Cloning and expression of thermophilic catechol 1,2-dioxygenase gene (catA) from Streptomyces setonii

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Abstract

Streptomyces setonii (ATCC 39116) degrades various single aromatic compounds such as phenol or benzoate via an ortho-cleavage pathway using catechol 1,2-dioxygenase (C12O). A PCR using degenerate primers based on the conserved regions of known C12O-encoding genes amplified a 0.45-kbp DNA fragment from S. setonii total DNA. A Southern hybridization analysis and size-selected DNA library screening using the 0.45-kbp PCR product as a probe led to the isolation of a 6.4-kbp S. setonii DNA fragment, from which the C12O-encoding genetic locus was found to be located within a 1.4-kbp DNA fragment. A complete nucleotide sequencing analysis of the 1.4-kbp DNA fragment revealed a 0.84-kbp open reading frame, which showed a strong overall amino acid similarity to the known high-G+C Gram-positive (but significantly less to the Gram-negative) bacterial mesophilic C12Os. The heterologous expression of the cloned 1.4-kbp DNA fragment in Escherichia coli demonstrated that this C12O possessed a thermophilic activity within a broad temperature range (up to 65°C) and showed a higher activity against 3-methylcatechol than catechol or 4-methylcatechol, but no activity against protocatechuate. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Catechol 1,2-dioxygenase; catA; Biodegradation; Streptomyces

1. Introduction

Streptomyces and its physiologically related actinomycetes are among the most abundant soil microorganisms playing major roles in the biosynthesis of many valuable secondary metabolites including antibiotics, anti-cancer drugs, immunosuppressors, enzyme inhibitors, and dyes [1]. In addition, some actinomycetes have also been recognized as ecologically valuable microorganisms due to their superior capabilities in degrading the diverse structures of various natural and unnatural organics including aromatic compounds [2–6]. Natural and unnatural aromatic compounds, some of them are quite recalcitrant or toxic when released into the environment, are widespread in nature. Due to the environmental concerns related to these hazardous aromatic compounds, various ways to eliminate or reduce their environmental presence have been pursued including bioremediation using soil microorganisms [7,8]. It has been well documented that certain Gram-negative soil bacteria, including Pseudomonas or Acinetobacter species, are capable of completely degrading relatively simple aromatic compounds including phenol and benzoate [8,9]. Furthermore, the enzymes and the genes involved in aromatic compound biodegradation have also been intensively elucidated at both biochemical and molecular genetic levels [10,11]. In general, most aromatic compounds are aerobically degraded through a common intermediate, catechol or protocatechuic acid depending on the chemical structure of the starting compound [10]. The catechol is further degraded either by cleavage between two hydroxyl groups by catechol 1,2-dioxygenase (C12O) via an ortho-pathway or by cleavage adjacent to the hydroxyl groups by catechol 2,3-dioxygenase via a meta-pathway [9,10], respectively. Recently, some Gram-positive soil bacteria including the Arthrobacter and Rhodococcus species were also isolated from various environments and characterized as containing a similar C12O-dependent catechol-degrading ortho-pathway like the one found in the Gram-negative bacteria [12–16]. Interestingly, however, the overall characteristics...
of the C12O-encoding gene (catA) found in the Gram-positive bacteria seem to be phylogenetically distinct from the ones in the Gram-negative bacteria, implying that these two systems probably originated from different evolutionary ancestors [12–16]. *Streptomyces setonii* (American Type Cell Collection (ATCC) 39116), originally isolated from vanilate-enriched Idaho soil, is a Gram-positive thermophilic actinomycete which degrades various single aromatic compounds including phenol or benzoate at the optimum temperature of 45°C through a catechol intermediate via an ortho-cleavage pathway using C12O [17,18]. In this manuscript, we present the complete nucleotide sequence of the thermophilic C12O-encoding gene, *catA* isolated from *S. setonii*, and enzyme characteristics of the heterologously expressed *S. setonii* C12O in *Escherichia coli.*

2. Materials and methods

2.1. Bacterial strains, plasmid, and cultivation conditions

*S. setonii* (ATCC 39116) was purchased from the ATCC (USA) and was routinely grown on a R2YE agar plate at 45°C for sporulation [19]. *S. setonii* spores were resuspended and stored in sterile 20% glycerol solution at −20°C. For total DNA isolation, *S. setonii* spore suspension was inoculated into the 25 ml of YEME liquid media and cultured for 2 days at 45°C [19]. The *Streptomyces* total DNA isolation method was previously described elsewhere [19]. *E. coli* DH5α strain and two plasmids (pUC19 and pGEMT-easy (Promega, USA)) were used for cloning experiments, and followed the standard molecular biology procedures described elsewhere [19].

