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# Regulation of genes in *Streptomyces* bacteria required for catabolism of lignin-derived aromatic compounds

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**Abstract** The major utilization pathway for lignin-derived aromatic compounds in microorganisms is the β-ketoadipate pathway. Through this pathway, the aromatic compounds protocatechuate and catechol are converted to acetyl coenzyme A and succinyl coenzyme A. The enzymes of the protocatechuate branch of this pathway are encoded by the *pca* genes. Here, we describe a gene cluster in *Streptomyces coelicolor* containing the *pca* structural genes and a regulatory gene required for the catabolism of protocatechuate. We found that transcription of the structural genes in *S. coelicolor* is induced by protocatechuate and *p*-hydroxybenzoate. We also observed inducible transcription of *pca* structural genes in the ligninolytic strain *Streptomyces viridosporus* ATCC 39115. Disruption of a gene encoding a putative MarR family transcription factor that is divergently transcribed from the *pca* structural genes resulted in constitutive transcription of the structural genes. Thus, the transcription factor encoded by this gene is an apparent negative regulator of *pca* gene transcription in *S. coelicolor*. Our findings suggest how *Streptomyces* bacteria could be engineered for and used in biotechnology for the utilization and degradation of lignin and lignin-derived aromatic compounds.

**Keywords** *Streptomyces* · Lignin · β-Ketoadipate · Protocatechuate · Aromatic catabolism

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## Introduction

There has been much attention focused on the exploitation of plant biomass as a renewable energy source (Ragauskas et al. 2006; Rubin 2008; van Wyk 2001). The bulk of plant biomass is lignocellulose, a complex of three polymers—lignin, cellulose, and hemicellulose (Rubin 2008). Cellulose, the major component of lignocellulose, is essentially a polymer of glucose molecules linked to one another via glycosidic bonds. Hemicellulose is a group of heteropolysaccharides derived from various 5- and 6-carbon monosaccharide units (e.g., arabinose, galactose, mannose, and xylose) (Breznak and Brune 1994; Rubin 2008). Lignin is a highly stable and heterogeneous polymer of phenylpropanoid units derived from aromatic phenols (Adler 1977). The cellulose microfibrils of lignocellulose are encapsulated in a matrix consisting of lignin covalently bound to hemicellulose (Adler 1977; Breznak and Brune 1994). The presence of lignin precludes accessibility of enzymes to the energy-rich and more easily metabolized cellulose and hemicellulose (Ljungdahl and Eriksson 1985). It is generally accepted that lignin degradation is a rate-limiting step in lignocellulose degradation (Bisaria and Ghose 1981; Himmel et al. 2007). Therefore, the breakdown of lignin is critical for the exploitation of plant biomass as a source of renewable energy (Chen and Dixon 2007).

Microbial bioconversion of lignin has emerged as a promising technology in the use of plant biomass as a feedstock for biofuels. Lignin degradation is best studied in white rot fungi (Hatakka 1994; Martinez et al. 2004). They secrete phenol oxidases (lignin peroxidase and laccase) that depolymerize lignin (Hatakka 1994; Martinez et al. 2004). The lignin is characteristically depolymerized into low molecular weight aromatic compounds such as coniferyl

alcohol, guaiacol, ferulate, *para*-coumarate, vanillate, *p*-hydroxybenzoate, and protocatechuate (Harwood and Parales 1996; Masai et al. 2007). Non-ligninolytic microorganisms and even some non-ligninolytic microorganisms are capable of catabolizing lignin-derived low molecular weight compounds (Harwood and Parales 1996). The major pathway for catabolism of aromatic compounds derived from lignin is the β-ketoadipate pathway (Harwood and Parales 1996; Masai et al. 2007). Enzymes from this pathway have been characterized in a variety of organisms (Harwood and Parales 1996). The first reaction in this pathway is a dioxygenase-catalyzed *ortho*-ring cleavage of protocatechuate or catechol to yield β-carboxymuconate or muconate, respectively (Fig. 1). In a sequence of five enzymatic reactions, either β-carboxymuconate or muconate is reductively converted to β-ketoadipate. Subsequently, the β-ketoadipate is enzymatically cleaved into acetyl coenzyme A and succinyl coenzyme A. Remarkably, this pathway enables the conversion of aromatic compounds into intermediates of the tricarboxylic acid cycle. Lignin-derived aromatic compounds are mostly fluxed into the β-ketoadipate pathway by enzymatic conversion to protocatechuate (Harwood and Parales 1996). Enzymes of the β-ketoadipate pathway have been predominantly found in soil-dwelling bacteria and fungi, especially those in symbiotic relationships with plants.

