Microbiological Research 209 (2018) 14–20

Contents lists available at ScienceDirect

Microbiological Research

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

Transcriptional control of the phenol hydroxylase gene *phe* of *Corynebacterium glutamicum* by the AraC-type regulator PheR

Can Chen[a,c,1], Yaoling Zhang[b,1], Lei Xu[c], Kaixiang Zhu[c], Yanyan Feng[c], Junfeng Pan[c], Meiru Si[c,d], Lei Zhang[c], Xihui Shen[c,*]

[a] Institute of Food and Drug Inspection, College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou, Henan 466001, China
[b] Agricultural Scientific Research Institute in Hanzhong City, Hanzhong, Shaanxi 723000, China
[c] Shaanxi Key Laboratory of Agricultural and Environmental Microbiology, College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China
[d] College of Life Sciences, Qufu Normal University, Qufu, Shandong 273165, China

ARTICLE INFO

Keywords:
AraC
Corynebacterium glutamicum
Phenol hydroxylase
Transcriptional regulator

ABSTRACT

Corynebacterium glutamicum can degrade phenol by a meta-cleavage pathway, which depends on *ncgl2588* (*phe*) of the *phe* operon encoding phenol hydroxylase. An additional gene, *ncgl2587* (*pheR*), is located upstream of *phe*. The *pheR* encodes an AraC/XylR-type regulator protein with 377 amino acid residues and is transcribed in the same direction as *phe*. Disruption of *pheR* by homologous recombination resulted in the accumulation of phenol in *C. glutamicum*. PheR demonstrates a low type of constitutive expression where phenol induces *phe* expression. PheR shares 75% sequence identity with AraC-type regulator of *Corynebacterium lubricantis* and 37 conserved residues, characteristic of AraC family, were located. A constructed pK18*mobsacB*-P_{phe}:*lacZ* transcriptional fusion plasmid was transformed into the wild-type, Δ*pheR*, and Δ*pheR*$^{+}$ strains, and the results indicated that PheR activates the expression of *phe* encoding phenol hydroxylase. Electrophoretic mobility shift assay (EMSA) demonstrated a direct interaction of PheR with the *phe* promoter region and binding site of PheR on the P_{phe} was located 109-bp upstream of *phe*, as indicated by foot printing analysis. Our research provides deep insight into PheR expression and its regulatory function on Phe in *C. glutamicum*.

1. Introduction

Existing ubiquitously in the environment, phenol and its derivatives come from many industrial processes in oil refineries, and petrochemical and phenolic resin plants. The most effective and economical method to remove these toxic pollutants from the environment is microbial degradation (Alexander, 1981). In recent years, with the development of genomics and biotechnology, there is interest in the degradation of aromatic compounds by microorganisms. To date, several catabolic pathways of aromatic compound degradation have been described (Shingler et al., 1989; Harwood and Parales, 1996; Tropel and van der Meer, 2004; Cao et al., 2009), especially of phenol. Consequently, understanding the mechanism of the evolution of catabolic pathways will facilitate the development of useful bacterial strains that can degrade recalcitrant synthetic compounds. Under aerobic conditions, phenol is normally converted by phenol hydroxylase (EC 1.14.13.7) into catechol, which is further degraded via ortho- or meta-cleavage pathway by catechol 1,2-dioxygenase or catechol 2,3-dioxygenase (Heinaru et al., 2000), respectively, to central metabolism intermediates of the Krebs cycle, such as pyruvate, succinate, and acetyl coenzyme A. In many bacteria, phenol hydroxylases are the key rate-limiting enzymes in the initial reaction of phenol catabolism (Shingler, 2003). Three different types of phenol hydroxylases have been identified: single- (Kalin et al., 1992; Kukor and Olsen, 1992; Kim and Oriel, 1995; Putrins et al., 2007), two- (Kirchner et al., 2003; Omokoko et al., 2008), and multi- component types (Nordlund et al., 1990; Ehrt et al., 1995; Arai et al., 1999; Yu et al., 2011). *Corynebacterium glutamicum* has been reported to have two-component flavin-dependent hydroxylases (Xiao et al., 2015).

