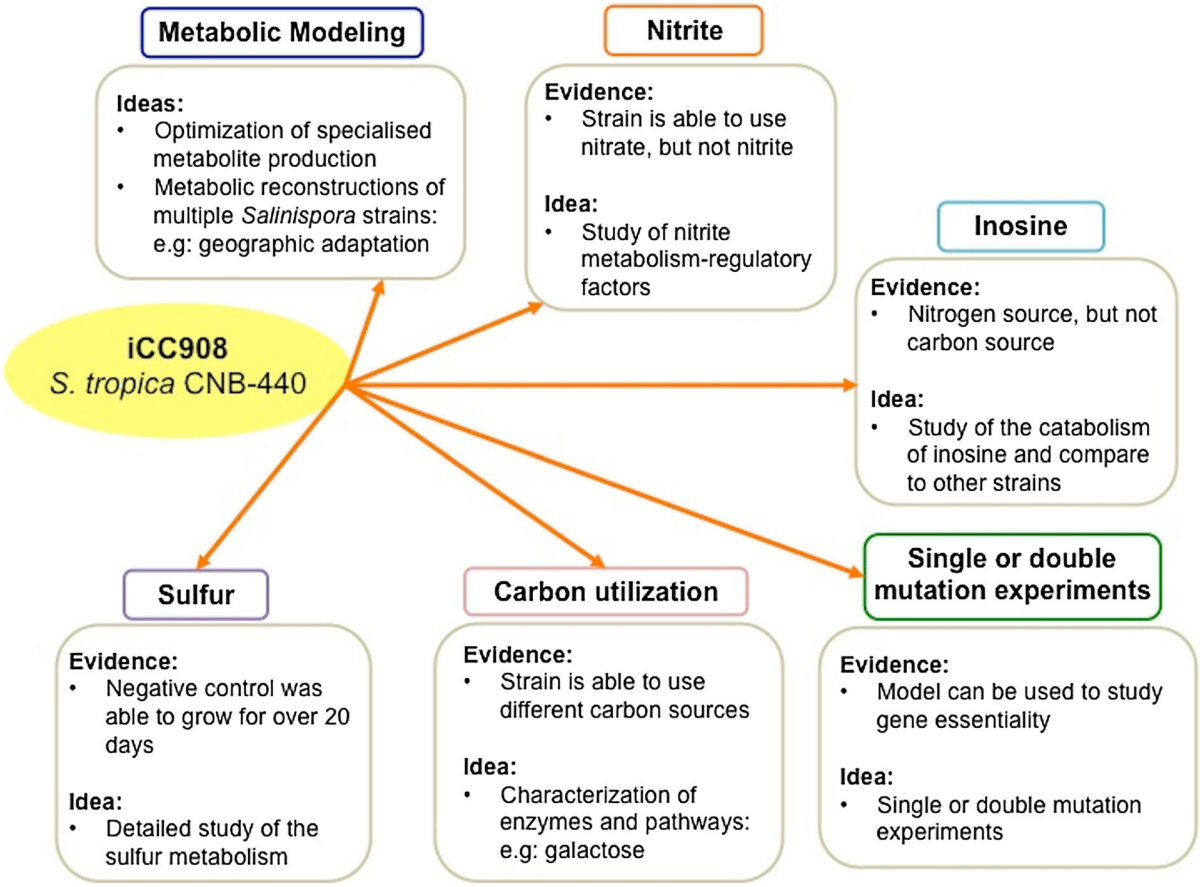


Fig. 5 Set of proposals to discover new metabolic functionalities of *Salinispora tropica* CNB-440 after analyzing the metabolic capabilities of the network with iCC908

catabolism can determine if a microorganism can grow on inosine as the sole carbon source. Another actinobacterium, *S. coelicolor* A3(2) can use glutamate as carbon source, but not glutamine. It has been mentioned that if glutamine is deaminated by glutaminase intracellularly, the glutamate formed cannot be further degraded providing only nitrogen for growth and not carbon (Borodina et al. 2005). Additional experiments are required to study if a similar behavior is observed in *S. tropica*.