![Restriction map of 6.4-kbp *S. setonii* DNA carrying a catechol catabolic locus. The approximate position of C12O (catA) and the muconolactone isomerase (catC) genes, plus the presence (+) or absence (−) of the C12O enzyme activity in *E. coli* containing the indicated plasmid. Abbreviation: B: BamHI; Bg: BglII; E: EcoRI; P: PstI.](https://academic.oup.com/femsle/article-abstract/195/1/17/521204)
2.2. Cloning and sequence analysis of S. setonii C12O gene

A pair of degenerate PCR primers were designed based on the central conserved regions of the known C12O-encoding genes (forward primer C12O-1: 5’-GA(G/A)T-GGCC(G/A/T/C)(T/C)(T/G/A/T/C)GT(G/G/A/T/C)-3’; reverse primer C12O-2: 5’-(G/A/T/C)(A/G/A)/(G/T/G/A/T/C)(A/G/A)/(G/A)/TG(G/G/A/T/C)(G/G/A/T/C)(G/G/A/T/C)(G/G/A/T/C)(G/G/A/T/C)-3’; Fig. 2). PCR with the degenerate primers using S. setonii total DNA as a template was performed according to the previously described procedure [20]. The Southern hybridization was performed using the DIG system (Roche Molecular Biochemicals, Germany) according to the manufacturer’s method. The DIG-labeled DNA was detected using CDP-Star®, which is an ultra-sensitive chemiluminescence substrate for alkaline phosphatase (Boehringer Mannheim Biotechnie, USA). In order to compare the deduced amino acid sequence alignment of catAs among Gram-positive bacteria, the computer-based sequence analyses were performed with Multiple Sequence Alignment with hierarchical clustering (Clustalw program by European Bioinformatics Institute). The GenBank accession number for the 1.4-kbp BamHI-EcoRI fragment of S. setonii is AF277051.

2.3. Crude lysate preparation and C12O enzyme assay

The E. coli containing plasmid pESK002-9(+) or pESK002-9(−) was cultivated overnight with constant shaking (200 rpm) at 37°C in Luria-Bertani medium supplied with 100 µg of ampicillin per ml. E. coli cells were harvested by centrifugation at 10 000 rpm for 5 min, washed with 10 ml of 1 mM MnSO₄ in 50 mM Tris–HCl (pH 7.5) buffer, disrupted by sonication by four 10-s bursts (35% amplitude and 0.9 pulse) with an ultrasonic homogenizer, and centrifuged at 10 000 rpm for 30 min. The clear supernatant (approximately 3.7 µg ml⁻¹ of total protein) was used as a crude lysate for both enzyme assay and SDS–PAGE. The in vitro C12O enzyme assay method was previously described elsewhere [13,17]. The C12O enzyme assay reaction mixture was composed of...
0.25 μg of total protein in 1 ml of a 50 mM Tris-EDTA (pH 7.5) buffer. The reaction was initiated by the addition of 30 mM catechol (or 3-methylcatechol, 4-methylcatechol, or protocatechuate) followed by measuring the absorbance at 260 nm at time intervals of 1 min [16,17]. SDS-PAGE (10%) was performed to verify the heterologous expression of S. setonii C12O using 5 μl of the crude lysate from the E. coli containing pESK002-9(+) or pESK002-9(−).

3. Results and discussions

3.1. Isolation of catechol catabolic locus from S. setonii

In order to clone and characterize a catechol catabolic locus including the C12O-encoding gene from S. setonii, a pair of degenerate PCR primers were designed based on the central conserved regions of the known C12O-encoding genes underlined in Fig. 2. A PCR using these degenerate primers amplified a DNA fragment of the expected size (0.45 kbp) [13]. This fragment was directly cloned into the pGEMT-easy (Promega, USA) vector and confirmed to possess a significant similarity to the previously reported C12O central regions by a nucleotide sequencing analysis (data not shown). The Southern hybridization with the S. setonii total DNA using the 0.45-kbp PCR fragment as a probe confirmed that this DNA fragment originated from S. setonii total DNA, and the PCR-amplified C12O central region was located in a 6.5-kbp PstI fragment of S. setonii total DNA (data not shown). Based on this information, an E. coli size-selected DNA library was constructed, which contained 6–7-kbp PstI-digested S. setonii total DNA cloned into a pUC19 plasmid, followed by colony hybridization screening using the same probe as in the Southern hybridization. Several positive

![Fig. 3. (A) C12O enzyme assay of in vitro conversion from catechol to cis,cis-muconic acid. The reaction was initiated by the addition of 30 mM catechol, followed by measuring the absorbance at 260 nm at time intervals of 1 min (up to 7 min). A: E. coli DH5α/pESK002-9(−); B: E. coli DH5α/pESK002-9(+). (B) SDS-PAGE: M, size marker (from the top, kDa), 95.5, 55.5, 43.0, 29.0, 20.4, 18.4, 14.4; 1, E. coli DH5α/pESK002-9(+); 2, E. coli DH5α/pESK002-9(−).]