Lignin depolymerization and catabolism have been studied in a number of bacterial species, including members of the *Streptomyces* genus (Grund and Kutzner 1998; McCarthy 1987; Zimmerman 1990). These Gram-positive, soil-dwelling bacteria are best known as producers of half of the 10,000 known antibiotics and of two thirds of the antibiotics used in clinical and veterinary medicine (Hopwood 2007). *Streptomyces viridosporus* ATCC 39115, *Streptomyces setonii* ATCC 39116, and *Streptomyces badius* ATCC 39117 are *bona fide* ligninolytic strains (McCarthy 1987). At least in the case of *S. viridosporus* ATCC 39115, extracellular enzymatic activity for the depolymerization of lignin has been observed (Ramachandra et al. 1987) and the underlying lignin peroxidases have been identified and characterized (Ramachandra et al. 1988). Interestingly, the lignin peroxidase, ALiP-P3, from *S. viridosporus* was the first bacterial enzyme reported to depolymerize lignin (Ramachandra et al. 1988). Both *S. viridosporus* and *S. setonii* are known to convert the lignin in corn stover into *p*-coumarate, ferulate, vanillate, *p*-hydroxybenzoate, and protocatechuate via formation of "acid-precipitable, polymeric lignin" intermediates (Borgmeyer and Crawford 1985; Crawford et al. 1983; Pometto and Crawford 1986). At least in *S. setonii*, it has been reported that these low molecular weight aromatics are catabolized, presumably via the β-ketoadipate pathway (Park and Kim 2003; Pometto et al. 1981; Sutherland et al. 1983). Davies and coworkers identified a seven-gene operon encoding homologs of β-ketoadipate pathway enzymes in *Streptomyces* sp. strain 2065 (Iwagami et al. 2000). They further predicted that enzymes of the β-ketoadipate pathway are widely distributed in the *Streptomyces* genus (Iwagami et al. 2000). Indeed, homologous gene clusters can be found in the genomes of *Streptomyces avermitilis* (Omura et al. 2001) and *Streptomyces coelicolor* (Bentley et al. 2002). In addition to demonstrating enzymatic activity of the protocatechuate dioxygenase encoded by *pcaH* and *pcaG* in *Streptomyces* sp. strain 2065, Davies and coworkers further

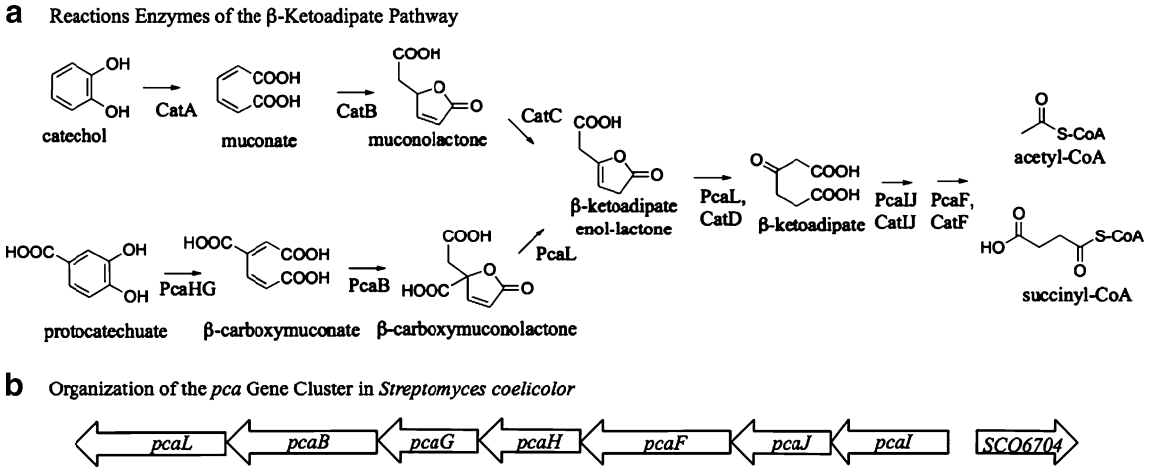


**Fig. 1** **a** The protocatechuate and catechol branches of the β-ketoadipate pathway. The enzyme names are shown below the *arrows*. **b** Organization of the *pca* gene cluster in *S. coelicolor*. Genes in the region spanning SCO6697 through SCO6704 are shown. The functions of the gene products are as follows: *pcaL* encodes putative γ-carboxymuconolactone decarboxylase/β-ketoadipate enol-lactone, *pcaB* encodes putative β-carboxymuconate cycloisomerase, *pcaGH* encode protocatechuate 3,4 dioxygenase, α and β-subunits, *pcaF* encodes β-ketoadipyl CoA thiolase, *pcaIJ* encode β-ketoadipate succinyl-CoA transferase α- and β-subunits, and SCO6704 encodes a putative MarR family transcriptional regulator

reported that its production is induced by *p*-hydroxybenzoate and vanillate, known products of lignin depolymerization (Iwagami et al. 2000). Inducible production of these enzymes is highly reminiscent of *Acinetobacter baylyi* (Siehler et al. 2007), *Sinorhizobium meliloti* (MacLean et al. 2006), and *Corynebacterium glutamicum* (Brinkrolf et al. 2006), where *pca* gene expression is tightly regulated by transcription factors that are sensitive to aromatic compounds.

