Regulation of gene expression for pterin degradation found in *Cupriavidus* sp. strain LA-1 and its application

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Research objective

Pterins function as pigments and cofactors in various organisms and are widespread in the environment. *Cupriavidus* sp. LA-1 uses 7-hydroxypterin (7-HP) and lumazine as the sole carbon sources. The gene cluster involved in 7-HP/lumazine degradation is substrate-inducible. This study aimed to clarify the transcriptional regulatory mechanisms of this gene cluster and produce a unique compound using pterin-degrading enzymes.

Methods

1. Bacterial strain and culture

Cupriavidus sp. LA-1, a bacterium that degrades 7-HP/lumazine, was used in the present study. The strain was precultured overnight at 28°C in modified M9 medium (M9) (10 mM K_2HPO_4, 10 mM KCl, 20 mM NH_4Cl, 10 mM $MgSO_4 \cdot 7H_2O$, and 0.1% Hunter's trace elements; pH 7.5) supplemented with 0.1% yeast extract. Cultured cells were collected by centrifugation, washed, and resuspended in sterilized saline before being transferred to primary culture in M9 containing either 7-HP or lumazine. *Escherichia coli* was cultured in Luria-Bertani medium (LB) (1% tryptone, 0.5% yeast extract, and 0.5% NaCl).

2. Expression and purification of recombinant proteins

Recombinant proteins were produced using the pET expression system. The *orf_*3 and *orf_*9 genes amplified by PCR using specific primers, KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan), and total DNA from *Cupriavidus* sp. LA-1 as the template, were digested with restriction enzymes and cloned into pET21a(+) and pET28a(+) vectors (Merck Millipore, Darmstadt, Germany). The resulting plasmids were transfected into *E. coli* BL21(DE3) for recombinant protein expression. After protein expression, the collected cells were washed twice with 50 mM HEPES buffer (pH 7.5) and lysed using a sonicator (20 W for five cycles of 1 min each) in the same buffer. After centrifugation, the supernatants were filtered through a 0.22-μm syringe filter and used as the cell-free extract (CFE). Recombinant proteins in the CFE were purified using a HisTrap™ FF column (Cytiva, Uppsala, Sweden) following the manufacturer's protocol, with 50 mM HEPES (pH7.5) as the base buffer. Imidazole was removed from the eluates by ultrafiltration (10 kDa), and the remaining recombinant protein solutions were stored at −80°C until use. Protein purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

3. Creation of a gene disruptant in *Cupriavidus* sp. LA-1

The *orf_3* and *orf_9* genes were disrupted by homologous recombination. Gene fragments (approximately 3 kb) of *orf_*3 and *orf_*9, including the upstream and downstream regions,

were amplified using PCR, digested with restriction enzymes, and cloned into pUC19 (Takara Bio Inc., Shiga, Japan). A chloramphenicol resistance gene was amplified by PCR using pNSGroE (GenBank: AY576605) as a template, digested with restriction enzymes, and inserted into *orf*_3 and *orf*_9 of each plasmid. The resulting plasmids were introduced into *Cupriavidus* sp. LA-1 cells by electroporation (1.6 kV for 5 ms), and transformants were selected on LB agar containing 100 μg mL^{-1} chloramphenicol. Gene disruption in the transformants was confirmed by PCR.

4. qRT-PCR

Total RNA from *Cupriavidus* sp. LA-1 was purified using the RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) and RNase-Free DNase Set (Qiagen). Synthesis of cDNA was performed from the extracted RNA (1 μg) using a QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA was subjected to qRT-PCR using the Light Cycler® 96 System (Roche Diagnostics GmbH, Mannheim, Germany), THUNDERBIRD Next SYBR qPCR Mix (Toyobo), and appropriate primers. Gene expression levels, represented by Cq values, were normalized to the 16S rRNA gene transcript levels.

5. Other analyses

Metabolites and enzyme products were detected and quantified using HPLC on an Agilent 1260 Infinity System (Agilent Technologies, CA, USA) equipped with a TSKgel ODS-120H column (Tosoh Co., Tokyo, Japan). The CFE of *Cupriavidus* sp. LA-1 was prepared using the same method used for *E. coli*.

Results

1. Expression of the pterin degradation gene cluster

Cupriavidus sp. LA-1 degrades 7-HP via xanthine (XA) using enzymes encoded by a pterin degradation gene cluster (Figure 1). The expression of *orf*_1 and *orf*_2 was significantly upregulated by 7-HP compared with 7-hydroxylumazine (7-HL), suggesting that the pterin form upregulates the expression of putative operon-1. In contrast, 7-HL significantly upregulated the expression of putative operon-2 and operon-3 compared with 7-HP. These results suggest that the transcriptional regulator (TR) of putative operon-1 responds to the pterin form, whereas those of putative operon-2 and operon-3 respond to the lumazine form.