C. glutamicum is a fast growing, aerobic, and non-pathogenic Gram-positive soil bacterium traditionally applied in biotechnological production processes, particularly in the fermentative production of amino acids and vitamins (Hermann, 2003; Leuchtenberger et al., 2005; Becker et al., 2009). Because of the industrial importance of *C. glutamicum*, extensive studies have already focused on its cellular physiology and metabolism (Ikeda, 2003). *C. glutamicum* is also capable of utilizing

* Corresponding author.
E-mail address: xihuishen@nwsuaf.edu.cn (X. Shen).
[1] These authors contributed equally to this work

https://doi.org/10.1016/j.micres.2018.02.001
Received 30 October 2017; Received in revised form 23 January 2018; Accepted 3 February 2018
Available online 06 February 2018
0944-5013/

a large variety of aromatic compounds as a sole source of carbon and energy for growth. After genome data mining, aromatic degradation and assimilation by this bacterium and relevant genetic clusters have rarely been explored, until recently (Shen et al., 2012), which provided the basis for identifying and analyzing the repertoire of transcriptional regulators in this species. Almost invariably, all catabolic pathways are objects of regulatory control at the level of gene expression. These catabolic pathways are connected to transcriptional regulation carried out by different types of regulatory proteins (Brinkrolf et al., 2006).

C. glutamicum is able to utilize phenol as a carbon source by *ncgl2588* (*phe*), which encodes a two-component phenol hydroxylase (Kalin et al., 1992). To date, very little information is available on the regulation of phenol hydroxylase in this bacterium. The expression of the *dmpKLMNOP* operon, which codes for a multicomponent phenol hydroxylase (*mPH*) in *Pseudomonas* sp. CF600, is strictly controlled by the NtrC-family transcriptional activator DmpR in response to aromatic effectors (Shingler et al., 1993). In addition to DmpR-type mediated regulation, the expression of *phcKLMNOP* operon encodes *mPH*, which is responsible for the activity of phenol hydroxylase. Furthermore, phenol hydroxylase is controlled by two transcriptional regulators, PhcS (GntR family) and PhcR (XylR/DmpR family) in *Comamonas testosteroni* R5 (Teramoto et al., 1999; Teramoto et al., 2001). Recently, a third transcriptional regulator (PhcT) for the *mPH* operon that belongs to the AraC/XylS family was identified (Teramoto et al., 2002). Besides that, phenol hydroxylase, which is encoded by *pheA2A1* genes, is regulated by PheR (AraC family) and *pheR* is involved in the first step of phenol degradation (Szokol et al., 2014). This is the first analysis of transcriptional regulation of two-component phenol hydroxylase.

This study focuses on *ncgl2587*, a gene upstream of the two-component phenol hydroxylase coding gene in *C. glutamicum*, which belongs to the AraC/XylS transcriptional regulator family. The aim of this investigation was to find the molecular mechanism of the regulation of phenol degradation by *ncgl2587*, which will have practical applications such as in treatment of soils contaminated by aromatic compound.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) broth or on LB plates containing 1.5% (w/v) agar. *C. glutamicum* strains were routinely grown at 30 °C on a rotary shaker (200 rpm) in LB or mineral salts (MS) medium containing the following ingredients per liter: Na_2HPO_4, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4$, 0.03 g; NH_4Cl, 1.07 g (pH 8.4). MS medium was supplemented with 3 mM phenol or glucose as carbon and energy source. Growth was monitored by measuring the Optical Density (OD) of the cultures at 600 nm. For preparation of competent cells, *C. glutamicum* was cultivated in brain-heart infusion (BHI) broth with 0.5 M sorbitol. For each experiment, three parallel cultures were analyzed. For selection of mutants, the media were supplemented with antibiotics at the following concentrations: kanamycin, 50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *C. glutamicum*; chloramphenicol, 20 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *C. glutamicum*.

