Characterization of a vanillic acid non-oxidative decarboxylation gene cluster from *Streptomyces* sp. D7

Kevin T. Chow, Margaret K. Pope and Julian Davies

Author for correspondence: Julian Davies. Tel: +1 604 221 8896. Fax: +1 604 221 8881. e-mail: jed@unixg.ubc.ca

Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z3

The genetics of non-oxidative decarboxylation of aromatic acids are poorly understood in both prokaryotes and eukaryotes. Although such reactions have been observed in numerous micro-organisms acting on a variety of substrates, the genes encoding enzymes responsible for these processes have not, to our knowledge, been reported in the literature. Here, the isolation of a streptomycete from soil (*Streptomyces* sp. D7) which efficiently converts 4-hydroxy-3-methoxybenzoic acid (vanillic acid) to 2-methoxyphenol (guaiacol) is described. Protein two-dimensional gel analysis revealed that several proteins were synthesized in response to vanillic acid. One of these was characterized by partial amino-terminal sequencing, leading to the cloning of a gene cluster from a genomic DNA lambda phage library, consisting of three ORFs, **vdcB** (602 bp), **vdcC** (1424 bp) and **vdcD** (239 bp). Protein sequence comparisons suggest that the product of **vdcB** (201 aa) is similar to phenylacrylate decarboxylase of yeast; the putative products of **vdcC** (475 aa) and **vdcD** (80 aa) are similar to hypothetical proteins of unknown function from various micro-organisms, and are found in a similar cluster in *Bacillus subtilis*. Northern blot analysis revealed the synthesis of a 2.5 kb mRNA transcript in vanillic-acid-induced cells, suggesting that the cluster is under the control of a single inducible promoter. Expression of the entire **vdc** gene cluster in *Streptomyces lividans* 1326 as a heterologous host resulted in that strain acquiring the ability to decarboxylate vanillic acid to guaiacol non-oxidatively. Both *Streptomyces* sp. strain D7 and recombinant *S. lividans* 1326 expressing the **vdc** gene cluster do not, however, decarboxylate structurally similar aromatic acids, suggesting that the system is specific for vanillic acid. This catabolic system may be useful as a component for pathway engineering research focused towards the production of valuable chemicals from forestry and agricultural by-products.

Keywords: actinomycetes, *Streptomyces*, vanillic acid, biotransformation, enzyme

INTRODUCTION

Chemical manufacture of benzenoid compounds from petroleum relies on abiotic, chemical catalysts. The use of petroleum poses a number of problems, as it is a non-renewable resource, a geopolitically volatile commodity, and a source of many environmentally toxic compounds. Therefore, there is growing interest in developing processes for enzymic conversion of renewable resources for the production of chemicals traditionally derived from petroleum. One such resource is lignin, which is the second most abundant structure from plant biomass (after cellulose). Lignin contains numerous phenyl-methylethers, which many micro-organisms have evolved the ability to degrade. Micro-organisms exhibiting such enzymic biotransformation potential could be harnessed for industrial use to supplement or replace traditional chemical synthesis methods (Frost & Draths, 1995).

Vanillic acid is an abundant component of solubilized...
lignin biomass, and degradative mechanisms by which this compound is catabolized have been elucidated for several prokaryotic organisms. The vanA and vanB genes responsible for vanillic acid demethylation have been cloned and sequenced from *Pseudomonas* sp. strain ATCC 19151 (Brunel & Davison, 1988), *Acinetobacter* sp. ADP1 (A. Segura & N. L. Ornston, accession no. AF009672) and, most recently, *Sphingomonas paucimobilis* (Nishikawa et al., 1998). In these organisms, vanillic acid is converted to protocatechuic acid by the enzyme vanillate demethylase; subsequently the protocatechuic acid is converted to protocatechuate by *Streptomyces* sp. strain D7 decarboxylates vanillic acid to **Fig. 1.** Catabolism of vanillic acid by *Streptomyces* sp. D7 yields guaiacol.

Huang et al., 1993; Santha et al., 1995; He & Wiegel, 1995, 1996; Zeida et al., 1998), this is, to our knowledge, the first report of the gene sequences associated with such processes. Further study of this system should allow these genes to be incorporated into metabolically engineered micro-organisms for the production of industrially useful chemical products such as guaiacol, catechol and adipic acid.

**METHODS**

**Soil sampling and strain isolation.** Soil samples were taken from various sites around the University of British Columbia campus as well as from several industrial sites. Soil samples were air-dried for several days, and 1 g of each sample was resuspended in 4 ml sterile distilled water. Each sample was vortexed for 10 s and then allowed to settle for approximately 15 min to remove large particulate matter. Each sample was diluted 10<sup>−2</sup>, and 0.1 ml of each diluted sample was spread on ISP4 agar (Difco) plates containing 75 µg cycloheximide ml<sup>−1</sup> (Sigma) to suppress fungal growth. Plates were incubated overnight at 30 °C lid-side up to allow liquid to soak into the agar before being inverted and incubated for an additional 4–7 d. After incubation, sporulating colonies presumed to be streptomycetes were picked with sterile toothpicks and cultivated on ISP4 plates. Confirmation of genus identity was performed by sequencing of a 505 bp 16S rDNA fragment produced using streptomycete-specific PCR primers. The primers consisted of the following sequences: forward, 5′-GAGATTGTGATCCTGCTCAG-3′; reverse, 5′-CGGACTGGTTGTTAGCACTTC-3′. Thermocycling was performed as follows: 1 min denaturation at 95 °C, 2 min annealing at 55 °C and 2 min extension at 72 °C. The cycle was repeated 30 times, with a final extension of 10 min at 72 °C.