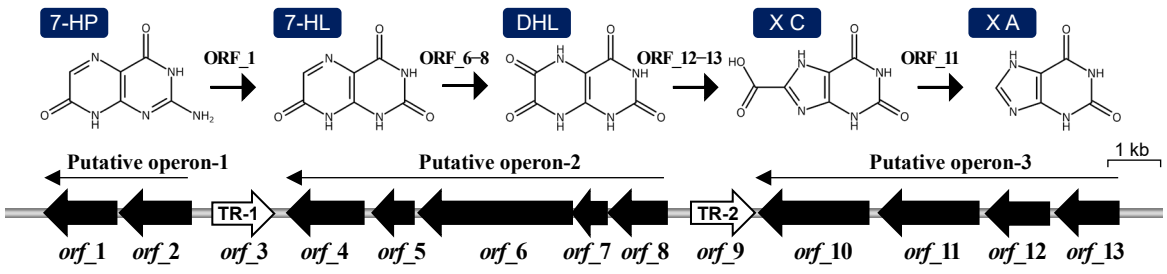

Fig. 1 Degradation of 7-HP and organization of the pterin degradation gene cluster in *Cupriavidus* sp. LA-1.

2. Putative operons regulated by TR-1 and TR-2

The pterin degradation gene cluster in *Cupriavidus* sp. LA-1 contains two TRs, TR-1 and TR-2, encoded by *orf*_3 and *orf*_9, respectively. The locations of TR-1 and TR-2 and the results of the gene expression analysis suggested that TR-1 regulates putative operon-1 and TR-2 regulates putative operon-2 and operon-3 (Figure 1). To clarify the responses of TR-1 and TR-2 to the substrate, we investigated the affinities of TR-1 and TR-2 to 7-HP and 7-HL. However, this could not be evaluated because the recombinant TR-1 and TR-2 proteins quickly aggregated after purification.

Accordingly, we disrupted TR-1 and TR-2 genes in *Cupriavidus* sp. LA-1 and created a TR-2 gene (*orf*_9) disruptant (ΔTR-2). The fabricated ΔTR-2 produced 7-HL from 7-HP via deamination but could not degrade the produced 7-HL or exogenous 7-HL. These findings indicate that TR-2 regulates putative operon-2 and operon-3 encoding 7-HL degradation enzymes.

3. Xanthine-8-carboxylic acid production using pterin degradation enzymes

A protein complex constructed using ORFs encoded by *orf*_12 and *orf*_13 catalyzes the conversion of 6,7-dihydroxylumazine (DHL) into xanthine-8-carboxylic acid (XC) (Figure 1). The CFE prepared from *Cupriavidus* sp. LA-1 cultured with 7-HP, 7-HL, or lumazine exhibited sufficient XC production from DHL, producing up to 2.5 mM XC at a 100% conversion rate (Figure 2). However, a further increase above 2.5 mM yielded an insignificant increase in XC production. Therefore, other factors, such as reaction volume, should be considered to improve the total production.

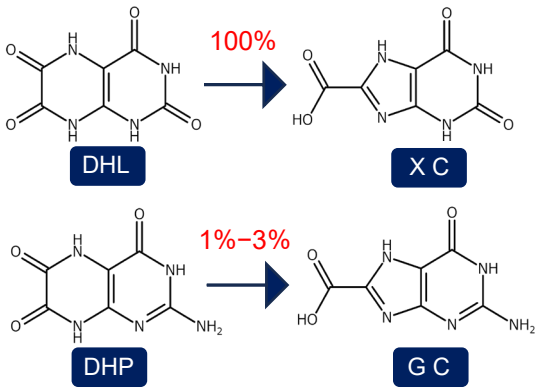

Fig. 2 Enzyme production of XC and GC using ORF12−13.

We also evaluated guanine-8-carboxylic acid (GC) production from 6,7-dihydroxypterin (DHP) using ORF12–13. ORF12–13 produced little GC from DHP (conversion rate: 1–3%) (Figure 2). Notably, this low productivity was not improved by changing the reaction conditions (e.g., pH, temperature, and buffer). This result indicates that ORF12–13 has high substrate specificity and that using this enzyme in GC production from DHP is difficult.

Conclusion

This study focused on the bacterial pterin degradation gene cluster and showed that pterin form-responsive TR (probably TR-1) and lumazine form-responsive TR-2 regulate gene cluster expression. We demonstrated that XC, which is difficult to produce using organic synthesis methods, can be produced from DHL using ORF12–13 at a high conversion rate.