Connecting Algal Polysaccharide Degradation to Formaldehyde Detoxification

Stefan Brott, François Thomas, Maike Behrens, Karen Methling, Daniel Bartosik, Theresa Dutschei, Michael Lalk, Gurvan Michel, Thomas Schweder, and Uwe T. Bornscheuer*
Supporting Information
Experimental Procedures

Reagents
d-Ribulose-5-phosphate and d-fructose-6-phosphate were purchased as disodium salts from Sigma Aldrich. The 37% formaldehyde solution was also purchased from Sigma Aldrich. All other chemicals were purchased at the highest purity from Sigma-Aldrich, Carl Roth, Alfa Aesar or Acros.

Gene deletions in Z. galactanivorans
Deletion mutants of genes encoding a cytochrome P450 monooxygenase (ZGAL_4677) and both, a putative 3-hexulose-6-phosphate synthase (ZGAL_3942, HxlA) and a 6-phospho-3-hexulose isomerase (ZGAL_3941, HxlB) were constructed using a sacB system described previously. All primers and strains are listed in Tables S2 and S3. To delete zgal_4677, a 2,049 bp fragment including the first 36 bp of zgal_4677 and 2,013 bp of upstream sequence was amplified using primers OFT0046 and OFT0048 on genomic DNA from Z. galactanivorans DsjT. The fragment was digested with BamHI and XbaI and ligated into pYT313 that had been digested with the same enzymes, to generate pFT14. A 2,222 bp fragment including the final 57 bp of zgal_4677 and 2,165 bp of downstream sequence was amplified using primers OFT0047 and OFT0049. The fragment was cloned into XbaI and PstI sites of pFT2 to generate the zgal_4677 deletion construct pFT15. To delete zgal_3941 and zgal_3942, a 2,013 bp fragment including the first 27 bp of zgal_3941 and 1,986 bp of upstream sequence was amplified using primers OFT0052 and OFT0054. The fragment was digested with XbaI and SalI and ligated into pYT313 that had been digested with the same enzymes, to generate pFT16. A 1,491 bp fragment including the final 27 bp of zgal_3942 and 1,464 bp of downstream sequence was amplified using primers OFT0053 and OFT0055. The fragment was cloned into SalI and PstI sites of pFT16 to generate the zgal_3941-3942 deletion construct pFT17. Plasmids pFT15 and pFT17 were introduced individually into the wild-type Z. galactanivorans DsjT by conjugation from E. coli S17-1. Conjugants with plasmids integrated in the genome were isolated on Cytophaga-agar containing 50 µg mL⁻¹ erythromycin. Single erythromycin-resistant colonies were grown overnight at 30 °C in Cytophaga medium without antibiotics. Cells that lost the plasmid through a second recombination were selected on Cytophaga-agar containing 5% sucrose. Isolated colonies were checked for erythromycin sensitivity. Deletions were confirmed by PCR and sequencing on isolated colonies using primer pairs OFT0050- OFT0051 to identify the zgal_4677 deletion mutant (mZG_0084), and primers OFT0056-OFT0057 to identify the zgal_3941-3942 deletion mutant (mZG_0082).

Bacterial growth
F. agariphila KMM 3901T and Z. galactanivorans DsjT strains were routinely grown from glycerol stocks in Zobell 2216E medium at 25 °C. Their resistance to formaldehyde was tested by inoculating them (initial OD₆₀₀ 0.05) in 50 mL flasks containing 5 mL Zobell 2216E medium with increasing initial formaldehyde concentration (10 µM – 1 mM). To test the effect of gene deletions, Z. galactanivorans WT, mZG0082 (Δzgal_3941-3942) and mZG0084 (Δzgal_4677) strains were grown in 5 mL Zobell 2216E medium with or without 500 µM formaldehyde. All tests were performed in triplicates and growth was followed by monitoring OD₆₀₀ on 180 µL of culture using a microplate spectrophotometer (Spark Tecan, Männedorf, Switzerland).