**3.2. Nucleotide sequence analysis of the 1.4-kbp DNA fragment**

The 1.4-kbp DNA insert of pESK002-9 was completely sequenced on both strands. Two complete open reading frames (ORF1 and ORF2) were identified in the 1.4-kbp region, the sizes of which were 0.84 kbp and 0.3 kbp, respectively (Fig. 1). The putative start codon (ATG) of ORF1 was located one nucleotide downstream of the BamHI restriction site. ORF1 and ORF2 were separated by 21 nucleotides and a putative ribosomal binding sequence (GGAGG) was found seven nucleotides upstream of the putative ORF2 start codon (ATG). The overall G+C contents of ORF1 and ORF2 were 69.8% and 60.9%, respectively. Both ORFs also exhibited a typical high-G+C content at the third position of the codon found in most Streptomyces ORFs (95.3% and 88.3%, respectively). A DNA database search using these ORFs revealed that ORF1 showed a significant similarity with the previously reported Gram-positive C12O-encoding gene (*catA*). The deduced amino acids of ORF1 showed overall amino acid identities with CatAs from *Arthrobacter* sp. mA3 (67%), *Rhodococcus opacus* 1CP (58%), *Rhodococcus erythropolis* AN13 (56%), and *Rhodococcus rhodochrous* NCIMB13259 (54%) (Fig. 2). However, a much less significant similarity was found between the *S. setonii* ORF1 (hereafter named *catA*) and other known Gram-negative bacteria *catA* genes; 31% and 19% overall amino acid identities with CatAs from *Pseudomonas putida* PRS1 and *Acinetobacter calcoaceticus*, respectively. As expected, the characteristic regions conserved in all known C12O genes (e.g. iron-binding site) were well preserved within the *S. setonii* catA (Fig. 2; histidine and tyrosine residues positioned at 163, 198, 222, and 224). The smaller ORF2 showed strong similarity to *catC* residues positioned at 163, 198, 222, and 224). The smaller ORF2 showed overall amino acid identities with CatCs from *Myco- bacterium smegmatis* mc2-155 (73%), *R. opacus* 1CP (68%), *Acinetobacter iwoffii* K24 (66%), and *P. putida* mt-2 (58%).

**Table 1**

<table>
<thead>
<tr>
<th>Substrate (assay temperature)</th>
<th>Specific activity (U)</th>
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<tr>
<td>Catechol (25°C)</td>
<td>5.96</td>
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<tr>
<td>Catechol (35°C)</td>
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<tr>
<td>Catechol (45°C)</td>
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</tr>
<tr>
<td>Catechol (55°C)</td>
<td>11.34</td>
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<td>Catechol (70°C)</td>
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</tr>
<tr>
<td>3-Methylcatechol (45°C)</td>
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</tr>
<tr>
<td>4-Methylcatechol (45°C)</td>
<td>8.23</td>
</tr>
<tr>
<td>Protocatechuate (45°C)</td>
<td>0</td>
</tr>
</tbody>
</table>

*(1 U = μmol min⁻¹ mg⁻¹ protein).*

3.3. **Heterologous expression and enzymatic characteristics of *S. setonii* C12O**

In order to show whether the 1.4-kbp insert of pESK002-9 contained a full length *S. setonii* C12O gene as well as a functional enzyme, the crude lysate supernatant of *E. coli* containing pESK002-9 was used for an in vitro C12O enzyme assay. The crude lysate supernatant was obtained by sonication and centrifugation from an overnight culture of *E. coli* containing pESK002-9. The crude lysate supernatant was used for the C12O enzyme assay, a spectrophotometric in vitro catechol to *cis,cis*-muconate conversion method [13,17]. As shown in Fig. 3A, only the crude lysate supernatant from the *E. coli* containing pESK002-9(+) showed a clear catechol to *cis, cis*-muconate conversion activity, but no such activity exhibited by the supernatant containing the plasmid with an oppositely cloned insert, pESK002-9(–). The *Kₐ* value of this C12O against catechol was approximately 1.8 μM. A putative C12O protein band was also clearly detected on SDS-PAGE only from the *E. coli* containing pESK002-9(+), but not from the *E. coli* containing the plasmid with an oppositely cloned insert, pESK002-9(–) (Fig. 3B). These results indicate that the *S. setonii* C12O gene in pESK002-9(+) is functionally expressed (probably as a translational fusion product) under the control of the Lac promoter located in pUC19. Interestingly, this C12O possessed a thermophilic enzyme activity within a broad temperature range from 25°C up to 65°C (highest at 45°C), and also showed a higher enzyme activity against 3-methylcatechol than catechol or 4-methylcatechol, but no activity against protocatechuate (Table 1). Although the protocatechue acid catabolic gene cluster was recently characterized for the first time from a newly isolated *Streptomyces* sp. strain 2065 [4], the C12O and its gene (*catA*) characterized in this communication is apparently the first C12O reported among streptomycetes and their physiologically related actinomycetes. Especially, unlike other known mesophilic C12Os, the *S. setonii* catA cloned and characterized in this work encodes a thermophilic C12O enzyme. It should be noted that *S. setonii* (ATCC 39116) recently has been suggested as a new member of the *Amycolatopsis* genus based on 16S rDNA sequences (personal communication with Prof. Donald Crawford).
Acknowledgements

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References