In the interest of uncovering the mechanism by which synthesis of enzymes in the protocatechuate branch of the β-ketoadipate pathway are regulated in *Streptomyces* bacteria, we have investigated the gene cluster encoding these enzymes in *Streptomyces coelicolor*, the model organism of the genus (Kieser et al. 2000). Presumably, *S. coelicolor*, a non-ligninolytic strain, uses the enzymes encoded in the *pca* gene cluster to scavenge lignin-derived aromatic compounds in its ecological niche. The fact that *S. coelicolor* is amenable to genetic manipulation and molecular genetic analyses facilitated characterization of the *pca* gene cluster. We found that the transcription of these *pca* genes and apparent activity of the cognate enzymes are only observed when *S. coelicolor* is grown in media containing aromatic compounds. Using reverse genetic analysis, we found that disruption of a divergently transcribed gene (SCO6704) encoding a putative transcription factor in *S. coelicolor* results in constitutive expression of the *pca* genes. Likewise, we demonstrate that *pca* gene transcription is inducible in the ligninolytic strain *S. viridosporus.* In addition, a gene homologous to the putative *S. coelicolor* transcriptional regulator (SCO6704) was identified in *S. viridosporus*. This is the first characterization of the regulated transcription of genes in *Streptomyces* bacteria encoding enzymes that catabolize lignin-derived aromatic compounds.

## Materials and methods

### Bacterial strains and culture conditions

The bacterial strains and plasmids employed in this study are listed in Table 1. *Escherichia coli* strains DH5α and ET12567/puZ8002 were grown on Luria–Bertani medium at 37°C (Sambrook et al. 1989), except BW25113/pIJ790 which was grown at 30°C to maintain selection of pIJ790. *Streptomyces* strains were grown on mannitol soya flour medium (SFM), Difco Nutrient Agar medium (DNA), yeast extract medium, and in minimal liquid medium (NMMP; Kieser et al. 2000) at 30°C. *Streptomyces* were grown in NMMP for Rothera tests and RNA isolations. SFM was used for *S. coelicolor*/*E. coli* conjugations and spore collection. For selection of *E. coli*, antibiotics were used at the following concentrations: ampicillin (100 μg/ml), apramycin (50 μg/ml), chloramphenicol (25 μg/ml), hygromycin (75 μg/ml), and kanamycin (50 μg/ml). For selection of *S. coelicolor*, apramycin (50 μg/ml) and hygromycin (75 μg/ml) were used. In conjugations with *S. coelicolor*, nalidixic acid (20 μg/ml) was used to counterselect *E. coli*.

### Construction of SCO6704 null mutant

*S. coelicolor* SCO6704 was replaced with an apramycin resistance cassette, *apr*, using polymerase chain reaction (PCR)-targeted mutagenesis (Gust et al. 2003). The *apr* resistance cassette was extended via Expand High Fidelity PCR (Roche) using SCO6704 KO FOR and SCO6704 KO REV primers (Table 2). The extended resistance cassette was introduced into *E. coli* BW25113/pIJ790 containing the St4C6 cosmid. Arabinose was added to induce expression of the λ RED recombinase. The resulting recombinant cosmid, St4C6 ΔSCO6704::*apr*, was transformed into ET12576/pUZ8002, a nonmethylating strain of *E. coli*, and introduced into wild-type *S. coelicolor* A3(2) M600 through conjugation as previously described (Jones et al. 1997), yielding *S. coelicolor* B760. Double cross-over exconjugants were selected by apramycin resistance and kanamycin sensitivity. The disruption of SCO6704 was confirmed via PCR on isolated genomic DNA from *S. coelicolor* B760 using the primers SCO6704 KO Det FOR and SCO6704 KO Det REV (Table 2). Phenotypic analysis was performed using reverse transcription-PCR (RT-PCR) and the Rothera test.

### Complementation of SCO6704 null mutant

A 1,145-bp fragment was excised from cosmid St4C6 containing the SCO6704 ORF and 294 bp upstream of the SCO6704 translational start site by restriction digestion with BlpI and TfiI. The fragment was treated with DNA polymerase I, Large (Klenow) Fragment (New England Biolabs). The blunt-ended fragment was then ligated into the SmaI site of pBluescript KS+ to yield pJS337. The fragment containing the SCO6704 locus was excised and ligated into pMS81 using EcoRV and SpeI restriction digestion to yield pJS339. pMS81 is a site-specific integrating vector that inserts into the ΦBT1 *attB* site of *S. coelicolor* (Gregory et al. 2003). pJS339 was transformed into ET12576/pUZ8002 and introduced into *S. coelicolor* via conjugation, yielding *S. coelicolor* B761 ΔSCO6704::*apr* pMS81-SCO6704 (Jones et al. 1997). Exconjugants were selected by hygromycin resistance. Phenotypic analysis of *S. coelicolor* B761 was accomplished through RT-PCR and the Rothera test.