Gene expression analysis
Expression data were retrieved from a previously published study with a publicly available GEO dataset GSE99940. Briefly, Z. galactanivorans DsjT was grown in marine minimum medium with 2 g L⁻¹ laminarin, agar or porphyran as a sole carbon source. After 48 h, RNA was retrieved from cells for cDNA synthesis and analyzed on a custom microarray. The effect of substrate on gene expression was tested by one-way ANOVA on normalized and log-transformed data, followed by a post-hoc Tukey test.
Cloning and expression in *E. coli*

Synthetic genes, codon optimized for expression in *E. coli*, encoding HPS and PHI from *Z. galactanivorans*, were synthesized and cloned into the pET-51b vector by BioCat GmbH (Heidelberg, Germany). The constructs encoded the recombinant proteins as fusions to a cleavable N-terminal Strep-tag for affinity purification. Chemically competent *E. coli* BL21 (DE3) cells were transformed with the plasmids harboring HPS or PHI and were spread on lysogeny broth (LB) agar plates containing 100 µg mL⁻¹ ampicillin. The agar plates were incubated overnight at 37 °C. One colony was picked and used to inoculate 5 mL LB medium which contained 100 µg mL⁻¹ ampicillin and was then incubated at 37 °C and 180 rpm overnight. For overexpression the cultivation was performed with 200 mL LB medium containing 100 µg mL⁻¹ ampicillin in a 1 L flask. The LB medium was inoculated with the overnight culture so that a starting optical density (OD₆₀₀) of 0.05 was obtained. Cells were then incubated at 37 °C and 180 rpm until an OD₆₀₀ of 1 was reached. Expression of target enzymes was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For the expression of HPS, 1 mM MgCl₂ was supplemented simultaneously and the cultivation was then continued at 25 °C and 180 rpm overnight. For PHI, cultivation was subsequently continued at 20 °C and 180 rpm overnight after the addition of IPTG. Cells were harvested by centrifugation at 10,000 x g and 4 °C for 30 min, washed with 50 mM sodium phosphate buffer pH 7.5, and subsequently stored at -20 °C until cell disruption.

Purification

For cell disruption, the cell pellet was resuspended in 10 mL of lysis buffer (100 mM TRIS-HCl buffer (pH 8.0) containing 500 mM NaCl, 5 mM MgCl₂, 0.1% Triton-X-100, and 1 mM phenylmethylsulfonyl fluoride). Cell disruption on ice was performed using a Sonoplus HD 2070 ultrasonic homogenizer (Bandelin electronic GmbH & Co. KG, Berlin, Germany) with the program: 2 x 3 min, 50% power, 50% cycle time. Cell debris was subsequently removed by centrifugation at 10,000 x g and 4 °C for 30 min. Purification utilizing gravity flow columns was performed using 10 mL of the Strep-Tactin® Sepharose® 50% suspension (IBA Lifesciences GmbH, Göttingen, Germany) as column material. After equilibration of the column with the wash buffer (100 mM TRIS-HCl buffer (pH 8.0), which contained 500 mM NaCl, 5 mM MgCl₂ and 0.1% Triton-X-100), the clarified lysate was applied. Unbound proteins were then removed from the column by excessive washing with the wash buffer. Elution of the target enzymes was then performed with the elution buffer 100 mM TRIS-HCl buffer (pH 8.0), which contained 2.5 mM d-desthiobiotin in addition to 500 mM NaCl and 5 mM MgCl₂. Elution fractions were pooled and concentrated using a Vivaspin 6 centrifugal concentrator with a 10 kDa molecular weight cut-off (Sartorius AG, Göttingen, Germany). PD-10 desalting columns (Cytiva Europe GmbH, Freiburg, Germany) were then used to desalt the sample and exchange the elution buffer to a 50 mM sodium phosphate buffer pH 7.5 supplemented with 5 mM MgCl₂.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the purity of the target enzymes. 20 µL protein sample was mixed with 5 µL of a 5-fold stock of SDS sample buffer (100 mM TRIS-HCl buffer (pH 6.8) containing 4% w/v SDS, 20% v/v glycerol, 2% (v/v) β-mercaptoethanol, 25 mM EDTA and 0.04% w/v bromophenol blue) and denatured by incubation at 95 °C for 10 min. For the SDS-PAGE a 12.5% acrylamide gel (separating gel) and a 4.0% loading gel were used. Electrophoresis was carried out at 200 V. Proteins were stained with Coomassie Blue (PhastGel® Blue R, Sigma Aldrich, Taukirchen, Germany). As reference the Pierce™ Unstained protein molecular weight marker (Thermo Scientific, Waltham, MA, USA) was used.

