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Identification of a repressor and an activator of azoreductase gene expression in *Pseudomonas putida* and *Xanthomonas oryzae*

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ABSTRACT

Genes responsible for the production of azoreductase enzymes in 2 gram-negative bacteria, the soil bacterium *Pseudomonas putida* (AzoP) and the plant pathogen *Xanthomonas oryzae* (AzoX), were identified. The deduced amino acid sequences of AzoP and AzoX, share 46% amino acid identity to each other. Two different bacterial transcription factors, a repressor (AzoPR) and an activator (AzoXR), in *P. putida* and *X. oryzae*, respectively, were found to be divergently oriented to their respective azoreductase genes. Both regulators are LysR-type transcriptional regulators (LTTR) that respond to the azo dye inducer, methyl red (MR). AzoPR represses transcription of *azoP* in *P. putida*, which is reversed when cells are exposed to MR. Interestingly, in *X. oryzae*, AzoXR positively regulates *azoX* transcription upon MR induction. Moreover, despite their similarity, with 51% amino acid sequence identity, *azoPR* and *azoXR* are expressed differently in response to MR. The transcription of *azoPR* is increased in a dye concentration-dependent manner, while *azoXR* transcription is constitutive and relatively higher than *azoPR*. Both regulators are autoregulatory. Gel mobility shift assays (EMSA) verified the binding between the regulators and their corresponding promoter regions. Additionally, binding only occurred under reduced conditions in the presence of 0.5 mM dithiothreitol (DTT), indicating that the proteins are active in their reduced form.

1. Introduction

Synthetic dyes are widely used in a number of industries such as textiles, food, cosmetics and paper printing. They are produced in large amounts annually. Nearly 1 million tons of synthetic dyes, comprised of an approximately 100,000 types, are estimated to be produced and used by industries worldwide with azo compounds making up 70% of production [1]. Azo compounds constitute the largest and the most diverse group of synthetic dyes. They are relatively low cost, easily synthesized and generally recalcitrant to biodegradation due to their xenobiotic nature. Azo dyes are mainly used in the textile industry where an estimated 2–50% of them is discharged in waste water or leaks into the ground water around the factories [2].

Azo dyes are of great concern due to their potential toxicity and carcinogenic properties [3]. In the body, the majority of azo dyes undergoes reduction catalyzed by enzymes of intestinal microorganisms and/or hepatic enzymes, including microsomal and soluble enzymes [4]. Many of the azo dyes in common use today contain highly charged substituents such as sulfonate and resist enzymatic attack. They are also poorly absorbed from the intestinal tract. Reduction of the carcinogenic dyes usually leads to loss of carcinogenic activity [4].

Microorganisms, being highly versatile, have evolved enzyme systems for the decolorization and mineralization of azo dyes under certain environmental conditions. Reductive cleavage of azo bonds, leading to the formation of aromatic amines, is the initial reaction during bacterial metabolism of azo dyes [4]. Recently, the enzymatic approach has attracted much interest in the decolorization/degradation of textile and other industrially important dyes from wastewater as an alternative strategy to conventional chemical, physical and biological treatments, which have serious

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limitation [4]. Enzymatic treatment is very useful due to the action of enzymes on pollutants even when they are present in very dilute solutions and recalcitrant to the action of various microbes participating in the degradation of dyes. Although a number of studies investigated the mechanism of action of bacterial azo reductases as well as explored the application of azoreductase enzymes in dye bioremediation [5–7], there is little or no information available concerning the genetic regulation of azoreductases.

We have been interested in studying the LysR type transcriptional regulator (LTTR) family in various bacteria particularly in the plant pathogen *Xanthomonas oryzae*. Oxidative stress responsive LTTR *oxyR* has been found and molecularly characterized in *X. oryzae* [8]. During genome database mining of *X. oryzae,* an ORF encoding the putative LTTR was found in front of the azoreductase gene and attracted our attention. However, *X. oryzae* is a plant pathogen, therefore it might not be a suitable host for biodegradation applications in the environment. Alternatively, we selected a soil bacterium *Pseudomonas putida* KT 2440, a versatile saprophytic organism capable of growth on several aromatic hydrocarbons. *P. putida* KT 2440 has been certified as a biosafety host and its genome has been sequenced. Interestingly, the genomes of both *X. oryzae* and *P. putida* encode azoreductase genes in close association with regulatory genes.

Here we report the identification of two different bacterial transcription factors controlling azoreductase gene transcriptions, a repressor and an activator, in *P. putida* and *X. oryzae*, respectively. Both regulators respond to azo dye as an inducer and transcriptionally control the expression of azoreductase genes via different mechanisms.

