Journal of Biotechnology 167 (2013) 123–134

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Establishment, *in silico* analysis, and experimental verification of a large-scale metabolic network of the xanthan producing *Xanthomonas campestris* pv. campestris strain B100

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A R T I C L E I N F O

Article history:
Received 2 November 2012
Received in revised form 28 January 2013
Accepted 28 January 2013
Available online 6 February 2013

Keywords:
Central carbon metabolism
Plant pathogen
Phosphogluconate dehydrogenase
Transketolase
Phosphofructokinase
Phosphoglucose isomerase

A B S T R A C T

The γ-proteobacterium *Xanthomonas campestris* pv. campestris (Xcc) B100 synthesizes the polysaccharide xanthan, a commercially important viscosifier. Since the complete genome of Xcc B100 is available, systems biology tools were applied to obtain a deeper understanding of the metabolism involved in xanthan biosynthesis. A large-scale metabolic network was reconstructed and manually curated. The reconstructed network included 352 genes, 437 biochemical reactions, 10 transport reactions, and 338 internal metabolites. To use this network for flux balance analysis, the biomass composition of Xcc B100 was determined. The comprehensive model obtained was applied for *in silico* analyses to predict biomass generation and gene essentiality. Predictions were extensively validated by analyzing batch culture performance and by carbon balancing including xanthan production. Single gene deletion mutants causing deficiencies in the central carbohydrate metabolism were constructed to enforce major flux redistributions. The impact of xanthan production was studied *in vivo* and *in silico*, comparing the physiology of a *gumD* mutant, negative in xanthan production, with the original strain. The results indicate a redistribution of resources from xanthan to biomass, rather than a reduction in carbon uptake. With this high quality metabolic model, both systems biology analyses and synthetic biology reengineering of Xcc gained an important tool.

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1. Introduction

The polysaccharide xanthan is an important biotechnological product (Hublik, 2012). Xanthan is an anionic heteropolysaccharide composed of repetitive pentasaccharide units with a β-1,4-linked main chain. Each pentasaccharide repeat unit consists of two glucose, two mannose, and one glucuronic acid residues. Pyruvate and acetate groups are non-stoichiometrically linked to the mannose residues (Shatwell et al., 1990). Due to its rheological properties, xanthan is widely used as a thickener in the food, cosmetics and oil drilling industries (García-Ochoa et al., 2000).

Xanthan is a natural product generated by the γ-proteobacterium *Xanthomonas campestris* pv. campestris (Xcc). As an exopolysaccharide (EPS), xanthan can be recovered easily from the culture broth by precipitation with ethanol or isopropanol (García-Ochoa et al., 2000). The molecular origins of xanthan biosynthesis are the sugar phosphates glucose 6-phosphate and fructose 6-phosphate, which are key intermediates of the central carbohydrate metabolism. These sugar phosphates which can be interconverted easily are linked to nucleotides to obtain nucleotide sugars which are then used as building blocks for the synthesis of the pentasaccharide subunits of the EPS xanthan. The assembly of the xanthan repeating units takes place at the inner membrane of Xcc (Vorhölter et al., 2008). In addition to its importance for biotechnology, Xcc is a model organism to study plant–pathogen interactions (Vicente and Holub, 2012). Bacteria of the genus *Xanthomonas* cause substantial losses in the cultivation of many crops like rice and citrus plants (Büttner and Bonas, 2010; Ryan et al., 2011). Xcc is the causal agent of the black rot disease of *Brassicaceae*, which include the model plant *Arabidopsis thaliana*. *X. campestris* pathovars are regarded to be among the top 5 plant pathogenic bacteria (Mansfield et al., 2012). Xanthan has been identified as a pathogenicity factor although functional details

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http://dx.doi.org/10.1016/j.jbiotec.2013.01.023

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Reference
Bacterial strains		
E. coli DH5α:MCR	[F-]*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ* M15 Δ(*lacZYA-argF*)U169 *endA1 recA1 deoR thi-1 supE44* λ-*gyrA96 relA1*	Gibco-BRL, CeBiTec strain collection
X. campestris pv. campestris B100	Wild type, Sm^r	Vorhölter et al., 2008
B100Δ*pfkA*	*pfkA* deletion mutant of strain B100, Sm^r, Δ*pfkA*::*loxP*	This work
B100Δ*pgi*	*pgi* deletion mutant of strain B100, Sm^r, Δ*pgi*::*loxP*	This work
B100Δ*edd*	*edd* deletion mutant of strain B100, Sm^r, Δ*edd*::*loxP*	This work
B100Δ*tkt*	*tkt* deletion mutant of strain B100, Sm^r, Δ*tkt*::*loxP*	This work
B100Δ*gumD*	*gumD* deletion mutant of strain B100, Sm^r, Km^r, Δ*gumD*::*nptII*::*loxP*	This work
Plasmids		
pFV7	Cloning vector, derivative of pZErO-1, *aacC1* from pHGW32, *lacZα-ccdB*, Gm^r	Vorhölter et al., 2001
pSAWloxP-K	Cloning vector, pFV7 derivative, *aacC1* from pHGW32, *lacZα-ccdB*, *nptII*, *loxP* Gm^r, Km^r	This work
pBBR1:mcs5:cre	pBBR1MCS-5 (Kovach et al., 1995) derivative, *aacC1* (Gm^r), carrying *cre* (Liu et al., 1998)	Zudi Uzeini, CeBiTec strain collection

are still subject to discussion (Aslam et al., 2008; Dunger et al., 2007).