### Transcriptional analysis of *pca* genes

Wild-type *S. coelicolor* M600, ΔSCO6704::*apr*, and ΔSCO6704::*apr* pMS81-SCO6704 shaken liquid cultures

**Table 1** Bacterial strains and plasmids used for this study

| Strain or Plasmid | Description | Source/Reference |
|---|---|---|
| Strains | | |
| *S. coelicolor* A3(2) | | |
| M600 | Prototroph SCP1-, SCP2- | (Kieser et al. 2000) |
| B760 | M600 ΔSCO6704::*apr* | This study |
| B761 | M600 ΔSCO6704::*apr*- pJS339 | This study |
| *S. viridosporus* | | |
| ATCC 39115 | | |
| *E. coli* | | |
| DH5α | F- ϕ80*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk-) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ- | Invitrogen |
| BW25113 | Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-4*) *lacIp-4000*(lacI$^{Q}$), λ-, *rpoS*369(Am), *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514 | (Datsenko and Wanner 2000) |
| ET12567 | *dam, dcm, hsdS, cat, tet* | (Gust et al. 2003) |
| Plasmids | | |
| pIJ790 | *[oriR101], [repA101(ts)], araBp-gam-bet-exo*, Chl$^{r}$ | (Gust et al. 2003) |
| pUZ8002 | RP4 Derivative, OriT$^{-}$, Kan$^{r}$ | (Gust et al. 2003) |
| pGem-T Easy | pUC-derived, *lacZ*, Amp$^{r}$ | Promega |
| pJS335 | *pcaH* cDNA from RT-PCR with *S. viridosporus* RNA using *pcaH* RT-PCR primers, cloned into pGEM-T Easy, Amp$^{r}$ | This study |
| pJS336 | *pcaI* cDNA from RT-PCR with *S. viridosporus* RNA using *pcaI* RT-PCR primers, cloned into pGEM-T Easy, Amp$^{r}$ | This study |
| pBluescript KS+ | pUC *ori*, MCS, Amp$^{r}$ | Agilent Technologies (Stratagene) |
| pJS337 | 1145 bp BlpI and TfiI fragment from cosmid St4C6 containing SCO6704 with 294 upstream bp in pBluescript KS+, Amp$^{r}$ | This study |
| pJS338 | 6704 fragment from PCR with *S. viridosporus* genomic DNA using SCO6704 PCR primers, cloned into pBluescript KS+, Amp$^{r}$ | This study |
| pMS81 | *OriT*, ΦBT1 *attB-int*, Hyg$^{r}$ | (Gregory et al. 2003) |
| pJS339 | 1145 bp BlpI and TfiI fragment from cosmid St4C6 containing SCO6704 with 294 upstream bp in pMS81, Hyg$^{r}$ | This study |

Resistance markers; kanamycin (Kan), chloramphenicol (Chl), ampicillin (Amp), hygromycin (Hyg)

were grown for 14 h (midexponential phase) after which either protocatechuate, *p*-hydroxybenzoate, or ethanol as a negative control were added. *S. viridosporus* shaken liquid cultures (30 mL) were grown in NMMP for 60 h followed by the addition of protocatechuate. The protocatechuate and *p*-hydroxybenzoate used in the experiments were dissolved in ethanol. The inducing concentration of the aromatic compounds was 2 mM. After a 1 h induction period, a 1.5 mL aliquot of cells from each culture was obtained. The cells were washed once with 10.3% (*w*/*v*) sucrose solution followed by the addition of 100 μL of 10 mg/mL lysozyme solution (50 mM Tris–HCl, 1 mM EDTA, pH 8.0). The cells were incubated at 37°C for 15 min. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The One Step RT-PCR Kit (Qiagen) was used for all RT-PCR reactions following the manufacturer's protocol. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer. A 215-bp complementary DNA (cDNA) corresponding to the *pcaI* (SCO6703) transcript and a 314-bp cDNA corresponding to the *pcaH* (SCO6700) transcript were detected with *pcaI* RT-PCR primers and *pcaH* RT-PCR primers, respectively. A 486-bp cDNA corresponding to the *hrdB* (SCO5820) transcript, encoding a vegetative sigma factor, was detected with *hrdB* RT-PCR primers, as a positive control. All primer sequences are listed in Table 2. The PCR program used for detection of transcripts in *S. coelicolor* was 50°C for 30 min, 95°C for 15 min, 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, followed by an elongation time of 10 min at 72°C. The *S. coelicolor* RT-PCR primers for *pcaH*, *pcaI*, and *hrdB* were used in the amplification of *S. viridosporus* cDNA. The PCR program used for detection of the *pcaH*, *pcaI*, and *hrdB* transcripts in *S. viridosporus* was the same, except the cycle number was increased to 30. The *S. coelicolor* specific primers were used in the reactions. Detection of possible contaminating DNA in RNA samples was accomplished by PCR with *Pfu* polymerase under the same conditions. No bands were