2. Materials and methods

2.1. Cloning of azoreductase structural and regulator genes

ORFs PP_2866 (denoted *azoP*) and PXO_01081 (*azoX)* that are predicted to encode azoreductases in *P. putida* KT2440 and *X. oryzae* PXO99 were amplified from the chromosomes of *P. putida* (BT4499 and BT4500) and *X. oryzae* (BT4495 and BT4496), respectively, using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The 600 bp-PCR fragments were digested with *Eco*RV and *Sma*I and inserted into pBluescript II KS vector to yield pKSAzoP and pKSAzoX.

azoPR and *azoXR* were amplified from the *P. putida* and *X. oryzae* chromosomes using the primer pairs BT4501 and BT4502, and BT4497 and BT4498, respectively. The sequences of the primers used are shown in Table 1. The blunt end PCR products of 903 bp and 1040 bp were ligated into pBluescript II KS that was digested with *Eco*RV and *Sma*I creating pKSAzoPR and pKSAzoXR, respectively.

2.2. Construction of full length azoP, azoX, azoPR and azoXR expression plasmids for complementation

Both *azoP* and *azoX* were excised as 600 bp *Bam*HI/*Hin*dIII fragments from pKSAzoP and pKSAzoX and then ligated into plasmid vector pBBRKm, generating pBBRAzoP and pBBRAzoX.

The complete full length genes, *azoPR* and *azoXR,* of *P. putida* and *X. oryzae* were PCR amplified using primers BT4499 and BT4500, and BT4495 and BT4496, respectively (primers shown in Table 1), and cloned into *Sac*I and *Hin*dIII digested pSRK-Gm, which is a Broad-Host-Range expression vector [9] to yield pSRKAzoPR and pSRKAzoXR. Plasmids were introduced into *P. putida* and *X. oryzae* mutant strains by electroporation.

2.3. Construction of P. putida and X. oryzae knockout strains

The *azoP* and *azoX* insertion mutants were constructed. The 340-bp part of *azoP* was amplified from the *P. putida* chromosome using primers BT4582 and BT4583 (Table 1). A 340-bp gene internal region of *azoX* was amplified from the *X. oryzae* chromosome using primers BT4584 and BT4585. The PCR products were ligated into pGEM-T Easy (Promega). The resulting clones were digested with *Spe*I and *Apa*I and inserted into pKNOCK-Gm digested with the same restriction enzymes, creating pGmAzoP and pGmAzoX. The broad-host-range plasmid, pKNOCK-Gm, is a mobilizable suicide vector that is routinely used for gene inactivation (Table 1). The plasmids, pGmAzoP and pGmAzoX, were then introduced into *P. putida* and *X. oryzae* by conjugation.

Gene internal fragments of *azoPR* and *azoXR* were cloned into pKNOCK-Km vector [10]. Gene internal fragments of *azoPR* (340 bp) were excised from pKSAzoPR by *Pvu*II and *Sal*I, and *azoXR* (340 bp) were excised from pKSAzoXR by *Bam*HI and *Sal*I, before ligation into pKNOCK-Km and transformation to *E. coli* BW20767 [10]. The pKNOCK-Km derivatives, pKNOCKAzoPR and pKNOCKAzoXR, were then introduced to *P. putida* and *X. oryzae*, respectively.

2.4. Transcription analysis of azoreductase structural and regulator genes

Total bacterial RNA was isolated using a hot phenol technique [11] from cultures grown in LB under either uninduced or induced conditions in the presence of MR dye at concentrations of 100 and 500 μM for 30 min. RNA (1 μg) was converted to cDNA using a ReverAid Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase kit (Fermentus) and random hexamer primers. Expression levels of *azoP* and *azoX* were then determined by end point RT-PCR and quantitative Reverse Transcription PCR (qRT-PCR) using a KAPA SYBR FAST (KAPA) kit and followed the manufacturers protocols. The primers used were: BT4582 and BT4583 for *azoP*, BT4584 and BT4585 for *azoX*, BT4644 and BT4645 for *azoPR* and BT4646 and BT4647 for *azoXR*. The primers used for RT-PCR of *azoPR* promoter and *azoXR* promoter were BT4431 and BT4501, and BT4429 and 4497 respectively. The Program StepOnePlus Real-Time PCR system (ABI, USA) was used for quantitative measurement of both genes using 16S rRNA as a control reference.

2.5. Azoreductase activity assays

E. coli JM110 strains carrying either pKSAzoP or pKSAzoX were grown in broth containing 0.02% (w/v) methyl red dye (MR). The absorbance of the culture supernatant was monitored at 430 nm every hour and the percentage of MR degradation was calculated using the equation: $(A_0 - A)/A_0 \times 100$ where A_0 is the absorbance of the control and A is the absorbance of the culture supernatant with dye [12].

2.6. Over expression of His-tagged AzoPR and AzoXR

A 900 bp *azoPR* fragment was amplified from the chromosome of *P. putida* using primers BT4853 and BT4854 and digested with *Eco*RV and *Hin*dIII before inserting into pETBlue2 to yield pETAzoPR in which a histidine tag is fused to the C-terminus of the protein. For *azoXR*, a 1014-bp PCR fragment was amplified from the *X. oryzae* chromosome using primers BT4851 and BT4852 and digested with *Nco*I and *Xho*I before inserting into plasmid pDuet digested with the same restriction enzymes to yield pDuetAzoXR, in which a histidine tag is fused to the N-terminus of *azoXR*. *E. coli* strain DE3 was used as the host strain for expression of His-tagged proteins, which was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG)

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