Biochimica et Biophysica Acta 1824 (2012) 433–442

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Functional annotation and characterization of 3-hydroxybenzoate 6-hydroxylase from *Rhodococcus jostii* RHA1

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ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form 9 December 2011
Accepted 14 December 2011
Available online 22 December 2011

Keywords:
Fingerprint
Flavoprotein
Functional annotation
3-hydroxybenzoate 6-hydroxylase
Monooxygenase
Rhodococcus jostii RHA1

ABSTRACT

The genome of *Rhodococcus jostii* RHA1 contains an unusually large number of oxygenase encoding genes. Many of these genes have yet an unknown function, implying that a notable part of the biochemical and catabolic biodiversity of this Gram-positive soil actinomycete is still elusive. Here we present a multiple sequence alignment and phylogenetic analysis of putative *R. jostii* RHA1 flavoprotein hydroxylases. Out of 18 candidate sequences, three hydroxylases are absent in other available *Rhodococcus* genomes. In addition, we report the biochemical characterization of 3-hydroxybenzoate 6-hydroxylase (3HB6H), a gentisate-producing enzyme originally mis-annotated as salicylate hydroxylase. *R. jostii* RHA1 3HB6H expressed in *Escherichia coli* is a homodimer with each 47 kDa subunit containing a non-covalently bound FAD cofactor. The enzyme has a pH optimum around pH 8.3 and prefers NADH as external electron donor. 3HB6H is active with a series of 3-hydroxybenzoate analogues, bearing substituents in *ortho*- or *meta*-position of the aromatic ring. Gentisate, the physiological product, is a non-substrate effector of 3HB6H. This compound is not hydroxylated but strongly stimulates the NADH oxidase activity of the enzyme.

1. Introduction

Rhodococcus jostii RHA1 is a Gram-positive soil actinomycete able to degrade a wide range of organic compounds [1–4]. It possesses one of the largest bacterial genomes ever sequenced and encodes an exceptional amount of oxygenases (203 putative genes), in particular flavoprotein monooxygenases (88 putative genes) [5].

Flavoprotein monooxygenases (EC 1.14.13.x) perform a wide range of regio- and enantioselective reactions and can be divided in six subclasses [6]. Subclass A comprises a family of single-component flavoprotein hydroxylases, which are crucially involved in microbial degradation of natural and anthropogenic aromatics [7–9], polyketide antibiotic biosynthesis [10–12], and antibiotic resistance [13–15].

Flavoprotein hydroxylases can be identified on the basis of three fingerprint sequences, first defined in 4-hydroxybenzoate 3-hydroxylase (PHBH) (Fig. 1) [16]. The GxGxxG sequence motif maps the ADP moiety of FAD [17], while the GD consensus motif represents the residues that interact with the riboflavin moiety of FAD [18]. Both of these FAD fingerprints are common for many flavoproteins [19]. The third, DG consensus motif, is specific for subclass A enzymes and serves a dual role of recognition of both FAD and NADPH [16].

Here we used the above mentioned fingerprints to detect putative flavoprotein hydroxylase sequences in the *R. jostii* RHA1 genome (Fig. 1; Table 1). Since most of the retrieved sequences are annotated without a particular function, we performed a multiple sequence alignment and phylogenetic analysis with a large set of known flavoprotein hydroxylases. Among the newly assigned functions, we present the biochemical characterization of 3-hydroxybenzoate 6-hydroxylase (3HB6H), a flavoprotein involved in the gentisate (2,5-dihydroxybenzoate) degradation pathway [20, 21]. Notably, the gene encoding for this flavoenzyme is mis-annotated as a salicylate (2-hydroxybenzoate) hydroxylase. *R. jostii* RHA1 3HB6H expressed in *Escherichia coli* is specific for 3-hydroxybenzoate derivatives and does not interact with salicylate.

2. Materials and methods

2.1. Chemicals

DNAseI was from Boehringer Mannheim GmbH (Mannheim, Germany). Restriction endonucleases and dNTPs were purchased from Invitrogen (Carlsbad, CA, USA). Phusion High Fidelity DNA polymerase was from Finnzymes (Espoo, Finland). In-Fusion PCR cloning System was purchased from Clontech (Mountain View, CA, USA). Oligonucleotides were synthesised by Eurogentec (Liege, Belgium). *E. coli* TOP10 was from Invitrogen (Carlsbad, CA, USA). The pBAD/*Myc*-His (Nde) expression vector was kindly provided by Prof. M.W. Fraaije (University of Groningen).

