Streptomycetes Inducible Gene Cluster Involved in Aromatic Compound Metabolism

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Abstract

Streptomyces setonii (ATCC 39116) is a Gram-positive thermophilic soil actinomycetes capable of degrading single aromatic compounds including phenol and benzoate via *ortho*-cleavage pathway. we isolated approximately 6.3-kb *S. setonii* DNA fragment containing a thermophilic catechol 1,2-dioxygenase(C12O) gene. Here we further revealed that the 6.3-kb *S. setonii* DNA fragment was organized into two putative divergently-transcribed clusters with 6 complete and one incomplete open reading frames (ORFs). The first cluster with 3 ORFs showed significant homologies to previously known *benA*, *benB*, and *benC*, implying a part of benzoate catabolic operon. The second cluster revealed an *ortho*-cleavage catechol catabolic operon with three translationally-coupled ORFs (*catR*, *catB*, *catA*). Each of these individually-cloned ORFs was expressed in *E. coli* and identified as a distinct protein band with a theoretical molecular weight in SDS-PAGE. The expression of the cloned *S. setonii* catechol operon was induced in a heterologous *S. lividans* by specific single aromatic compounds including catechol, phenol, and 4-chlorophenol. The similar induction pattern was also observed using a luciferase gene-fused reporter system, implying that *S. setonii* employs an inducer-specific regulatory mechanism for aromatic compound metabolism.

Introduction

Streptomycetes and their physiologically-related actinomycetes are ubiquitous Gram-positive soil microorganisms and a group of the most important industrial microorganisms for the biosynthesis of many valuable secondary metabolites, including antibiotics, anti-cancer drugs, immunosuppresors, and enzyme inhibitors. From an environmental and ecological perspective, they are also valuable microorganisms due to

their capabilities in degrading the diverse structures of various natural and unnatural organic compounds like other well-characterized Gram-negative and some Gram-positive bacteria. Interestingly, the overall characteristics of certain catabolic enzymes involved in aromatic compound metabolism such as catechol 1,2-dioxygenase (C12O) found in Gram-positive bacteria seem to be phylogenetically distant from those found in Gram-negative bacteria, implying that the two metabolic systems probably originate from different evolutionary ancestors. *Streptomyces setonii* (ATCC 39116), originally isolated from vanilate-enriched Idaho soil, has been proved to be able to degrade various single aromatic compounds including phenol and benzoate through a catechol intermediate via an *ortho*-cleavage pathway using C12O. Previously, we constructed a *S. setonii* DNA library and isolated a 6.3-kb *Pst*I fragment, within which a 1.4-kb fragment containing the full length of the C12O gene, *catA,* was completely sequenced and characterized. In this manuscript, we further continued to investigate the flanking regions of *S. setonii* C12O gene, revealing for the first time among streptomycetes and their physiologically-related actinomycetes, the presence of divergently-transcribed two operons; benzoate and catechol catabolic gene clusters. Our results also suggested that the expression of *S. setonii ortho*-cleavage catabolic pathway was induced by specific aromatic compounds, implying the presence of an inducer-specific regulatory system for catechol catabolism.

Materials and Methods

1. Bacterial strains, plasmid, and cultivation conditions

Streptomyces setonii (ATCC39116) was purchased from the American Type Cell Collection (ATCC, USA) and was routinely grown on R2YE agar plate at 45C for sporulation. *S. setonii* spores were resuspended and stored in sterile 20% glycerol solution at -20 C. *E. coli* DH5, BL21(DE3), pUC19, pET21b, and a streptomycetes-*E. coli* shuttle vector pWHM3 were used for cloning and expression experiments.

2. Heterologous expression in *E. coli*

Six complete ORFs (*benA, benB, catR, catA, catB, catC*) identified in pESK003 were individually cloned and heterologouslly expressed in *E. coli*. The *E. coli* containing a pET21-based construct was cultured in a Luria-Bertani (LB) liquid media containing 100 ug/ml of ampicillin at 37 C for 90 min, followed by the addition of 1 mM IPTG and 3 hr incubation. The *E. coli* containing a pUC19-based construct was cultured in LB liquid

media containing 100 ug/ml of ampicillin at 37 C for overnight. *E. coil* cells were harvested by centrifugation at 10,000 rpm for 5 min, washed with 10ml of 1 mM MnSO4 in 50 mM Tris-HCl (pH 7.5) buffer, disrupted by sonification by four 10-sec burst (35% amplitude and 0.9 pulse) with an ultrasonic homogenizer, and centrifuged at 10,000 rpm for 30 min. The clear supernatant was used as a crude lysate for 10% SDS-PAGE.

3. Inducible and heterologous expression of C12O in *S. lividans*

The 6.3-kb *Pst* I fragment was first cloned in a streptomycetes-*E. coli* shuttle vector pWHM3 in *E. coli* (named pESK003), followed by the PEG-transformation into *S. lividans*. The *S. lividans* containing the plasmid pESK003 was cultivated with constant shaking (200 rpm) at 30 C in 50 ml of a minimal medium supplied with 0.5% of glucose and 5 ug/ml thiostrepton for 28 hrs. The culture was further incubated for 8 hrs after the addition of an inducing compound. The clear supernatant was used as a crude lysate for the C12O enzyme and the reaction was initiated by the addition of 30mM catechol followed by measuring the absorbance at 260 nm at time intervals of 1min.