**Isolate catabolic-screening procedures.** Screening for the catabolism of aromatic acids was carried out on agar minimal medium containing aromatic acid (0–3 g l<sup>−1</sup>), trace elements, phosphate buffer and the pH indicator bromothymol blue, pH 7.2 (Grund et al., 1990). At pH values 7.2 and below, the indicator appears green to yellow, while at pH values above 7.2 the indicator is blue. Catabolism of the aromatic acid being tested results in decreased acidity in the medium; this rise in pH can be scored visually by a change of the medium colour from green (at pH 7.2) to blue (at pH >7.2). Isolates that caused the medium to turn blue were also observed to grow well in the presence of the aromatic acid being tested. Organisms that expressed agarase also grew on assay plates, but did not cause a colour change in the medium. Isolates which were positive in the bromothymol blue plate assay were grown in liquid minimal medium in the presence of the appropriate aromatic acid, and culture supernatants sampled during time-course studies were analysed by UV spectrophotometry to monitor substrate disappearance. Decrease in absorbance at the wavelength for the particular aromatic acid being studied (e.g. 250 nm for vanillic acid) as compared to abiotic controls was evidence that the aromatic compound tested was being transformed or specifically degraded.

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are shown in Table 1. *Streptomyces* sp. D7 was isolated from forest soil on the University of British Columbia campus, as described above. Subcloning of *Streptomyces* DNA was performed in *Escherichia coli* DH5α with pUC19. Expression studies of the vdc genes in *Streptomyces lividans* 1326 were performed using pJK680.

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### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH5α MCR</td>
<td>Host for pUC19 and derivatives</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td><em>Streptomyces sp. D7</em></td>
<td>Wild-type vanillate decarboxylase isolate</td>
<td>This study</td>
</tr>
<tr>
<td><em>Streptomyces lividans 1326</em></td>
<td>Wild-type <em>Streptomyces</em> heterologous expression host</td>
<td>John Innes Collection, Norwich</td>
</tr>
<tr>
<td><strong>Plasmid vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>2.7 kb Ap’ <em>E. coli</em> cloning vector</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>pIJ702</td>
<td>7.2 kb Ts’ <em>Streptomyces</em> cloning vector with mel promoter</td>
<td>Katz et al. (1983)/Terragen Diversity, Inc.</td>
</tr>
<tr>
<td>pIJ680</td>
<td>5.3 kb Ts’ <em>Streptomyces</em> expression vector with aph promoter</td>
<td>Hopwood et al. (1985)/Dr L. Sandercock, UBC Biotechnology Laboratory</td>
</tr>
<tr>
<td>pKCE1</td>
<td>pUC19 carrying 4.4 kb <em>Streptomyces</em> sp. D7 BamHI genomic DNA fragment with vdc gene cluster</td>
<td>This study</td>
</tr>
<tr>
<td>pKCS1</td>
<td>pIJ702 carrying 4.4 kb BamHI insert from pKCE1 inserted in same orientation as mel promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pKCS2</td>
<td>pIJ702 carrying 4.4 kb BamHI insert from pKCE1 inserted in opposite orientation as mel promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pKCS3</td>
<td>pIJ680 carrying PCR-amplified vdc gene cluster inserted downstream of aph promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

(Hopwood et al., 1985), a vector that places target genes under the control of the aminoglycoside phosphotransferase (*aph*) constitutive promoter, and pIJ702 (Katz et al., 1983), which provides the weaker constitutive tyrosinase (*mel*) promoter. *S. lividans* 1326 was converted to protoplasts and transformed according to published methods (Bibb et al., 1978; Thompson et al., 1982). Protoplasts were plated on R5 solid medium (Thompson et al., 1980) and allowed to regenerate for 14 h before transformants were selected by an overlay of soft nutrient agar containing thiostrepton to achieve a final concentration of 50 µg thioestrepton ml⁻¹ per plate. Chromosomal DNA was extracted from *Streptomyces* strains by the method of Fisher (Hopwood et al., 1985). *Streptomyces* plasmids were isolated by the alkaline lysis method (Kieser, 1984) and *E. coli* plasmid DNA was routinely isolated using the Qiaprep Spin miniprep kit (Qiagen) or the NucleoSpin miniprep kit (Clontech) for sequencing and routine manipulations.