Nickel nitrilotriacetic acid (Ni-NTA) agarose was purchased from Qiagen (Valencia, CA, USA) and Bio-Gel P-6DG was from Bio-Rad (Hercules, CA, USA). HiLoad 26/10 Q-Sepharose HP, Superdex 200 HR10/30, low molecular weight protein marker, prestained kaleidoscope

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doi:10.1016/j.bbapap.2011.12.003

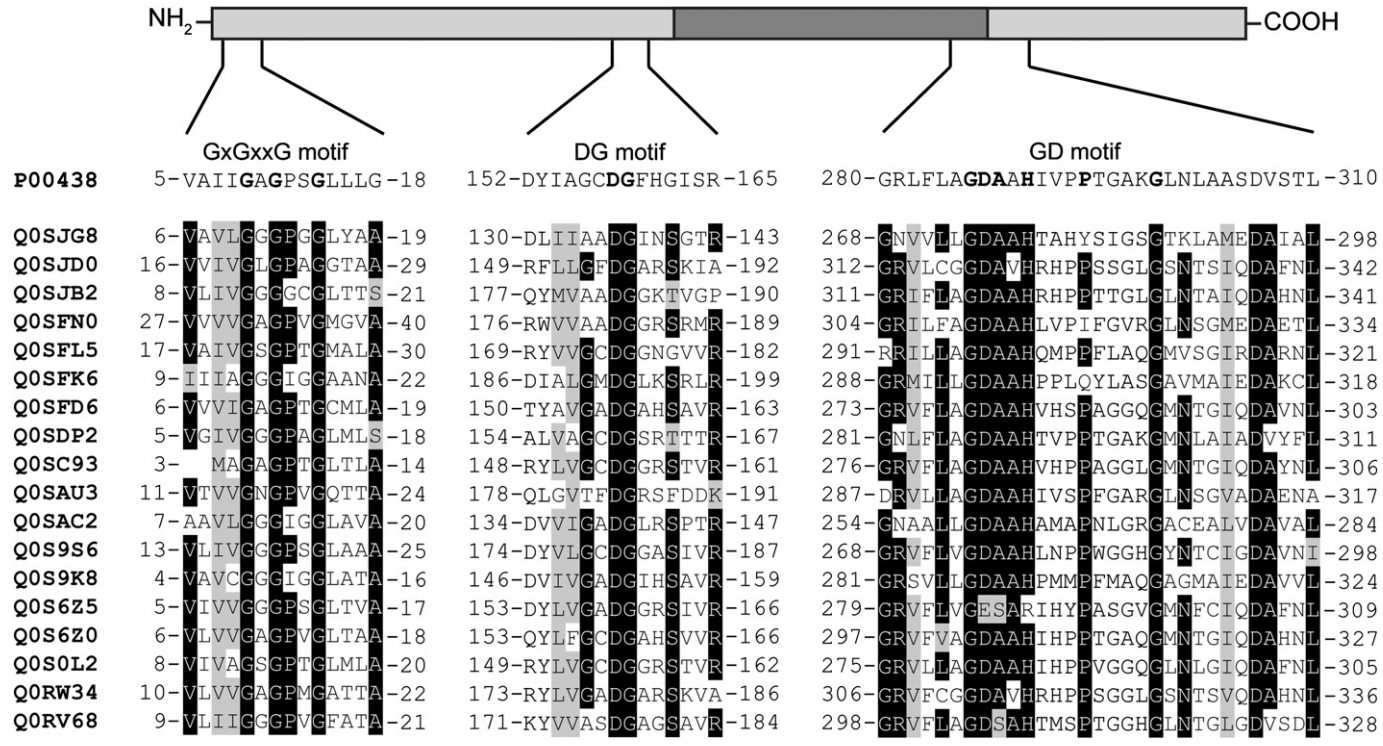

Fig. 1. Sequence comparison of putative flavoprotein hydroxylases from *Rhodococcus jostii* RHA1. Upper panel: Schematic representation of the primary structure of PHBH from *P. fluorescens* (UniProt ID: P00438) with the FAD-binding domain in light gray and the substrate binding domain in dark gray. Middle panel: Schematic representation of the flavoprotein hydroxylase fingerprints of PHBH from *P. fluorescences* used in this study. Bold characters represent residues used for fingerprint acronyms. Lower panel: Sequence alignment of flavoprotein hydroxylase fingerprint regions among putative flavoprotein hydroxylases from *R. jostii* RHA1. Identical residues are shaded in black, similar residues are shaded in gray.

protein standards, and catalase (232 kDa), aldolase (158 kDa), BSA (68 kDa) and ovalbumin (43 kDa) were obtained from Pharmacia Biotech (Uppsala, Sweden).