**Table 2** Oligonucleotides used for this study

| Primer Name | Application/Function | Sequence |
|---|---|---|
| SCO6704 KO FOR | PCR-targeting/disruption of SCO6704 | 5′-TCAGTGCACCTACGAAAGTGACCACCGGGAGTG CACATGACTAGTATTCCGGGGATCCGTCGACC-3′ |
| SCO6704 KO REV | PCR-targeting/disruption of SCO6704 | 5′-GGCCGGGTTGCGGAGCCCCTCCGCGGCGTCCG CGACCCGACTAGTTGTAGGCTGGAGCTGCTTC-3′ |
| SCO6704 KO Det FOR | Verification of SCO6704 disruption | 5′-GTGAGGCGTTCTCGGATG-3′ |
| SCO6704 KO Det REV | Verification of SCO6704 disruption | 5′-GGACGGCACGACCTACAC-3′ |
| SCO6704 PCR 1 FOR | Sequencing of *S. viridosporus* 6704 fragment | 5′-TGCTGTGGAACACGATGG-3′ |
| SCO6704 PCR 1 REV | Sequencing of *S. viridosporus* 6704 fragment | 5′-CGGATCAGGTCGAAGAAC-3′ |
| *pcaI* RT-PCR FOR | Detection of the *pcaI* (SCO6703) transcript | 5′-GAGCGGTGTGCCGAAC-3′ |
| *pcaI* RT-PCR REV | Detection of the *pcaI* (SCO6703) transcript | 5′-GGGGATCATCTCCACCTC-3′ |
| *pcaH* RT-PCR FOR | Detection of the *pcaH* (SCO6700) transcript | 5′-ACGGCTTCTACCGCTTCAC-3′ |
| *pcaH* RT-PCR REV | Detection of the *pcaH* (SCO6700) transcript | 5′-GTCCTTCTTCGATCCAGGTG-3′ |
| *hrdB* RT-PCR FOR | Detection of the *hrdB* (SCO5820) transcript | 5′-CTCGAGGAAGAGGGTGTGAC-3′ |
| *hrdB* RT-PCR REV | Detection of the *hrdB* (SCO5820) transcript | 5′-TGCCGATCTGCTTGAGGTAG-3′ |

observed indicating that all RT-PCR products correspond to the amplification of RNA transcripts.

### Assays for β-ketoadipate formation

β-Ketoadipate formation was assessed using the Rothera test, a colorimetric assay. *S. coelicolor* shaken liquid cultures (30 mL) were grown in NMMP at 30°C for 14 h followed by the addition of 2 mM protocatechuate for induction. *S. viridosporus* shaken liquid cultures (30 mL) were grown in NMMP for 60 h followed by the addition of protocatechuate. Three hours after induction, 10 mL of the cultures were harvested, treated with 1 mL of 10 mg/mL lysozyme solution (50 mM Tris–HCl, 1 mM EDTA, pH 8.0), and incubated at 37°C for 1 h. A modified Rothera test was performed on these lysates (Ottow and Zolg 1974; Stanier et al. 1966). To the lysates was added 2.5 mL of 0.02 M Tris buffer (pH 8.0), 2.0 mL of 4 mM protocatechuate (in $dH_20$), and 0.5 mL of toluene. Samples were incubated while shaking at 30°C for 18 h. After incubation, 1.0 g ammonium sulfate, 100 μL of aqueous 2% solution sodium nitroprusside, and 0.5 mL of concentrated ammonia were added. The development of a purple color, indicating the presence of the *ortho* pathway intermediate β-ketoadipate, after 5 min was considered a positive test (Ottow and Zolg 1974; Stanier et al. 1966).

### Sequencing of *pcaH*, *pcaI*, and a SCO6704 homolog in *S. viridosporus*

*S. coelicolor* primers specific for *pcaH*, *pcaI*, and SCO6704 were used to amplify homologous genes in *S. viridosporus* genomic DNA and RNA. A 336-bp PCR product was amplified using *S. viridosporus* genomic DNA as template with SCO6704 PCR 1 FOR and SCO6704 PCR 1 REV and the *Pfu* polymerase (Table 2). The fragment was cloned into pBluescript KS+ to yield pJS338. cDNAs corresponding to fragments of *S. coelicolor pcaI* and *pcaH* were amplified via RT-PCR (as described previously) from RNA isolated from *S. viridosporus*, using *pcaI* RT-PCR primers and *pcaH* RT-PCR primers (Table 2). The cDNAs were gel-purified and cloned into pGEM-T Easy to yield pJS335 and pJS336, respectively. All plasmids were sequenced by Davis Sequencing (Davis, California).

### Accession numbers

The aforementioned partial sequences of the *S. coelicolor pcaV*, *pcaI*, and *pcaH* orthologs identified in *S. viridosporus* ATCC 39115 were submitted to GenBank. The accession numbers for these genes are GU180197, GU180198, and GU180199, respectively.

## Results

### *S. coelicolor* has a gene cluster encoding enzymes of the β-ketoadipate pathway

Bioinformatic analysis of the *S. coelicolor* genome sequence revealed the presence of a cluster of genes (SCO6697–SCO6703) encoding enzymes that are homologous to those of the protocatechuate branch of the β-ketoadipate pathway (Bentley et al. 2002; http://streptomyces.org.uk; Fig. 1b). Divergently transcribed from the structural genes is a putative MarR family transcription factor (SCO6704). *S. coelicolor* has the homologs of the *pca* genes, yet it is not

capable of depolymerizing lignin. The seven structural genes in the *pca* cluster encode enzymes that are necessary and sufficient for the conversion of protocatechuate to acetyl coenzyme A and succinyl coenzyme A (Fig. 1a). The structure of the gene cluster suggests that the seven genes are co-transcribed from a single promoter as a polycistronic message. All of the *pca* genes are transcribed from the same strand with a small number of, if any, intervening base pairs. The divergently transcribed gene (SC06704) was deemed a likely regulator of the *pca* gene cluster.