**Media and growth conditions.** *Streptomyces* sp. D7 and *S. lividans* 1326 were routinely cultivated in tryptic soy broth (TSB) or on mannitol soy flour agar plates at 30 °C. Catabolic tests and growth experiments were performed using mineral salts medium supplemented with 0.5% yeast extract [MSMYE: (NH₄)₂SO₄, 0.1 g l⁻¹; NaCl, 0.1 g l⁻¹; MgSO₄·7H₂O, 0.2 g l⁻¹; CaCl₂, 0.01 g l⁻¹; yeast extract, 0.5 g l⁻¹; K₂HPO₄, 1.0 g l⁻¹; KH₂PO₄, 0.5 g l⁻¹; pH 7.2] and aromatic compounds of interest at concentrations of 3.6–6 mM. When appropriate, thiostrepton at 50 µg ml⁻¹ was included for selection and maintenance of plasmid-containing strains. For DNA extraction or protoplast preparation, strains were cultivated in YEME (liquid medium) supplemented with 0.5% glucose and 5 mM MgCl₂ at 30 °C. *E. coli* DH5α was grown in Luria–Bertani (LB) medium (supplemented with 100 µg ampicillin ml⁻¹ when maintaining pUC-based plasmids) at 37 °C.

**Time-course analysis of gene expression.** To analyse gene expression during growth in the presence of aromatic acids, the following strategy was employed. Ten millilitres of MSMYE supplemented with 6 mM vanillic acid in a 50 ml baffled flask (Bellco) was inoculated with one colony of sporulating *Streptomyces* sp. D7 grown on ISP4 agar. The seed culture was incubated at 30 °C, 260 r.p.m., for approximately 21 h, at which time the cells were centrifuged, washed twice and then resuspended in 10 ml sterile distilled water. One millilitre of the seed suspension was used to inoculate 50 ml MSMYE in separate 250 ml baffled flasks. One flask was treated as a control, uninduced culture; the second flask was supplemented with 3.6 mM vanillic acid after early exponential growth was reached. Immediately prior to induction of the test culture, 1 ml of the control culture was removed and radiolabelled with 100 µCi (37 MBq) [³⁵S]methionine/cysteine (DuPont-NEN) at 30 °C for 30 min in a 15 ml polypropylene tube with shaking. After incubation, the labelled sample was pelleted, quick frozen in a dry ice ethanol bath and stored at −70 °C. One millilitre aliquots of both induced and uninduced cultures were labelled as above at 1, 2, 5, 12.5 and 18 h after addition of vanillic acid to the experimental culture. These frozen pellets were lysed (as
described in the next section) to obtain protein extracts for proteomic analysis by 2D-PAGE.

**Protein extraction and sample preparation.** Cell pellets were thawed on ice and washed twice in 2 ml lysis buffer [10 mM Tris/HCl pH 7.5, 5 mM EDTA, 100 µg PMSF ml\(^{-1}\) (Sigma), 1 µg pepstatin A ml\(^{-1}\), then resuspended in 0.5 ml of the same buffer. Samples were sonicated on ice, with a microtip set at medium power setting, in three bursts, 15 s each, with 15 s delays to prevent samples from overheating. Immediately after sonication, samples were centrifuged at 14000 \(g\) for 15 min at 4 °C. The pellet was discarded, and DNase/RNase solution (24 mM Tris base, 476 mM Tris/HCl, 50 mM MgCl\(_2\), 1 mg DNase I ml\(^{-1}\), 0.25 mg RNase A ml\(^{-1}\) was added to 10 \(^{-1}\) the total volume of each supernatant, followed by incubation for 10 min on ice. Total cell extracts (approx. 1 ml per time-point sample) were concentrated by ultrafiltration at a molecular mass cut-off of 10000 Da, then resuspended in 20 µl IEF sample buffer. In addition to concentrating the sample, the ultrafiltration step removed unincorporated \(^{35}\)S-labelled amino acids.

**Protein 2D-PAGE.** Protein two-dimensional (2D) gel electrophoresis was performed according to Garrels (1979) using the protocols, chemicals and equipment of the Investigator system (Genomic Solutions). First dimension IEF tube gels incorporated ampholytes in the pH range 3–10, optimized for analysis of total cell extracts. Second dimension high-tensile-strength slab gels contained 12.5 % acrylamide and an acrylamide to \(\text{N}{^\text{2}}\text{N}{^-}\text{methylene-bisacrylamide ratio of 30:0.65 (Duracryl, Genomic Solutions). Ten microliths of each sample containing} 1 \times 10^6 \text{c.p.m. was applied to each IEF tube and focused for} 18000 \text{V h}^{-1} (17.5 \text{h total run time). After second dimension electrophoresis, slab gels were agitated in fix solution (40 % methanol, 10 % glacial acetic acid) for 1 h, followed by treatment with a fluor solution (Enhance; DuPont-NEN), and then dried. Proteins were visualized by exposure of dried gels to Kodak Bio-Max MR film for 7–10 d at \(-70° \text{C).}

**Computer-aided analysis of 2D-PAGE gels.** Autoradiograms were scanned and analysed using PDQuest 2D analysis software version 5.0 (PD1) for 2D gels. Automated spot detection was performed using the PDQuest standard algorithms for \(^{35}\)S-labelled gels. Spot quantification was performed by computer-generated 2D Gaussian modelling. Gels were compared by a process of landmarking and matching spots among all gels in the experiment. A correction factor for each 2D gel protein spot was calculated as the total optical density units detected in the standard gel (a master reference gel created by merging all gels in a time-course matchset) divided by the total optical density units detected in the particular gel to which each protein spot belonged. The PDQuest gel analysis software was run on a SparcStation 5 (Sun Microsystems).