4. Construction of *lux* gene-fused reporter system and inducible luciferase assay

In order to construct *lux* gene-fused reporter system, the previously-reported pESK004 was digested with *Hind* III and ligated with about 3-kb DNA fragment containing a putative promoter region, *catR, catB, and catA* genes which was amplified by PCR using a pair of *Hind* III-containing primers. The *S. lividans* containing pESK006 was cultured in a minimal media at 30 C with 200 rpm for 24 hrs, followed by the addition of various aromatic compounds including phenol, 2-chlorophenol, 4-chlorophenol, benzoate, catechol. The one ml of samples were taken at every 2 hr for the optical density (OD600nm) measurement and luciferase enzyme assay.

Results and Discussion

A total of six complete and one partial open reading frames (ORFs) were identified in the previously isolated 6.3-kb *S. setonii* DNA fragment (Figure.1). A DNA database search using these ORFs revealed two putative divergently-transcribed gene clusters with four ORFs to the right and two and half ORFs to the left (Figure.1) The first gene cluster (named benzoate cluster) with two translationally-coupled ORFs and one partial ORF were aligned in same direction, and assigned in order of their amino acid similarities as *benA*, *benB*, and a part of *benC*. The second gene cluster (named catechol cluster) located

downstream of the benzoate cluster consists of four ORFs aligned in an opposite direction with three translationally-coupled ORFs. These four ORFs were then assigned in order of their deduced amino acid similarities as *catR*, *catB*, *catA*, and *catC*. Each of the putative ORFs was individually amplified from the predicted start to the stop codons using PCR method, and independently cloned into *E. coli* expression vector. As shown in Figure 2, each of the *E. coli* transformants containing putative ORF expressed a distinct protein band with an expected molecular weight in SDS-PAGE.

To investigate the regulatory mechanism of catechol catabolism in *S. setonii*, the plasmid pESK003 (a 6.3-kb *Pst* I fragment cloned in pWHM3) was heterologously expressed in *S. lividans* and assayed for C12O activity after induction with several aromatic compounds. The C12O activity was detected only when the pESK003-containing *S. lividans* was induced by 1 mM of catechol, yet no such C12O activity detected without any induction. Interestingly, a similar C12O induction pattern was also observed when the phenol or 4-chlorophenol was used as the inducing compound (Figure. 3a). The induced C12O activities by these three compounds also increased proportionally to the concentration of the inducing compounds (Figure. 3b).

In order to further confirm the inducible expression of catechol catabolic operon by specific aromatic compounds, a part of the operon containing only a putative promoter region and 3 translationally-coupled genes (*catR, catB,* and *catA*) fused with *lux* reporter genes was cloned into the pWHM3 shuttle vector (named pESK006, Fig. 1). As expected, the luciferase activity was detected when the pESK006-containing *S. lividans* was induced by 1 mM of catechol, or 4-chlorophenol. (Figure. 5a). Like the inducible C12O activity shown in the pESK003-containing *S. lividans*, the induced luciferase activity was also proportionally dependent on the concentration of the 4-chlorophenol in the pESK006-containing *S. lividans* (Figure. 5b).

References

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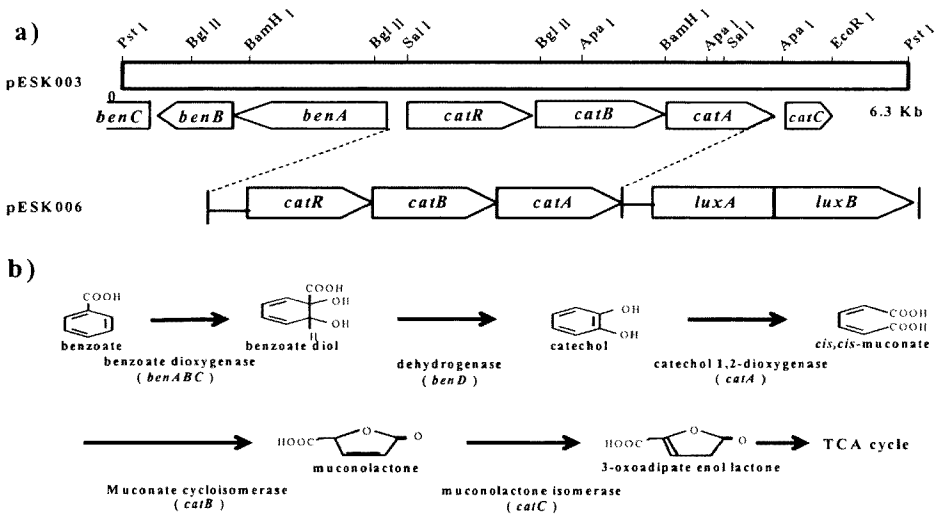

Figure 1. a) Restriction map of a 6.3-kb *S. setonii* DNA carrying benzoate/catechol catabolic locus (pESK003) and *lux* gene fused construct (pESK006) The approximate position and direction of each gene are indicated by an open arrow. b) Proposed genes, enzymes, and pathways involved in benzoate/catechol metabolism of *S. setonii*.