## Lignin-derived aromatic compounds induce transcription of the *pca* gene cluster in *S. coelicolor*

It was reported that production of enzymes from the β-ketoadipate pathway in *Streptomyces* sp. strain 2065 is induced by *p*-hydroxybenzoate and vanillate (Iwagami et al. 2000). By analogy, we hypothesized that the transcription of the *pca* gene cluster in *S. coelicolor* would be induced by *p*-hydroxybenzoate and by protocatechuate. Initially, RT-PCR was used to determine if transcription of the genes is inducible. Indeed, we found that transcription of the *pcaH* and *pcaI* genes were only detected in shaken liquid cultures of *S. coelicolor* to which either protocatechuate or *p*-hydroxybenzoate were added (Fig. 2). The level of induction could not be assessed as semi-quantitative RT-PCR was used. In any case, the aromatic compound-dependent transcription of both the *pcaI* and *pcaH* genes (the first and fourth genes in the cluster) is suggestive of co-regulated transcription of all seven genes in the *pca* gene cluster as a polycistronic message.

While both *p*-hydroxybenzoate and protocatechuate are inducers, it is unclear if *p*-hydroxybenzoate is an inducer only because it is converted to protocatechuate by one or both putative hydroxylases (SCO3084 and SCO1308) encoded on the *S. coelicolor* chromosome (Bentley et al. 2002) (http://streptomyces.org.uk). Interestingly, we found that both genes are transcribed in the presence of *p*-hydroxybenzoate (data not shown).

## Biochemical assays for β-ketoadipate formation corroborate inducible expression of the *pca* genes

A biochemical assay for β-ketoadipate formation was used to corroborate the apparent aromatic compound-dependent transcription of the *pca* genes. Specifically, we used the Rothera test to detect β-ketoadipate, the key intermediate in the catabolism of protocatechuate (Ottow and Zolg 1974; Stanier et al. 1966). The Rothera test is a colorimetric assay in which sodium nitroprusside and ammonia specifically react with ketones to yield a visibly purple product. Given its specific reactivity with ketones, the Rothera test has been extensively used in studies of aromatic catabolism to detect β-ketoadipate (Ottow and Zolg 1974; Stanier et al. 1966; Iwagami et al. 2000). In this case, formation of the purple color is indicative of the *ortho*-cleavage of protocatechuate (Ottow and Zolg 1974; Stanier et al. 1966). In these experiments, the inducers (protocatechuate and *p*-hydroxybenzoate) were added to *S. coelicolor* cultures, and after 3 hours, the corresponding cell lysates were tested for the presence of enzymes that convert protocatechuate into β-ketoadipate using the Rothera test. Indeed, cell lysates from *S. coelicolor* cultures induced with protocatechuate gave positive Rothera tests, while cell lysates from cultures that were not induced gave negative Rothera tests (Table 3). The immediate formation of a purple color in the Rothera test indicated that *ortho*-cleavage, and not *meta*-cleavage, of protocatechuate occurred. Furthermore, the results of the biochemical experiments validate the inducible *pcaH* and *pcaI* gene transcription observed in the RT-PCR experiments (Fig. 2).

## Transcription of the gene of the *pca* gene cluster is negatively regulated by SCO6704

Adjacent to the *pca* structural genes is a divergently transcribed gene (SCO6704) encoding a putative MarR family transcription factor. Based on the annotation and its proximity to the structural genes, we hypothesized that the SCO6704 gene regulates expression of the *pca* gene cluster. To test our hypothesis, we constructed a SCO6704 null mutant using a PCR-targeting procedure in which the gene was replaced by an apramycin resistance marker (Gust et al. 2003), yielding *S. coelicolor* B760 ΔSCO6704::*apr*. Interestingly, the cell lysates of the null strain grown in the absence and presence of protocatechuate yielded positive Rothera tests (Table 3). Moreover, the *pcaH* and *pcaI* genes were constitutively transcribed in the SCO6704 null mutant grown in the absence and presence of protocatechuate (Fig. 3). The deregulated transcription of

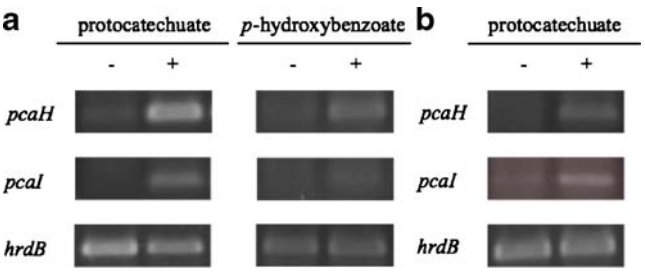


**Fig. 2** Transcriptional responses of *S. coelicolor* M600 (**a**) and *S. viridosporus* ATCC 39115 (**b**) to protocatechuate and *p*-hydroxybenzoate. RT-PCR was used to detect the transcription of *pcaH* and *pcaI*. The *hrdB* control transcript was detected in all of the RNA samples. The *hrdB* gene, which encodes a vegetative sigma factor, is constitutively transcribed (Kieser et al. 2000)