**Protein amino-terminal sequencing.** Soluble cell protein from a non-radio-labelled, vanillic-acid-induced sample was run in 10 replicate gels, each containing 150 µg protein, and stained post-electrophoretically by the zinc-imidazole method (Ortiz et al., 1992). The zinc-imidazole stain results in a white background with clear spots that correlate with protein locations. These protein spots are not fixed in the gel matrix. Protein spots of interest were cut from each gel and pooled for loading in a single 15-mm-thick well of a 12.5 % acrylamide slab gel. SDS-PAGE sample buffer (Laemmli, 1970) was added on top of the gel pieces in the well, and the protein was electrophoresed into the slab gel. The slab gel was blotted to Immobilon-P™ PVDF membrane (Millipore) using the semi-dry graphite blotter supplied with the Investigator 2D system. A three-buffer protocol was used according to the manufacturer’s instructions, in which \(\varepsilon\)-amino-n-caproic acid was substituted for glycine. The membrane was stained with a Coomassie blue R-250 solution for several seconds, destained in 40 % methanol, then washed with 18 MΩ cm\(^{-1}\) distilled water several times. The purified, blotted protein band was excised from the membrane and air-dried prior to amino-terminal sequencing by the Nucleic Acid Protein Sequencing (NAPS) Unit at the University of British Columbia.

**DNA sequencing and analysis.** Automated DNA sequencing was performed using the AmpliTaq PRISM kit (Applied Biosystems) with a standard thermocycling program provided by the manufacturer, with variations in the annealing temperature to match the melting temperature of the sequencing primer being used. Sequence reaction products were sent to the NAPS Unit at the University of British Columbia and electrophoresed on an ABI model 377 DNA sequencing apparatus (Applied Biosystems). Nucleic acid sequence was analysed by the Wisconsin Package Version 10 (Genetics Computer Group) on a Sun Microsystems SparcStation S.

**Library construction and gene cloning.** A Lambda DASH II (Stratagene) genomic DNA phage library of chromosomal *Streptomyces cacao* sp. D7 fragments was constructed by ligating 9–22 kb *SalI*AI partially digested chromosomal DNA fragments into the BamHI site of the phage arms. This library was screened by high-stringency hybridization at 60 °C with [\(^{32}\)P]ATP-labelled oligonucleotide probes derived from protein amino-terminal sequencing data. The *vdcB, vdcC* and *vdcD* genes were subcloned as a 4.4 kb BamHI fragment into pUC19 in *E. coli* DH5α MCR (Gibco-BRL).

**Chemical analytical methods.** Culture supernatants were filtered through 0.45 µm syringe filters and processed through a C-18 hydrophobic interaction column attached to a HPLC system (Hewlett Packard, model 1050). Conditions for separation were 30 % phosphoric acid/water, 70 % methanol, with a flow rate of 1 ml min\(^{-1}\). Retention times for vanillic acid and guaiacol under these conditions are 5 min and 4 min, respectively. Integrated peak areas corresponding to compounds in supernatant samples were calibrated against known concentrations of vanillic acid and guaiacol standards. Additional analysis of supernatant samples was performed using a Cary 1 Bio ultraviolet/visible spectrophotometer (Varian). For UV analysis, vanillic acid characteristically displays a primary absorbance at 230 nm and a secondary absorbance at 285 nm, while guaiacol absorbs at 275 nm.