2.2. DNA manipulation and plasmid construction

The *pheR* deletion mutant (Δ*pheR*) was constructed by using homologous recombination. An appropriate DNA fragment (800 bp with *Bam*HI site) upstream of *pheR*, and another fragment (900 bp with *Sal*I site) downstream of *pheR* were amplified by PCR using primers D*pheR* upF/D*pheR* upR and D*pheR* lowF/D*pheR* lowR (Table S2). These two fragments were used in the second round of PCR with primer pair D*pheR* upF/D*pheR* lowR (Table S2) to generate the *pheR* deletion fragment, which was subsequently inserted into the vector pK18*mobsacB* to obtain pK18*mobsacB*-Δ*pheR*. To construct P*phe* promoter fragments with PheR-binding site mutations, overlapping PCR was performed to replace the consensus binding sites (20 bp) with a 20 bp DNA fragment from the *phe* encoding region. Briefly, to replace the PheR-binding site, the primer pairs DPphe-upF/DPphe-upR and DPphe-lowF/DPphe-lowR (Table S2) were used to amplify the up-fragment and down-fragment of the P*phe* promoter, respectively. Overlapping PCR was used to incorporate a 20 bp overlap of the DNA fragment ATGCGCGCGGAAGGT AGCGG, which replaced a part of the PheR-binding site (AGGCGGAC ATCATCTTGCCT), and was performed using the up-fragment and down fragment as the template and DPphe-upF/DPphe-lowR as the primer pair to obtain the DNA fragment P*pheM* (370 bp). And the fragment was confirmed by DNA sequencing. For complementation, *ncgl2587* fragment was amplified by primer pair P19-*pheR*-F/P19-*pheR*-R (Table S2) and then ligated to pXMJ19 to obtain pXMJ19-*pheR* plasmid. The *pheR* was cloned into the pET28a vector containing a *N*-terminal hexahistidine tag followed by a TEV cleavable small ubiquitin-like modifier (SUMO) fusion protein with primer pair PheR-F/PheR-R (Table S2).

2.3. Construction of deletion mutants and complemented strains

To construct the *pheR* in-frame deletion mutant, the pK18*mobsacB*-*pheR* plasmid was transformed into *C. glutamicum* wild-type (WT) by electroporation. Integration of the introduced plasmid into *C. glutamicum* chromosome by single crossover was selected on BH (brain-heart broth) plates containing 25 μg/ml kanamycin and 40 μg/ml nalidixic acid. The kanamycin-resistant (Km^R) colonies were grown overnight in LB allowing a second cross-over to occur. Selection for loss of the genome integrated *sacB*-containing plasmid was performed on LB plates containing 20% sucrose and 40 μg/ml nalidixic acid. Strains growing on this plate were tested for kanamycin sensitivity (Km^S) by parallel picking on LB plates containing either kanamycin or sucrose. Kanamycin-sensitive and sucrose-resistant strains were tested for deletion by PCR using the D*pheR* upF/D*pheR* lowR primer pair (Table S2) and confirmed by PCR and DNA sequencing as previously described (Shen and Liu, 2005). For complementation, pXMJ19-His_6-*pheR* was transformed into Δ*pheR*, by electroporation, and expression of each gene in *C. glutamicum* was induced by the direct addition of 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures (Shen and Liu, 2005).

2.4. Construction of lacZ transcription fusion vector and β-galactosidase assays

The *lacZ* fragment was inserted into pK18*mobsacB* by using the primer pair *lacZY*-F/*lacZY*-R. The promoter fragments of *phe* and *pheR* were amplified by PCR with primer pairs P_{phe}-F/P_{phe}-R and P_{pheR}-F/P_{pheR}-R, respectively, and ligated upstream of *lacZY* to obtain the plasmids pK18*mobsacB*-P_{phe}:*lacZ* and pK18*mobsacB*-P_{pheR}:*lacZ*. The pK18*mobsacB*-P_{phe}:*lacZ* transcriptional fusion was transformed into WT, Δ*pheR* and Δ*pheR*$^+$ (with pXMJ19-*pheR*) strains while pK18*mobsacB*-P_{pheR}:*lacZ* was transformed into the WT strain only. These strains were incubated at 37 °C in MS medium containing 3 mM phenol or glucose as the sole carbon source. The β-galactosidase activity was determined for cells harvested at the end of logarithmic growth (OD_{600} = 0.7) according to a method described previously (Miller, 1992).

2.5. Reverse transcription (RT) PCR and quantitative reverse transcription-PCR (RT-PCR)

C. glutamicum WT, Δ*pheR* and Δ*pheR*$^+$ strains were maintained on 3 mM each of glucose or phenol. Total RNA were obtained from cells in late logarithmic phase at about 28 h of growth. RNA concentration was determined spectrophotometrically and its integrity was assessed by agarose gel electrophoresis. RNA was transcribed to cDNA using ReverTra Ace (Toyobo, Japan) following the manufacturer's instructions. The obtained cDNA was stored at −20 °C.