Aromatic compounds were purchased from Sigma-Aldrich (St Louis, MO, USA) and Acros Organics (New Jersey, US). Catalase, FAD, FMN, riboflavin and arabinose were from Sigma-Aldrich (St Louis, MO, USA). Pefabloc SC was obtained from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were from commercial sources and of the purest grade available.

2.2. Sequence analysis

The genome of *R. jostii* RHA1 was analysed for the presence of flavoprotein hydroxylases at the European Bioinformatic Institute (www.ebi.ac.uk). FASTA analysis (www.ebi.ac.uk/Tools/sss/fasta) was performed to determine protein sequence homology. Multiple sequence alignments were made using CLUSTALW [22]. DNA cluster comparison and database searches were carried out using Nucleotide and Protein resources from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and UniProt Database (www.uniprot.org). Phylogenetic analysis was performed using FigTree (tree.bio.ed.ac.uk).

2.3. Cloning, expression and purification of 3-hydroxybenzoate 6-hydroxylase in E. coli

A 1.2 kb DNA fragment encoding a putative salicylate hydroxylase (Gene ID: 4218663) was PCR amplified from *R. jostii* RHA1 genomic DNA, using the oligonucleotides SM_1869fwd (5′AGGAGGAATTACAT**ATG**TCGAATCTGCAGGACGCAC3′) and SM_1869rev (5′GTTCGGGCCCAAGCTTTGACGCGCGATCGGACG3′), introducing *Nde*I and *Hind*III restriction sites (underlined), respectively and containing the start codon (bold). The amplified fragment was cloned into the pBAD/Myc-His expression vector containing a C-terminal His_6-tag by using the In-Fusion PCR Cloning System. The resulting construct (pBAD-3HB6H-His_6) was verified by automated sequencing of both strands and electroporated to *E. coli* TOP10 cells for recombinant expression.

The Åkta Explorer FPLC system (Pharmacia Biotech) was used for protein chromatography. For enzyme production, *E. coli* TOP10 cells, harbouring a pBAD-3HB6H plasmid, were grown in TB medium supplemented with 100 μg mL^{-1} ampicillin until an optical density ($OD_{600\ nm}$) of 0.8 was reached. Expression was induced by the addition of 0.02% (w/v) arabinose and the incubation was continued for 16 h at 37 °C. Cells (36 g wet weight) were harvested by centrifugation, resuspended in 120 mL of 20 mM potassium phosphate, 300 mM NaCl (pH 7.4), containing 1 mM Pefabloc SC, 1 mg DNAse, 100 μM $MgCl_2$ and subsequently passed twice through a precooled French Pressure cell (SLM Aminco, SLM Instruments, Urbana, IL, USA) at 16,000 psi. The resulting homogenate was centrifuged at 25,000 *g* for 45 min at 4 °C to remove cell debris, and the supernatant was applied onto a Ni-NTA agarose column (13×1.6 cm) equilibrated with 20 mM sodium phosphate, 300 mM NaCl (pH 7.4). The column was washed with two volumes of equilibration buffer. The enzyme was eluted with 300 mM imidazole in equilibration buffer. The active pool, containing an added excess of free FAD, was desalted using a Biogel column (14×2.6 cm), running in 50 mM BisTris–HCl, 0.1 mM EDTA (pH 7.2). The desalted protein was loaded onto a HiLoad 26/10 Q-Sepharose HP column equilibrated with the same buffer. After washing with two column volumes of starting buffer, the protein was eluted with a linear gradient of NaCl (0–1 M) in the same buffer. Active fractions were pooled and concentrated to 8 mg/mL by using Amicon filters (30 kDa cutoff) and dialysed at 4 °C against 50 mM BisTris–HCl (pH 7.2). Purified 3HB6H was frozen in liquid nitrogen and stored at −80 °C.

2.4. Protein analysis

SDS/PAGE was performed using 12.5% acrylamide slab gels essentially as described by Laemmli [23]. Proteins were stained using