**RNA isolation and transcript detection.** Total RNA was isolated from cells grown under two different conditions. Primary cultures were grown in 25 ml YEME for 48 h before cells were pelleted by centrifugation, washed twice with sterile water and resuspended in minimal media (MSMYE). For induced cultures, the media were supplemented with 3·6 mM vanillic acid, while no additional substrates were added to the uninduced control. These cultures were then allowed to grow an additional 3 h after which cells were pelleted and washed as before. Total RNA was isolated using standard RNA isolation techniques (Hopwood et al., 1985; Kirby et al., 1967). For transcript detection, Northern gel electrophoresis and transfer were performed according to the manufacturer’s recommendations for the NorthernMax kit (Ambion). Fifteen micrograms of total RNA was loaded on the gel and 1 µg of an RNA standard ladder (NEB) was included for size comparison. After electrophoresis was complete, the RNA ladder lane was excised and stained with ethidium bromide to allow for visualization and to confirm RNA integrity. Transcript was
Table 2. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length (nt)</th>
<th>Sequence (5' → 3')</th>
<th>Purpose</th>
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<tr>
<td>vdcC.F</td>
<td>20</td>
<td>GGCGACGCCGCCTGAAGTCC</td>
<td>Forward amplification of vdcC gene for Northern blot probe</td>
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<tr>
<td>vdcC.R</td>
<td>20</td>
<td>GGGTCCGGTCGGTGTCAGACG</td>
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<td>padx.F</td>
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<td>Forward amplification of vdcB gene for Northern blot probe</td>
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<td>padx.R</td>
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<tr>
<td>vdcBCD.F</td>
<td>26</td>
<td>CGGATCCAGTGACAGGTTTGAGGTGG</td>
<td>Forward amplification of vdc gene cluster for expression in pIJ680</td>
</tr>
<tr>
<td>vdcBCD.R</td>
<td>28</td>
<td>AGTCTAGACCAGCGGTCGGAGGTGACC</td>
<td>Reverse amplification of vdc gene cluster for expression in pIJ680</td>
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</table>

detected using a probe specific for the vdcC gene. To generate the probe, traditional double-stranded PCR was performed on pKCE1 using oligonucleotides vdcC.F and vdcC.R (Table 2). Following amplification, excess dNTPs and oligonucleotides were removed using a QIAquick PCR Purification kit (Qiagen). This product was then used as template for asymmetric PCR with only the vdcC.R primer. This resulted in a single-stranded PCR product that was complementary to the predicted RNA transcript. During chain elongation, [$^{32}$P]dCTP was provided in place of dCTP in the dNTP mix to allow for direct incorporation of radiolabel. The extension product was purified and allowed to hybridize with the immobilized RNA at 60°C for 24 h. Excess probe was removed by washing as directed by the NorthernMax protocol, and the hybridizing transcript was visualized by exposure to film for autoradiography. The membrane was then stripped and rehybridized using a vdcB-specific single-stranded PCR product generated in a similar manner, using the padx.F and padx.R primers (Table 2).

Enzyme assays. Late-exponential-phase mycelia harvested from YEME cultures were washed with phosphate buffer (10 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT) and resuspended in the same buffer, containing protease inhibitors (100 µg PMSF ml⁻¹, 1 µg pepstatin A ml⁻¹), at a ratio of 0.1 ml buffer per 1 ml original culture. Samples were sonicated as previously described and centrifuged at 12,000 g for 15 min to remove insoluble cell debris. Soluble cell extracts were tested for decarboxylase activity by adding vanillic acid or comparative substrates to a final concentration of 1 mM. Samples were incubated for 15 min at 25°C, at which time they were analysed by scanning the UV range from 300 nm to 200 nm. Soluble cell extract without substrate was used as a background in the reference cuvette. To test enzyme activity under anaerobic conditions, nitrogen gas was slowly bubbled through assay sample tubes prior to addition of substrate.

Reagents and enzymes. All reagents used were of the highest quality, and purchased from Sigma unless noted otherwise. Restriction endonucleases and other modification enzymes were obtained from Gibco-BRL, New England Biolabs, Boehringer Mannheim or Pharmacia.

RESULTS

Isolation and characterization of Streptomyces sp. D7

Although a natural substrate, vanillic acid did not support efficient growth as a sole source of carbon for the majority of soil isolates that we screened. In fact, the compound appeared to be toxic in some cases at the levels used in selection media (3–6 mM). Of 70 soil isolates tested for vanillic acid utilization, only 10% showed noticeable growth on minimal medium agar. Streptomyces sp. D7 was isolated from forest soil sampled from the campus of the University of British Columbia in Vancouver, Canada. On ISP4 agar medium the organism formed colonies that consisted of dark grey substrate and aerial mycelium with lighter grey spores. The organism secreted a yellow pigment when grown in MSMYE liquid medium and during growth on ISP4 agar, but the pigment appeared red on mannitol soy agar, possibly due to pH effects.

Streptomyces sp. D7 conversion of vanillic acid to guaiacol

When mycelia of Streptomyces sp. D7 were grown in the presence of vanillic acid, a strong odour characteristic of guaiacol was apparent several hours after inoculation. This biotransformation was confirmed by spectroscopic and chromatographic analysis of culture supernatants as described in Methods. By HPLC analysis of culture supernatants, it was observed that vanillic acid was
Decarboxylation of vanillic acid (■) to guaiacol (●) by *Streptomyces* sp. D7. Concentrations of aromatic compounds in culture supernatants were measured using HPLC and standard solutions of known concentrations.

Proteins synthesized by *Streptomyces* sp. D7 upon exposure to vanillic acid

2D-PAGE analysis of protein extracts from mycelia exposed to vanillic acid revealed a number of changes to the proteome of the organism. Most significantly, a protein of 52 kDa with an isoelectric point of 4.9 was synthesized in large amounts only in the presence of vanillic acid. The protein was most abundant during the early stages of vanillic acid catabolism, and was reproducibly expressed upon repeating the entire growth induction experiment four times (Fig. 3). The 52 kDa protein was catalogued as protein 3717 in our proteome database.

