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Functional coupling between vanillate-*O*-demethylase and formaldehyde detoxification pathway

Makoto Hibi, Tomonori Sonoki, Hideo Mori *

Biofrontier Laboratories, Kyowa Hakko Kogyo Co. Ltd., 3-6-6 Asahimachi, Machidashi, Tokyo 194-8533, Japan

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Abstract

Pseudomonas putida vanillate-*O*-demethylase consisting of VanA and VanB was expressed in *Escherichia coli* strain K-12. Recombinant *E. coli* strain K-12 cells expressing VanAB efficiently converted vanillate into protocatechuate with glucose consumption. Mutant lacking either *pgi* or *zwf* showed higher or lower converting activity than the parental strain, respectively. Formaldehyde, which is the by-product of the demethylation, was converted into formate in the cellular reaction. Formate accumulation was blocked by gene disruption of the *E. coli frmA* that coded glutathione-dependent formaldehyde dehydrogenase.

Keywords: Protocatechuate; Formaldehyde; Vanillate-*O*-demethylase; Formaldehyde dehydrogenase; *S*-Formylglutathione hydrolase; Bioconversion

1. Introduction

Lignin is a highly polymerized and complex compound common in woody plants. In the pulp industry, lignin is burnt as fuel and not used for further application because of its complex structure. However, lignin is the most abundant aromatic compound in nature, and is thought to be the promising compound to substitute for petrochemicals [1,2]. Efficient conversion of lignin to small molecular weight key chemicals will open the way for industrial usage of lignin chemistry. Biological degradation of lignin mainly consists of two phases. Firstly white rot fungi initiate the degradation of lignin [3], and secondly bacteria decompose lignin-derived compounds to smaller molecular weight. Protocatechuate and vanillate are important intermediate metabolites in the degradation pathway of lignin-derived compounds such as ferulic acid and vanillin by soil microbes.

The vanillate-*O*-demethylase of *Pseudomonas* sp. is a heterodimeric enzyme consisting of the terminal oxygenase VanA and the reductase VanB [4–7]. This enzyme reductively catalyzes the conversion of vanillate into protocatechuate and formaldehyde. Both NADH and NADPH serve as electron donors for the enzyme, with preference depending on the species of the enzyme origin [4]. Heterologous expression of *vanA* and *vanB* has made *Escherichia coli* able to some extent to convert vanillate to protocatechuate [6].

Formaldehyde produced by demethylation of vanillate is highly toxic. The detoxification pathway of formaldehyde includes glutathione-dependent formaldehyde

* Corresponding author. Tel.: +81 42 726 2555; fax: +81 42 726 8330.

E-mail address: hmori@kyowa.co.jp (H. Mori).

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dehydrogenase [8–12] and *S*-formylglutathione hydrolase [13–16], and is widely conserved from prokaryotes to humans. Formaldehyde spontaneously binds to glutathione and becomes *S*-hydroxymethyl glutathione that is the substrate for formaldehyde dehydrogenase and oxidized to *S*-formylglutathione. *S*-formylglutathione hydrolase hydrolyses *S*-formylglutathione into formate and reduced glutathione. In *E. coli*, glutathione-dependent formaldehyde dehydrogenase was firstly isolated as the mammalian glutathione-dependent formaldehyde dehydrogenase homologue [12], and its N-terminal amino acid sequences correspond to FrmA of strain K-12. Secondly the formaldehyde resistance gene from formaldehyde-resistant strain *E. coli* VU3695 was revealed to encode glutathione-dependent formaldehyde dehydrogenase and shows 90% similarity to *frmA* of *E. coli* strain K-12 [17,18]. However, it has not been confirmed whether *frmA* is actually involved in formaldehyde detoxification of strain K-12. And also, little has been known about *S*-formylglutathione hydrolase of *E. coli*. Though two putative *S*-formylglutathione hydrolase structural genes, *frmB* and *yeiG,* were found in the *E. coli* strain K-12 genome [19], experimental analysis of the two genes have not been done. A recent report showed that *frmR*, *frmA*, and *frmB* were induced as an operon by formaldehyde [20].