**Table 3** Biochemical assays for conversion of protocatechuate into β-ketoadipate

| Strain | Induced with Protocatechuate (2mM) | Rothera Test Result |
|---|---|---|
| WT *S. coelicolor* M600 | − | − |
| WT *S. coelicolor* M600 | + | + |
| *S. coelicolor* B760 ΔSCO6704::*apr* | − | + |
| *S. coelicolor* B760 ΔSCO6704::*apr* | + | + |
| *S. coelicolor* B761 ΔSCO6704::*apr*- pJS339 | − | − |
| *S. coelicolor* B761 ΔSCO6704::*apr*-pJS339 | + | + |
| *S. viridosporus* ATCC 39115 | − | − |
| *S. viridosporus* ATCC 39115 | + | + |

The characteristic purple color of the sample was deemed to be indicative of the presence of β-ketoadipate (a positive Rothera test). All reactions were performed in quadruplicate

these genes suggests that the product of the SCO6704 represses transcription of the *pca* genes. To confirm that disruption of the SCO6704 gene was necessary and sufficient for deregulation of *pca* transcription, a complementation strain was constructed. A derivative of the integrative plasmid, pMS81 (Gregory et al. 2003), harboring a fragment containing the SCO6704 open reading frame with >200 bp of flanking DNA, was introduced into the null strain, yielding *S. coelicolor* B761 ΔSCO6704::*apr*- pJS339. The phenotype of the SCO6704 null mutant was suppressed by complementation. The complementation strain *S. coelicolor* B761 exhibited fully inducible transcription of *pcaH* and *pcaI* (Fig. 3). Moreover, *S. coelicolor* B761 only gave a positive Rothera test when grown in the presence of protocatechuate. The phenotype of the null strain suggests that the SCO6704 gene encodes a repressor of *pca* gene transcription.

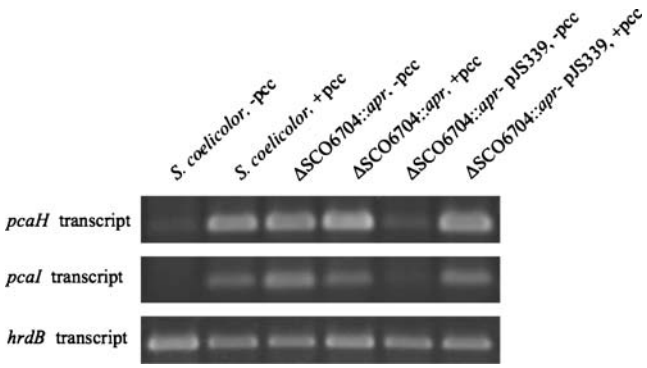


**Fig. 3** Transcription profile of *pca* gene expression in wild-type *S. coelicolor* and mutant strains. RT-PCR was used to detect the transcription of *pcaH* and *pcaI*. The *hrdB* control transcript was detected in all of the RNA samples. The *hrdB* gene, which encodes a vegetative sigma factor, is constitutively transcribed (Kieser r. 2000). The *pcaH* and *pcaI* genes are transcribed in wild-type *S. coelicolor* M600 cultures grown in NMMP media supplemented with protocatechuate (pcc). The *pcaH* and *pcaI* genes are transcribed independently of pcc in the null strain, *S. coelicolor* B760 ΔSCO6704::*apr*. This transcriptional defect was suppressed in the complementation strain, *S. coelicolor* B761 ΔSCO6704::*apr*- pJS339

## A ligninolytic *Streptomyces* species is capable of catabolizing lignin-derived aromatics

*S. viridosporus* ATCC 39115 is the best characterized ligninolytic streptomycete (Crawford et al. 1983; Pometto and Crawford, 1986). Based on the detection of the *pcaHG* genes in *S. viridosporus* by Southern blotting (Iwagami et al. 2000), it was hypothesized that this organism had a functional gene cluster encoding enzymes of the protocatechuate branch of the β-ketoadipate pathway. To test this hypothesis, we used RT-PCR to determine if transcripts corresponding to the *pcaH* and *pcaI* genes were present in RNA isolated from *S. viridosporus*. Because the sequences of the *pca* genes in this species were not available, primers specific for the *pcaH* and *pcaI* genes from *S. coelicolor* were used. We amplified cDNA products corresponding to *pcaH* and *pcaI* using RNA isolated from *S. viridosporus* grown as shaken liquid cultures in the presence of protocatechuate as template (Fig. 2). Cloning and sequencing of the cDNAs from the RT-PCRs revealed that their nucleotide sequences were more than 92% identical to the *S. coelicolor* *pcaH* and *pcaI* genes. We did not amplify cDNA products by RT-PCR using RNA isolated from *S. viridosporus* grown in the absence of protocatechuate as template (Fig. 2). These results are suggestive of inducible transcription of the *pca* genes, as was the case for *S. coelicolor*. Based on the inducible transcription of the genes, we sought out an ortholog of the *S. coelicolor* SCO6704 gene in *S. viridosporus*. Using primers specific for the SCO6704 gene, we successfully amplified an ortholog from the genomic DNA of *S. viridosporus* whose product is 99% identical in amino acid sequence to the product of SCO6704.