Cloning of the gene encoding protein 3717

Edman degradation sequencing of protein 3717 yielded the following amino acid data: AYDDLRYFLDTLEK-EGQLLRIT. This sequence matched well (70% similarity) with the amino-terminal sequence of p-hydroxybenzoate carboxylase from the anaerobe *Clostridium hydroxybenzoicum* (He & Wiegel, 1995; GenBank accession no. AAB34313). From the amino-terminal sequence of protein 3717, a 56-mer degenerate oligonucleotide of the sequence $5’$-$GT(CT)AGACCT(GC)TACCT(GC)GCAT(CT)GACAT(CT)GAGAAGGAGGG(GC)CAGCT(GC)CT’$ was hybridized against a Lambda DASH II phage library of *Streptomyces* sp. D7 genomic DNA. A phage clone carrying an approximately 13 kb genomic DNA insert hybridized strongly to the probe, and *BamHI* digestion products of this insert were subcloned into pUC19 for further manipulations and sequencing.

Sequence analysis revealed that the gene encoding protein 3717 was contained on a 4.4 kb *BamHI* genomic DNA fragment, and was determined to be the second gene in a cluster of at least three genes, designated vdcB (602 bp), vdcC (1424 bp) and vdcD (239 bp), as depicted in Fig. 4. **BlastX** sequence analyses (Altschul *et al.*, 1990) revealed that the gene cluster, in whole or in part, is
Non-oxidative decarboxylation by Streptomyces

Fig. 5. Dendrograms depicting the sequence-based relationships between proteins and hypothetical proteins similar to the product of the vdcB gene (a) and the vdcC gene (b). These dendrograms are derived from sequence alignments produced using the PILEUP program, which is part of the Wisconsin Package Version 10 bioinformatics software package (GCG).

present in a variety of micro-organisms. The vdcB translation product is highly similar to phenylacrylate decarboxylase (PAD) from Saccharomyces cerevisiae. The yeast PAD contains a putative membrane-binding domain close to the amino-terminus, which is highly conserved among other hypothetical PAD homologues from various micro-organisms, as revealed by genome projects. The vdcC translation product is also highly similar to hypothetical proteins from various microbial genome projects, in addition to the amino-terminal similarity to 4-hydroxybenzoate carboxy-lyase as revealed from the Edman degradation sequencing of protein 3717. Dendrograms of the vdcB and vdcC translation products in comparison to other microbial homologues are shown in Fig. 5. Unlike the first two genes in the cluster, the vdcD translation product shows similarity only to a hypothetical protein from Bacillus subtilis. Although these genes have homologues in a number of microbial genomes, they are not always clustered. These genes encode catabolic enzymes for the degradation of a number of organic chemicals.

Northern blot analysis

mRNA was isolated from Streptomyces sp. D7 under both uninduced and vanillic-acid-induced conditions, blotted to a positively charged nylon membrane, then probed separately with either 32P-labelled vdcC or vdcB PCR amplification products. The resulting autoradiograms revealed that a transcript of approximately 2.3 kb was synthesized in vanillic-acid-induced cells (data not shown). A transcript of this size corresponds to the expected combined length of all three vdc genes and their associated intergenic regions. This result indicates that the gene cluster may be transcribed from a single, inducible promoter.

Expression of the gene cluster in S. lividans 1326

The 4.4 kb BamHI DNA fragment containing the vdcBCD gene cluster was inserted into the Streptomyces cloning vector pIJ702 at the unique BglII site. Insertion at this site places the cluster downstream of the mel promoter, thereby disrupting transcription of the tyrosine gene that serves as a colour selection marker for transformants. pIJ702 carrying the insert in the same orientation as the mel promoter was designated pKCS1; conversely, a vector construct with the insert in the opposite orientation to the promoter was designated pKCS2. S. lividans 1326 carrying pKCS1 acquired the ability to efficiently decarboxylate vanillic acid to
guaiacol, while *S. lividans* 1326 carrying pKCS2 produced extremely low amounts of guaiacol (Fig. 6). *S. lividans* 1326 wild-type cells did not decarboxylate vanillic acid. These results suggest that transcription of the genes required for vanillic acid decarboxylation by *S. lividans* 1326 carrying pKCS1 is being driven by the constitutive *mel* promoter. There is possibly a low level of transcription from a natural promoter, however, as observed for *S. lividans* 1326 carrying pKCS2, which contains the gene cluster in the opposite orientation from the *mel* promoter (Fig. 6b).