Since VanAB requires cofactor and generates biologically toxic formaldehyde, it was thought to be difficult to perform the efficient demethylation in recombinant *E. coli*. This reaction would be one of the limiting steps of the prospective bioconversion from lignin-derived compounds to valuable products in recombinant *E. coli*. In this paper *Pseudomonas putida* vanillate-*O*-demethylase was expressed in *E. coli* strain K-12 and this recombinant enzyme showed strong activity both in vitro and in vivo. The recombinant strain was used to analyze electron donor supply and formaldehyde detoxification pathways in *E. coli* strain K-12. The results indicate the potential of *E. coli* strain K-12 for the prospective bioconversion machinery for producing valuable chemicals from lignin-derived compounds.

2. Materials and methods

2.1. Plasmid construction

P. putida vanA and *vanB* genes (GenBank Accession No. AE016788) [21] were gifts of Yoshihiro Katayama (Tokyo University of Agriculture and Technology). The entire *vanAB* operon was inserted to downstream of *lacPO* in pMW118 (GenBank Accession No. AB005475, obtained from Nippon Gene, Japan) so that its expression was induced by addition of isopropyl-β-D-thiogalactopyranoside, and the resulting plasmid was designated as pMQVanAB.

2.2. Bacterial strains and culture

E. coli strains used in this study are listed in Table 1. Knock-out mutants of *pgi, zwf, frmA,* and *frmB* were obtained from Hirotada Mori (Nara Institute of Science and Technology) [22,23]. Deletion mutant of *yeiG* was constructed by Wanner's method [24] in this study. Gene deletions were transferred into the wild type strain W3110 by P1-phage transduction [25]. Used strains were pre-cultured in Luria–Bertani (LB) medium (Difco, Detroit, USA) overnight and inoculated at 1% volume into fresh LB medium or M9 minimal medium (Difco, Detroit, USA) with proper supplements indicated elsewhere in this paper and cultivated at 30 °C with vigorous agitation.

2.3. Preparation of whole cell extracts and assay of cofactor dependency of VanAB

Strains were cultured in 50-ml LB medium until optical density at 660 nm reached to 1.2. Then IPTG was added at the final concentration of 2 mM for vanAB induction. The culture was incubated for further 3 h. Harvested cells were washed twice in distilled water and collected by centrifugation (2000*g*, 10 min). Cells were processed by three freeze–thaw cycles [26]. Demethylating activity was not recovered in cleared lysate samples. Since it was difficult to re-activate the enzyme activity from packed debris, whole cell extract before centrifugation was used for enzymatic assay. The protein concentration of whole cell extract was measured by Protein Assay Kit (Biorad) using immunoglobulin G as standard. The VanAB reaction was carried out at 30 °C in 0.1 ml solution containing 50 mM vanillate, 50 mM reduced glutathione, 50 mM NAD(P)H or distilled water, and 70 μl whole cell extract. Protocatechuate concentration of the reaction mixture was analyzed by high-performance liquid chromatography (HPLC) system equipped with Asahipak GS-320 HQ column

Table 1
Strains and plasmid

Strains and plasmid	Description	Reference
Strains		
W3110	*E. coli* strain K-12, F^- λ^- *IN (rrnD-rrnE)* 1 rph-1	[27]
WV1181	W3110 (pMQVanAB)	This study
WK1841	WV1181, Δ*zwf*::*kan*	This study
WK3985	WV1181, Δ*pgi*::*kan*	This study
WK0347	WV1181, Δ*frmA*::*kan*	This study
WK0346	WV1181, Δ*frmB*::*kan*	This study
WC2141	WV1181, Δ*yeiG*::*cat*, Δ*frmB*::*kan*	This study
Plasmid		
pMW118	pSC101 derivative	[28]
pMQVanAB	*P. putida vanAB* genes in pMW118	This study