Consistent with inducible transcription of the *pca* genes were the results of Rothera tests of *S. viridosporus* grown as shaken liquid cultures in the presence and absence of protocatechuate. Positive Rothera tests were only observed

in *S. viridosporus* cultures that had been induced with protocatechuate (Table 3).

## Discussion

It is estimated that polymeric lignin represents 25% of the land-based biomass on Earth (Harwood and Parales 1996). Although lignin is a huge reservoir of carbon, most of it goes unused. Harnessing the catabolic capabilities of microorganisms to degrade lignin could enable the conversion of this abundant form of biomass into high value chemicals or fuels (Crawford 1981; Crawford et al. 1984). Given the potential of bioconversion of lignin, there has been much interest in the metabolic pathways that depolymerize lignin and catabolize the resulting low molecular weight aromatics compounds. A number of microbial enzymes that depolymerize lignin have been identified and characterized (Martinez et al. 2004). Enzymatic depolymerization of lignin is catalyzed by extracellular, oxidative metalloenzymes (Martinez et al. 2004). The simple aromatic compounds resulting from lignin depolymerization are intracellularly catabolized mostly via the enzymes of the β-ketoadipate pathway. Enzymes from the β-ketoadipate pathway are widely distributed among soil-dwelling bacteria and fungi. Decades of painstaking research have yielded a great deal of information about the mechanisms of enzymes of this pathway (Harwood and Parales 1996).

Exploitation of microorganisms for the bioconversion of lignin and lignin-derived aromatic compounds requires an understanding of how the catabolic pathways are regulated. Regulation of the β-ketoadipate pathway has been extensively studied (Harwood and Parales 1996). In many cases, the expression of β-ketoadipate pathway genes is induced by aromatic compounds or by β-ketoadipate (Harwood and Parales 1996). Typically, there is a transcriptional factor that positively or negatively regulates gene transcription. In *A. baylyi*, there is a transcriptional activator called PcaU that upregulates transcription of the *pca* genes in the presence of aromatic compounds (Gerischer et al. 2008; Siehler et al. 2007). *C. glutamicum* has a transcription factor encoded by *pcaR* that negatively regulates the expression of *pca* structural genes in the absence of aromatic compounds (Brinkrolf et al. 2006). Similarly, the *pca* gene cluster in *Agrobacterium tumefaciens* is under the control of a transcriptional repressor encoded by the *pcaU* gene (Parke 1995). While it has been reported that synthesis of the first enzyme in the β-ketoadipate pathway is induced in the presence of aromatic compounds in *Streptomyces* species strain 2065, the genetic basis of *pca* structural gene regulation in *Streptomyces* bacteria has not been investigated. For the first time, we demonstrate that transcription of these genes is inducible in both the non-ligninolytic species *S. coelicolor* and in the ligninolytic species *S. viridosporus*. Biochemical assays of β-ketoadipate formation in the presence of aromatic compounds corroborate the induced transcriptional response.

In the case of *S. coelicolor*, we ascribe the regulation of the *pca* structural genes to a putative MarR family transcription factor encoded by SCO6704 (Iwagami et al. 2000). Constitutive expression of the *pca* structural genes in the SCO6704 null strain suggests that the gene encodes a transcriptional repressor. It is noteworthy that a homolog of this gene was detected in the ligninolytic strain *S. viridosporus*, which also exhibits inducible transcription of the *pca* genes. Interestingly, the *S. coelicolor* SCO6704 gene product is distinct from PcaR (an IclR family member) that negatively regulates *pca* gene transcription in *C. glutamicum*, a close relative of *Streptomyces* bacteria (Bentley et al. 2002; Brinkrolf et al. 2006). To the best of our knowledge, this is the first time that a MarR family transcription factor has been implicated in the regulation of *pca* genes (Gerischer 2002). Most regulators of *pca* structural genes are in either the LysR or the IclR families (Gerischer 2002). However, there are precedents for MarR-type transcription factors as regulators of other pathways for aromatic catabolism where mostly they act as repressors (Tropel and van der Meer 2004). Based on its apparent function as a transcriptional repressor, we suggest that SCO6704 be named *pcaV* (Gerischer 2002; Tropel and van der Meer 2004). Orthologs of *pcaV* can be found in other streptomycete genomes. *S. avermitilis* has an ortholog of *pcaV* divergently transcribed from its *pcaI* ortholog (Omura et al. 2001). Since the sequence of the *pca* gene locus in *S. viridosporus* is not known, the location of its *pcaV* ortholog can only be speculated.

The findings reported here provide further evidence that *Streptomyces* bacteria may be useful in the utilization and bioconversion of lignin and lignin-derived aromatic compounds in biotechnological applications. They also suggest that upregulation of the biochemical pathways for aromatic catabolism can be achieved via deletion of a transcriptional repressor.

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