**Expression of the vdc gene cluster using the aph promoter**

pIJ680 (Hopwood *et al.*, 1985) is a derivative of pIJ702 that contains the *Streptomyces fradiae* aminoglycoside phosphotransferase (*aph*) constitutive promoter, and allows for high rates of transcription of genes inserted downstream of the promoter. The *vdc* genes were amplified by PCR using primers, vdcBCD.F and vdcBCD.R, that incorporated a *BamHI* site upstream and a *XbaI* site downstream of the ORF(s) (Table 2). A PCR product of the entire gene cluster was cloned into *BamHI* + *XbaI*-cut pIJ680, creating pKCS3; this plasmid was used to transform *S. lividans* 1326. The resulting recombinant *S. lividans* 1326 strain converted vanillic acid to guaiacol at the same rate as wild-type *Streptomyces* sp. D7. Sonicated cell extracts of *S. lividans* 1326 expressing the *vdc* system via pKCS3 catalysed decarboxylation of vanillic acid under both aerobic and anaerobic conditions. These results confirm the involvement of the cloned gene products in a non-oxidative system.

**Substrate specificity**

Sonicated cell extracts of *S. lividans* 1326(pKCS3) were used to test the specificity of the *vdc* system towards aromatic acids similar to vanillic acid. The following compounds were tested: 4-methoxy-3-hydroxybenzoate (isovanillic acid), 3,4-dimethoxybenzoate (veratrate), 3,4-dihydroxybenzoate (protocatechulate), 4-hydroxy-3,5-dimethoxybenzoate (syringate), 3,4,5-trihydroxybenzoate (gallate), 3-phenylpropenoate (trans-cinnamate). No decarboxylation of any of the substrates tested was observed, as assayed by UV spectrophotometry.

**DISCUSSION**

Although there are many reports of microbial non-oxidative decarboxylation of aromatic acids in the literature, only recently have enzyme purifications been successful, as these proteins appear to be unstable in cell-free extracts. Of the enzymes purified thus far, all share one common feature: they are all single polypeptides that form multi-subunit enzyme complexes. However, depending on substrate and organism, the molecular mass of the polypeptide, as well as the number of subunits, is variable. Several examples of aromatic acid non-oxidative decarboxylases and their subunit configurations are listed in Table 3. We have demonstrated that *Streptomyces* sp. D7 produces a protein of approximately 52 kDa when grown in the presence of vanillic acid, and suggest that two additional proteins, of 36 kDa and 9 kDa, are also synthesized. The functions of these proteins remain unknown, and one question that arises is: which gene, *vdcB* or *vdcC*, encodes the catalytic protein. The product of *vdcB* has primary amino acid sequence highly similar to phenylacrylate decarboxylase from yeast. In light of this functional implication, it may be possible that the *vdcB* product is the subunit of a multi-component enzyme complex, in a similar manner to those listed in Table 3. However, transcription of all three genes on a single mRNA molecule suggests that the products of *vdcC* and *vdcD* are also necessary for vanillic acid decarboxylation. In fact, experiments in which each of the *vdc* genes was expressed separately, or in combinations of two, under the control of the *aph* promoter in pIJ680 failed to produce the vanillic acid decarboxylating phenotype (data not shown). While *vdcD* encodes a protein that is

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**Fig. 6.** Decarboxylation of vanillic acid (■) to guaiacol (●) by recombinant *S. lividans* 1326 strains. (a) *S. lividans* 1326 carrying pKCS1; (b) *S. lividans* 1326 carrying pKCS2; (c) *S. lividans* 1326 wild-type.
likely too small to be the catalytic unit, we note that the amino-terminus of the vdcC product was highly similar to the limited amino acid sequence obtained from the purified p-hydroxybenzoate carboxy-lyase of C. hydroxybenzoicium. The Clostridium enzyme was purified and characterized, but only limited amino acid sequence was obtained (He & Wiegel, 1995). The enzyme is responsible for decarboxylation of p-hydroxybenzoate to phenol, a dead-end metabolite. With the exception of the amino acid sequence similarity between the vanillic-acid-induced protein of Streptomyces sp. D7 and the C. hydroxybenzoicium enzyme, it is difficult to speculate about the exact function(s) of the vdcC or vdcD gene products in the reaction. Neither polypeptide shows a relationship to any characterized enzyme in the databases.

The apparent high substrate specificity of the vdc system is perhaps not surprising in light of other non-oxidative decarboxylase studies. Most decarboxylases are, like gallate decarboxylase from Pantoea agglomerans T71, highly substrate specific (Zeida et al., 1998); however, p-hydroxybenzoate carboxy-lyase from C. hydroxybenzoicium is active against both p-hydroxybenzoate and protocatechuate (He & Wiegel, 1995). K. pneumoniae was biochemically demonstrated to produce a number of non-oxidative decarboxylases, each enzyme specific for a different aromatic acid substrate (Grant & Patel, 1969).

Microbial non-oxidative decarboxylase systems characterized in the literature (Grant & Patel, 1969; Yoshida & Yamada, 1982; Nakajima et al., 1992; Huang et al., 1993; Santhan et al., 1995; He & Wiegel, 1995, 1996; Zeida et al., 1998) have minimal or no cofactor requirements for activity. The vanillate decarboxylase system of Streptomyces sp. D7 is active in the absence of oxygen, and amino acid sequence analysis of all three vdc gene products failed to reveal any cofactor binding motifs, e.g. for NAD and FAD, characteristic of oxidative enzymes.