Determination of protein concentration

Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme assays

Activity of HPS was assayed by the d-ribulose-5-phosphate-dependent disappearance of formaldehyde and by the formation of F6P. A protein concentration of 10 µg mL⁻¹ for HPS and PHI were used in the biocatalysis. As substrates, 0.75 mM d-ribulose-5-phosphate disodium salt and 0.5 mM formaldehyde were used. The reaction volume was 0.2 mL and the reactions were performed in a 50 mM sodium phosphate buffer (pH 7.5) supplemented with 5 mM MgCl₂ for 5 min at an incubation temperature of 30 °C and an agitation of 1000 rpm. The formaldehyde concentration was then determined using the Nash reagent.[5]

In the reverse reaction a protein concentration of 50 µg mL⁻¹ for each enzyme and 20 mM d-fructose-6-phosphate disodium salt as substrate were used. The reaction volume was 0.2 mL and the reactions were performed in a 50 mM sodium phosphate buffer pH 7.5 supplemented with 5 mM MgCl₂ for 10 minutes at an incubation temperature of 30 °C and an agitation of 1000 rpm. Formaldehyde formation was then detected using the Nash reagent.[5]

Additionally, F6P formation was detected by coupling HPS and PHI with the phosphoglucone isomerase (PGI) from yeast (Roche Holding AG, Basel, Switzerland) and the glucose-6-phosphate dehydrogenase (G6pDHG) from baker’s yeast Type XV (Sigma Aldrich, St. Louis, MO, USA).[5] A protein concentration of 10 µg mL⁻¹ was used for HPS and PHI, and 5 U mL⁻¹ were used for PGI and G6pDHG. For substrates, 0.75 mM d-ribulose-5-phosphate disodium salt, 0.5 mM formaldehyde and 0.5 mM NADP⁺ were used. The reaction was carried out in a 50 mM TRIS-HCl buffer (pH 7.5) containing 5 mM MgCl₂ at 30 °C. The absorbance at 340 nm was measured every 2 min using the Infinite® M200 pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland).
**Formaldehyde quantification**

For the determination of the formaldehyde concentration, 0.2 mL freshly prepared Nash reagent was added immediately to the reaction mixture, this mix was then immediately incubated at 50 °C at an agitation of 1,000 rpm for 10 min. After centrifugation at 17,000 x g for 2 min to remove precipitated proteins, the mixture was transferred to a microtiter plate and absorbance was measured at 420 nm. The formaldehyde concentration was then determined by a formaldehyde standard curve, which was prepared in parallel to the reaction mixture.

**Computational analysis**

RefSeq assemblies of genomes deposited in MarRef (v1.5) and MarDB (v1.5) were downloaded from NCBI to create a target database. Translated coding sequences were compared to TIGRFAM profiles TIGR03127.1 (6-phospho-3-hexulose isomerase) and TIGR03128.1 (3-hexulose-6-phosphate synthase) as well as to PFAM models PF07992.17 together with PF14759.9 (to aim for ferredoxin reductase homologs, ZOBGAL_RS21970), PF00111.30 (for putative ferredoxins, ZOBGAL_RS21975) and PF00067.25 (for putative cytochrome P450 monooxygenases, ZOBGAL_RS21980) using the hmmscan function of HMMER v3.3.2 with model-specific noise cutoff threshold (--cut_nc). Results were then compared to *Z. galactanivorans* *Dsj* sequences ZOBGAL_RS18540 (HPS), ZOBGAL_RS18545 (PHI), ZOBGAL_RS21970, ZOBGAL_RS21975, and ZOBGAL_RS21980 using Protein-Protein BLAST v2.11.0+ with default settings. Circos was used to visualize similarity on protein level.
Supporting Figures

Figure S1: Structure of porphyran. The porphyran backbone consists of chains composed mainly of the alternating monosaccharide units 4-linked-α-L-galactose-6-sulfate (L6S) and 3-linked-β-D-galactose (Gal) or 3,6-anhydro-α-L-galactose (LA). In addition, O-methylation of D-galactose results in the presence of the methoxy sugar 6-O-methyl-D-galactose (G6Me).