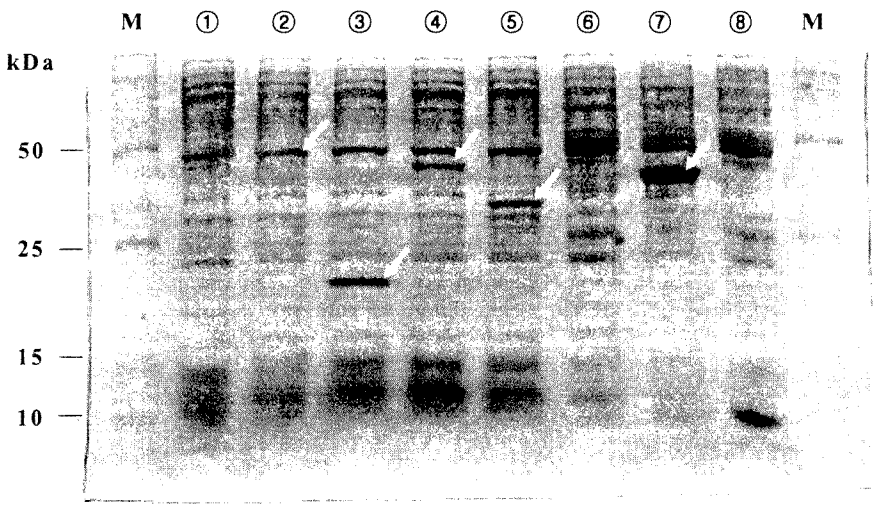

Figure 2. SDS-PAGE of heterologous expression of each ORF identified from 6.3kb *Pst* I fragment in *E. coli*. Lane M, molecular weight markers; lane 1, pET21b; lane 2 BenA; lane 3, BenB; lane 4, CatB; lane 5, CatR; lane 6, pUC19; lane 7, CatA; lane 8, CatC.

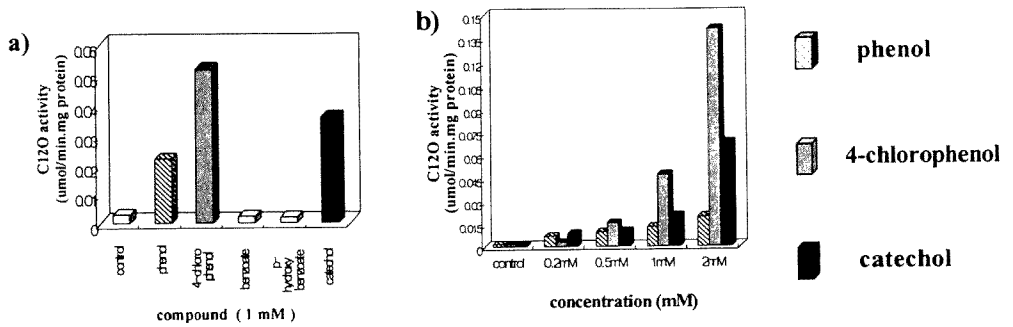

Figure 3. a) C12O activities induced by 1 mM of various aromatic compounds; control (no inducer compound), phenol, 4-chlorophenol, benzoate, p-hydroxy benzoate, and catechol. b) Concentration-dependent C12O induction by catechol, phenol, and 4-chlorophenol

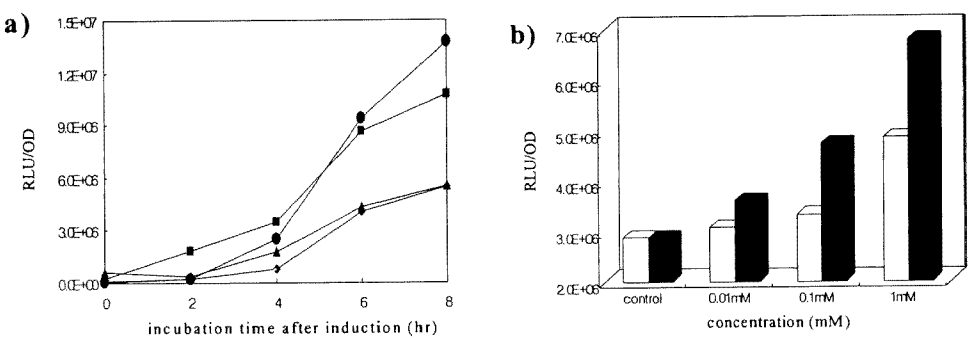

Figure 4. a) Luciferase activities induced by 1 mM of various aromatic compounds: ◆, control (no inducer compound); ▲, benzoate; ■, catechol; ●, 4-chlorophenol. b) Concentration-dependent luciferase induction by catechol (open bar) and 4-chlorophenol (closed bar)