The distribution of homologues of the vdc genes throughout the microbial world, mostly on chromosomes, but also plasmid-borne as in the case of pNL1 in Sphingomonas aromaticivorans, suggests that these gene products provide useful metabolic abilities for their hosts. However, non-oxidative decarboxylation of most aromatic acids yields toxic phenolic compounds and, in many cases cited in the literature (e.g. He & Wiegel, 1996), the micro-organisms do not possess appropriate mechanisms to further degrade the compounds produced by these dead-end pathways. In this study, Streptomyces sp. D7 was able to rapidly convert vanillic acid to guaiacol, but was unable to further degrade the guaiacol, which is a toxic phenol. In another example, C. hydroxybenzoicium decarboxylated p-hydroxybenzoate to phenol, and protocatechuate to catechol, without further metabolism. The functions of these seemingly toxic metabolic reactions of micro-organisms are not readily apparent; however, in natural ecosystems, it can be imagined that other organisms in a consortium would mineralize and remove these toxins from the environment. For example, Streptomyces setonii 75Vi2 (Pometto et al., 1981; Sutherland, 1986) and a Moraxella sp. (Sterjiades et al., 1982) demethylate methoxylated aromatic compounds such as guaiacol to catechol, leading to subsequent mineralization. Evidence implicating cytochrome P-450 systems was provided in both cases. S. setonii 75Vi2 was also observed to degrade phenol (Antai & Crawford, 1983). Indeed, there is a growing focus on biodegradation, not from the standpoint of an individual micro-organism, but rather as a coordinated function of the entire gene pool (Wackett et al., 1999). Efficient biodegradation is therefore likely the result of natural consortiums of micro-organisms. The observation that Streptomyces sp. D7 converts vanillic acid to the toxic guaiacol but is unable to remove the guaiacol from its environment appears to support this view.

The proteomics analysis presented in this work demonstrates that genes encoding proteins linked to vanillic acid decarboxylation are induced by vanillic acid itself. This is supported by the Northern blot data, indicating that the vdc gene cluster is transcribed in one polycistronic mRNA product upon induction by vanillic acid. These results suggest that transcription of the gene cluster is under tight regulatory control. In fact, preliminary nucleotide sequence analysis upstream of the vdc gene cluster is highly indicative of a divergent regulatory gene. BLAST-X analysis of the region matched the translation product strongly to a putative positive

### Table 3. Variations in subunit size and configuration – characteristics of some microbial aromatic acid non-oxidative decarboxylases

<table>
<thead>
<tr>
<th>Decarboxylase enzyme</th>
<th>Organism</th>
<th>Configuration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxybenzoate</td>
<td>Clostridium hydroxybenzoicum</td>
<td>350 kDa (six subunits of 57 kDa)</td>
<td>He &amp; Wiegel (1995)</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate</td>
<td>Clostridium hydroxybenzoicum</td>
<td>270 kDa (five subunits of 57 kDa)</td>
<td>He &amp; Wiegel (1996)</td>
</tr>
<tr>
<td>4,5-Dihydroxyphthalate</td>
<td>Pseudomonas testosteroni</td>
<td>150 kDa (four subunits of 38 kDa)</td>
<td>Nakazawa &amp; Hayashi (1978)</td>
</tr>
<tr>
<td>4,5-Dihydroxyphthalate</td>
<td>Pseudomonas fluorescens</td>
<td>420 kDa (six subunits of 66 kDa)</td>
<td>Pujar &amp; Gibson (1985)</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoate</td>
<td>Aspergillus niger</td>
<td>120 kDa (four subunits of 28 kDa)</td>
<td>Kamath et al. (1987)</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoate</td>
<td>Trichosporon cutaneum</td>
<td>66 kDa (two subunits of 36-5 kDa)</td>
<td>Anderson &amp; Dagley (1981)</td>
</tr>
<tr>
<td>3,4,5-Trihydroxybenzoate</td>
<td>Pantoea agglomerans T71</td>
<td>320 kDa (six subunits of 57 kDa)</td>
<td>Zeida et al. (1998)</td>
</tr>
</tbody>
</table>
transcriptional activator from the *Streptomyces coelicolor* A3(2) genome.

Cloning the genes for vanillic acid non-oxidative decarboxylation expands our knowledge of this reaction. Non-oxidative decarboxylases represent “crosstalk” between the two major branches of aromatic acid catabolism, characterized by either catechol or protocatechuic acid decarboxylase from *Clostridium* and *Rhodotorula rubra*. It will be interesting to determine if the numerous sequence homologues of vanillic acid decarboxylase from *Streptomyces* sp. D7 from microbial genome databases are indeed other non-oxidative decarboxylases. Knowledge gained from such endeavours should ultimately allow this class of enzyme to be used for a variety of metabolic engineering applications.

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**REFERENCES**


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