Figure S2: Effect of increasing concentrations of formaldehyde on the growth of F. agariphila and Z. galactanivorans. Growth was performed with Zobell 2216E medium with increasing initial formaldehyde concentration at 25 °C. Values are mean ± s.e.m. (n = 3).
Figure S3: SDS-PAGE of purified proteins. The purified proteins (P) and the crude cell extract (C) were separated on a 12.5% gel and stained with Coomassie blue. 7.5 µg of the proteins were loaded onto the gel. As reference (M) the Pierce™ Unstained protein molecular weight marker (Thermo Scientific, Waltham, MA, USA) was used. The experiment was repeated independently with similar results.

Figure S4: Coupled enzyme assay for the detection of F6P formation. Formed F6P is converted by PGI to glucose-6-phosphate, which is then oxidized by G6pDHG under NADP⁺ consumption to d-glucono-1,5-lactone-6-phosphate. A protein concentration of 10 µg mL⁻¹ was used for HPS and PHI, and 5 U mL⁻¹ was used for PGI and G6pDHG. For substrates, 0.75 mM d-ribulose-5-phosphate, 0.5 mM formaldehyde and 0.5 mM NADP⁺ were used. The reaction was carried out in a 50 mM TRIS-HCl buffer (pH 7.5) containing 5 mM MgCl₂ at 30 °C. The absorbance at 340 nm was measured every 2 min using the Infinite® M200 pro microplate reader. Mean values are shown, error bars present ± s.d. (n = 3).
Figure S5: Formaldehyde formation in the reverse reaction. A protein concentration of 50 µg mL\(^{-1}\) for each enzyme and 20 mM D-fructose-6-phosphate as substrate were used. The reaction volume was 0.2 mL and the reactions were performed in a 50 mM sodium phosphate buffer pH 7.5 supplemented with 5 mM MgCl\(_2\) for 10 min at an incubation temperature of 30 °C and an agitation of 1,000 rpm. Mean values are shown, error bars present ± s.d. (n = 3).
Supporting Tables

Table S1: Annotated genes found in the NCBI database for *F. agariphila* KMM3901 and *Z. galactanivorans* Dsij, which encode enzymes involved in known formaldehyde detoxification pathways. The accession number is given for each gene.

| Annotated enzyme activity                                      | *F. agariphila* KMM3901 | *Z. galactanivorans* Dsij |
|----------------------------------------------------------------|--------------------------|---------------------------|
| 3-Hexulose-6-phosphate synthase                               | -                        | WP_013995270.1            |
| 6-Phospho-3-hexuloseomerase                                    | -                        | WP_013995269.1            |
| Serine hydroxymethyltransferase                               | WP_038533459.1           | WP_013996064.1            |
| Bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase | WP_038527446.1           | WP_013993916.1            |
| Formyltetrahydrofolate deformylase                            | WP_038527618.1           | WP_215931961.1            |

Table S2: Primers used in this study.

| Primers | Sequence and Description | Description |
|---------|--------------------------|-------------|
| OFT0046 | 5’ TTTTTTGGATCCTCCTCTTATAGTCGGGTATATCAAGG 3’; forward primer used in construction of pFT14; BamH1 site underlined |
| OFT0047 | 5’ GAGTTGGACCGACAGAACCAAACCATAACC 3’; reverse primer used in construction of pFT15; PstI site downstream in amplified fragment |
| OFT0048 | 5’ TTTTTTTCTAGATTTTTCAAACGGGTCTGGAAGC 3’; reverse primer used in construction of pFT14; XbaI site underlined |
| OFT0049 | 5’ TTTTTTTCTAGACAGCGTAAAGTAGTTCATTTTCATAAC 3’; forward primer used in construction of pFT15; XbaI site underlined |
| OFT0050 | 5’ GGCTCTAATATGGGTTGCATCCG 3’; forward primer to confirm deletion of zgal_4677 |
| OFT0051 | 5’ ATATCGGTCTCTATCTCACTGGC 3’; reverse primer to confirm deletion of zgal_4677 |
| OFT0052 | 5’ TTTTTTTCTAGAACTTTAAAGCTGTAGG 3’; forward primer used in construction of pFT16; XbaI site underlined |
| OFT0053 | 5’ TTTTTTTCTGAGACCAAGTAAAAATCCCAATGACTTTTAGG 3’; reverse primer used in construction of pFT17; PstI site underlined |
| OFT0054 | 5’ TTTTTTTGTCGACCTTGCTTTCATCAAGTATTGTTTCC 3’; reverse primer used in construction of pFT16; SalI site underlined |
| OFT0055 | 5’ TTTTTTTTGCAGAGGTGCTAAAGGAATTATTGGAAGGCC 3’; forward primer used in construction of pFT17; SalI site underlined |
| OFT0056 | 5’ CGGACGAGGGGTCTATAATGC 3’; forward primer to confirm deletion of zgal_3941-3942 |
| OFT0057 | 5’ TTCGTCTTTTGAATTATGAGGAGGC 3’; reverse primer to confirm deletion of zgal_3941-3942 |
### Table S3: Bacterial strains and plasmids used in this study.