It is known that *S. tropica* can grow on nitrate, but not on nitrite as nitrogen source. However, nitrite is produced during nitrate reduction. In this case, it may be regulatory constraints that do not allow nitrite utilization rather than the lack of metabolic capabilities. The pyrophosphate transport reaction was removed due to lack of evidence. After all modifications, the accuracy of iCC908 increases to 93 %. This result reflects the increased scope of iCC908 to analyze a wider range of growth conditions and helps validate the content of the reconstruction. Figure 5 summarizes new hypotheses generated after analyzing the metabolic capabilities of the network with iCC908 and proposed strategies. These new experiments will allow us to gain new biological knowledge of this seawater-requiring marine actinomycete and improve the metabolic representation of this organism.

Conclusions

Modeling of *S. tropica* is a challenging task since, although marine bacteria have been studied for years (Macleod 1965), bacteria inhabiting marine sediments remain largely uncharacterized (Fenical and Jensen 2006). In this study, we reconstructed a genome-scale

model for *S. tropica* CNB-440, iCC908, to gain new insights into the metabolic capabilities of this organism. A model derived from MODEL SEED was used as a starting point for the reconstruction of iCC908 (Alam et al. 2011). Extensive literature about *Salinispora* strains, phylogenetically related organisms such as bacteria of the family *Micromonosporaceae* and other actinomycete reconstructions were used during the manual curation process. The model describes growth on minimal and complex medium. Despite poor or non-existent characterization of some enzymes in the biosynthetic pathways of salinosporamides and sporolides, the model was able to represent production media, gene deletions, different production ratios and chemical complementation studies.

Growth experiments were performed for carbon, nitrogen, phosphorous, and sulfur sources to evaluate the accuracy of the model. The ability of the strain to use sole growth supporting sources was examined on defined minimum medium (DMM). DMM was designed to provide reproducibility of chemical composition, and to meet the experimentally determined ionic strength required by the cells (Tsueng and Lam 2010). Discrepancies with published results (Maldonado et al. 2005; Ahmed et al. 2013) about the utilization of fructose, mannose, mannitol and xylose as carbon sources are reported in this study. These findings could change the current description of the metabolism of *S. tropica* (Goodfellow et al. 2012). Additionally, experimental evidence suggests that the cells may be able to store sulfur. In silico simulations predicted the use of alternative carbon, nitrogen, phosphorous and sulfur sources. Comparison with the experimental results revealed that 76 % of model predictions agree with experiments. All false negative predictions were resolved by adding reactions from the KEGG database to gap fill the missing knowledge or by relaxation of directionality constraints. False positives give some insight about the missing context-specific information such as regulatory factors that were not included in the reconstruction. The overall accuracy of the model was improved to 93 %. The performance of iCC908 cannot be compared to the predecessor model (Alam et al. 2011) since all the estimations were done for minimal growth conditions. The model generated by Alam et al. (2011) only includes growth in complex medium, lacks specialised metabolism pathways, the biomass reaction is not organism specific, and the model predicted a strain-specific auxotrophy for methionine, which was tested experimentally and is not present in the strain.

iCC908 has been reconstructed based on the current knowledge of *S. tropica*. However, our understanding of *S. tropica* is constantly evolving and new data can be incorporated into the reconstruction. Single gene mutation experiments could be used to capture missing or erroneously present metabolic functions in the model and improve gene protein reaction annotations (Zomorrodi and Maranas 2010). As shown in this study, experimental data is essential for curation and validation of genome scale reconstructions. A limited biological knowledge means a limited coverage of the reconstruction. New hypotheses were generated to drive new experiments and gain new biological knowledge of this seawater-requiring marine actinomycete.

The definition of the metabolic capabilities of this organism will help with the design of fermentation conditions, the study of fermentation profiles, and production of specialised metabolites. In order to use the reconstruction as a tool to improve specialised metabolite production, it is necessary to represent the nutritional requirements of the target microorganism. The use of metabolic tools such as metabolic models is fundamental both at academic and industrial levels for strain design of an organism of practical interest.

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Conflict of interest The authors have declared no conflict of interest.

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