Xanthan production could be enhanced by a better understanding of the Xcc central metabolism, from which the xanthan precursors are derived. Today, genome data are a promising starting point for metabolic analyses. Reconstructions of metabolic networks based on genome data were already conducted for many bacteria, archaea, and eukaryotes including industrially relevant organisms (Milne et al., 2009). A review on the reconstruction process can be found elsewhere (Feist et al., 2009).

For Xcc, genome data are available for the strains ATCC 33913 (da Silva et al., 2002), 8004 (Qian et al., 2005), B100 (Vorhölter et al., 2008), and JX (Tao et al., 2012). The complete genome of Xcc B100 was already used for the draft reconstruction of part of the central carbohydrate metabolism and nucleotide sugar biosynthesis (Vorhölter et al., 2008). Furthermore, a good deal of metabolic data is available for Xcc or related xanthomonads from diverse studies, ranging from radiorespirometric experiments (Zagallo and Wang, 1967) to DNA microarray-based analyses (Serrania et al., 2008) and including besides further techniques the genetic and biochemical characterization of key enzymes (Köplin et al., 1992; Pielken et al., 1988; Whitfield et al., 1982) as well as profound analyses of uptake systems (Blanvillain et al., 2007; de Crécy-Lagard et al., 1995).

For Xcc B100, genome data have been utilized to reconstruct the amino acid biosynthetic pathways for all proteinogenic amino acids in conjunction with ^{13}C-isotopologue profiling *via* NMR (Schatschneider et al., 2011). The genome data revealed that Xcc is capable of importing and metabolizing a huge variety of carbohydrates (Vorhölter et al., 2008). For the catabolism of glucose, genes are present in Xcc to constitute three catabolic main pathways, the pentose phosphate (PP) pathway, the Entner–Doudoroff (ED) pathway and the Embden–Meyerhof–Parnas (EMP) pathway, which is also called glycolysis. It was previously shown that Xcc catabolizes glucose mainly *via* the ED pathway and to a small extent *via* the PP pathway (Zagallo and Wang, 1967). Since no activity has been shown for the phosphofructokinase reaction, it is assumed that the EMP pathway has no functional role in Xcc (Pielken et al., 1988; Whitfield et al., 1982). Pyruvate derived from the imported glucose *via* the ED pathway, or *via* glyceraldehyde-3-phosphate from the PP pathway is subsequently catabolized by the tricaboxylic acid cycle.

Constraint-based modeling is an important tool in systems biology that allows a more detailed analysis of the metabolism. Because of lacking information on important aspects like the kinetics for many metabolic reactions, the constraint-based modeling approach is advantageous as it solely requires data on the reaction stoichiometries (Orth et al., 2010; Wiback et al., 2004). Constraint-based modeling analyzes the network structure to reveal a feasible flux distribution space. In such a complex distribution space, a constraint-based modeling technique like flux balance analysis (FBA) searches for possible flux solutions by optimizing an objective function, usually the generation of biomass (Blank and Kuepfer, 2010; Orth et al., 2010; Reed and Palsson, 2003). It is applied to gain a better understanding of cellular metabolism, to predict cellular behavior in different environments, or to predict consequences of perturbations on the network. Additionally, metabolic networks support the analyses of high-throughput data and provide a scaffold for hypothesis and their testing (Durot et al., 2009; Oberhardt et al., 2009).

The ultimate aim of a systems biology approach would be to model how metabolic resources are allocated for xanthan biosynthesis. A first FBA had been conducted for *X. campestris* pv. campestris (Xcc) by means of a concise model describing the core of the central carbohydrate metabolism (Letisse et al., 2002). In this initial modeling approach, the influence of the pentose phosphate pathway, the periplasmatic pathway and glyceraldehyde-3-phosphate recycling were analyzed and the resulting variations in flux were examined. In this study, we made use of the now available genome data to introduce a first large scale metabolic model for Xcc that considerably expands our capacity for metabolic analysis. To facilitate network modeling, the biomass composition of Xcc was elucidated and deletion mutants were established to validate the model-based predictions. This model will enable more comprehensive mathematical and experimental studies of metabolic properties of the Xcc metabolism.

2. Materials and methods

2.1. Strains and media

Strains and plasmids applied in this study are listed in Table 1. TY medium (5 g tryptone, 3 g yeast extract, 0.7 g $CaCl_2$, per l; Beringer, 1974) was used as rich medium for cultivation of *X. campestris* pv. campestris B100 (Hötte et al., 1990; Vorhölter et al., 2008) at 30 °C. *Escherichia coli* strains were grown in Luria broth (Luria and Delbrück, 1943) at 37 °C. Antibiotics were added to the media when required in the following concentrations: for *X. campestris* pv. campestris kanamycin (Km) 80 μg/ml, gentamicin (Gm) 30 μg/ml and streptomycin (Sm) 800 μg/ml and for *E. coli* Km 40 μg/ml, Gm 10 μg/ml.

2.2. Recombinant DNA methods

DNA manipulation was conducted following standard protocols (Sambrook and Russell, 2001). Restriction enzymes and DNA ligase were purchased from Fermentas (St. Leon-Rot, Germany). The PCR primers (Suppl. Table 1) were ordered from Metabion (Martinsried, Germany). Deletion mutants were generated with deficiencies in key steps of the carbohydrate metabolism (*pfkA*, *pgi*, *tkt*, *edd*, *gumD*).

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