| Description                                                                 | Ref.                                                                 |
|------------------------------------------------------------------------------|----------------------------------------------------------------------|
| **E. coli strains**                                                          |                                                                     |
| NEB5α Strain used for general cloning                                         | New England Biolabs (Ipswich, MA, USA)                              |
| Genotype: fhuA2 (argF-lacZ)U169 phoA glnV44 80 (lacZ)M15 gyRA96 recA1 relA1   |                                                                     |
| EndA1 thi-1 hsdR17                                                           |                                                                     |
| S17-1 λ pir Strain used for conjugation with Z. galactanivorans              | [10]                                                                 |
| Genotype: Apir hsdR pro thi; chromosomal integrated RP4-2 Tc::Mu Km::Tn7    |                                                                     |
| **Marine strains**                                                           |                                                                     |
| F. agariphila KMM 3901 wild type F. agariphila strain                         | [11]                                                                 |
| Z. galactanivorans DsjT (DSM 12802) wild type Z. galactanivorans strain     |                                                                     |
| mZG0082 Δzgal_3941-3942 in Z. galactanivorans DsjT                           | This study                                                          |
| mZG0084 Δzgal_4677 in Z. galactanivorans DsjT                                 | This study                                                          |
| **Plasmids**                                                                 |                                                                     |
| pYT313 Suicide vector carrying sacB under F. johnsoniae ompA promoter; Ap' (Em') | [1]                                                                 |
| pFT14 2,049 bp region upstream of Z. galactanivorans zgal_4677 amplified     | This study                                                          |
| with primers OFT0046 and OFT0048 and cloned into BamHI and XbaI sites of    |                                                                     |
| pYT313; Ap' (Em')                                                           |                                                                     |
| pFT15 Construct used to delete Z. galactanivorans zgal_4677 2,222 bp        | This study                                                          |
| region downstream of zgal_4677 amplified with primers OFT0047 and OFT0049   |                                                                     |
| and cloned into PstI and XbaI sites of pFT14; Ap' (Em')                     |                                                                     |
| pFT16 2,013 bp region upstream of Z. galactanivorans zgal_3941 amplified     | This study                                                          |
| with primers OFT0052 and OFT0054 and cloned into XbaI and SalI sites of     |                                                                     |
| pYT313; Ap' (Em')                                                           |                                                                     |
| pFT17 Construct used to delete Z. galactanivorans zgal_3941-3942 1,491 bp   | This study                                                          |
| region downstream of zgal_3942 amplified with primers OFT0053 and OFT0055   |                                                                     |
| and cloned into PstI and SalI sites of pFT16; Ap' (Em')                     |                                                                     |

*Antibiotic resistance phenotypes: ampicillin, Ap'; erythromycin, Em'. Antibiotic resistance phenotypes are those expressed in E. coli. The antibiotic resistance phenotypes given in parentheses are those expressed in Z. galactanivorans but not in E. coli.*

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### Author Contributions

M.G., T.S. and U.T.B. initiated the study and directed the project. F.T. conducted the growth studies and created the knock-out strains. S.B. and M.B. expressed and purified the enzymes and performed the biocatalysis. D.B. performed the computational analysis. K.M. and M.L. performed metabolite analysis, S.B. prepared the main manuscript, which was revised by F.T., T.D, M.G, T.S. and U.T.B. and approved by all authors.