PROTEIN PURIFICATION AND GENETIC CHARACTERIZATION OF A STREPTOMYCETE PROTOCATECHUATE 3,4-DIOXYGENASE

by

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ABSTRACT

A previously uncharacterized *Streptomyces* sp. isolate 2065 was found to degrade vanillic acid and *p*-hydroxybenzoic acid, utilizing these compounds as a sole carbon source. Induction of protocatechuate 3,4-dioxygenase (3,4-PCD [EC 1.13.11.3]), a ring-cleavage dioxygenase, when the strain was grown in the presence of vanillic acid or *p*-hydroxybenzoic acid was observed, indicating that this streptomycete isolate catabolizes both these lignin model compounds through the protocatechuic acid branch of the β-ketoadipate pathway. The 3,4-PCD was purified from cells grown in the presence of *p*-hydroxybenzoic acid. Two proteins, the α- and β-heterologous subunits of 3,4-PCD, were observed in approximately equimolar amounts on denaturing SDS-PAGE. The α- and β-subunits were found to correlate to ring fission activity. N-terminal protein sequence information was obtained, from which DNA oligonucleotides were designed and used in amplification of an 800-bp PCR product from isolate 2065 genomic DNA. The protein sequence of this 800-bp DNA fragment was found to be similar to the amino acid sequence for PcaH, the β-subunit for 3,4-PCD. A bacteriophage λ genomic library of 2065 was constructed and screened using the PCR product as a streptomycete *pcaH* gene probe; a 4.5-kb DNA fragment containing the structural genes for the α- and β-subunits of a protocatechuate 3,4-dioxygenase was cloned and the *pcaG* and *pcaH* genes were sequenced. The *pcaG* and *pcaH* genes encode 201 and 257 amino acid polypeptides with predicted sizes of 21,763 Da and 29,262 Da, respectively. The *pcaGH* genes and their predicted protein sequences were found to be similar to those from *Burkholderia cepacia*, *Rhodococcus opacus, Pseudomonas putida*, and *Acinetobacter calcoaceticus*.

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ABBREVIATIONS AND SYMBOLS

A	adenine
Ala	alanine
Arg	arginine
Asn	asparagine
benzoic acid	benzenecarboxylic acid
bp	basepair
catechol	1,2-dihydroxybenzene
cinnamic acid	3-phenyl-2-propenoic acid
C	cytosine
cm	centimeter
C-terminal	carboxy terminal
p-coumaric acid	3-(4-hydroxyphenyl)-2-propenoic acid
DIG-11-dUTP	digoxygenin-11-deoxyuracil triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
ferulic acid	3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid
g	gravity
G	guanine
gentisate	2,5-dihydroxybenzoic acid
Gln	glutamine
Gly	glycine
guaiacol	2-methoxyphenol
His	histidine
p-hydroxybenzoic acid	4-hydroxybenzoic acid
Ig	immunoglobulin
Ile	isoleucine
ISP	International *Streptomyces* Project
isovanillic acid	3-hydroxy-4-methoxybenzoic acid
kDa	kilodaltons
λ	wavelength
λ_{max}	maximum wavelength
Leu	leucine
µg	microgram
µM	micromolar
µl	microliter
M	molar
ml	milliliter
mM	millimolar
N-terminal	amino terminal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
Phe	phenylalanine
PMSF	phenylmethyl sulfonyl fluoride

Pro	proline
protocatechuic acid	3,4-dihydroxybenzoic acid
p.s.i	pounds per square inch
PVDF	poly(vinylidene difluoride)
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulfate
Ser	serine
T	thymine
Tiron	4,5-dihydroxy-*m*-benzenedisulfonic acid
Tris-HCl	Tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tyr	tyrosine
U	units
Val	valine
vanillic acid	4-hydroxy-3-methoxybenzoic acid
vanillin	4-hydroxy-3-methoxybenzaldehyde
veratric acid	3,4-dimethoxybenzoic acid

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I would like to thank my supervisor Julian Davies for all the guidance and support he has given me throughout my graduate studies. I will never forget all the patience and understanding he has shown me; from him I have learned invaluable lessons about life as well as research. Thanks to all the current and past members of the Davies Laboratory for their useful advice, especially Kevin Chow for his help and guidance throughout the gestation of my thesis and Rumi Asano for her friendship. Keqian Yang assisted with the DNA cloning and sequencing work and Chris Radomski from Terragen Diversity, Inc. provided DNA sequencing consultation and helped with 16s rDNA sequencing. A final thanks to my thesis committee members William Mohn and J. Thomas Beatty for all their constructive criticism.

DEDICATION

This thesis is dedicated in loving memory of my late husband Stephen Christopher Hayward. He always provided me with all the encouragement I ever needed; he was my greatest advocate and my best friend. I will always remember his strength, his courage, and his spirit for life.

INTRODUCTION

Streptomyces is a large genus of Gram-positive, filamentous eubacteria that is important both ecologically and medically. This genus of bacteria has been well studied for their ability to produce secondary metabolites particularly antibiotics, while their biodegradative properties have not been thoroughly investigated. Despite this *Streptomyces* spp. are known to play a major role in mineralization of many compounds that are resistant to decomposition including pectin, lignin, chitin, keratin, latex, and aromatic compounds. They are natural soil microorganisms and may constitute up to 20% of the culturable soil population (Prescott *et al.*, 1993), and their hyphal growth and saprophytic lifestyle allows them to penetrate through and gain access to plant tissues. Of their biodegradative properties, their ability to degrade lignin and low molecular weight aromatic compounds derived from lignin, called lignin model compounds, has received the most attention.

Lignin is a complex aromatic polymer and is second to cellulose as the most abundant organic compound on earth (Kirk & Farrell, 1987). There is interest in lignin biodegradation for potential use of ligninases in the pulp and paper industry to help soften, decolour, and/or delignify pulp without damaging the cellulose fibres, or to treat mill effluents (Crawford & Crawford, 1980; Wick, 1994). Lignin is considered a waste material in such industries and is therefore a potential source of aromatic compounds for use as feedstocks in biochemical transformation reactions (Crawford & Crawford, 1980; Glazer & Nikaido, 1995). Previous studies have indicated that *Streptomyces* spp. degrade lignin by a poorly understood primary metabolic process that results in modification and solubilization rather than significant depolymerization (Godden *et al.*, 1992). Crawford *et al.* (1983) have described in cultures of *Streptomyces* spp. grown on lignin production of carbon dioxide and soluble material consisting

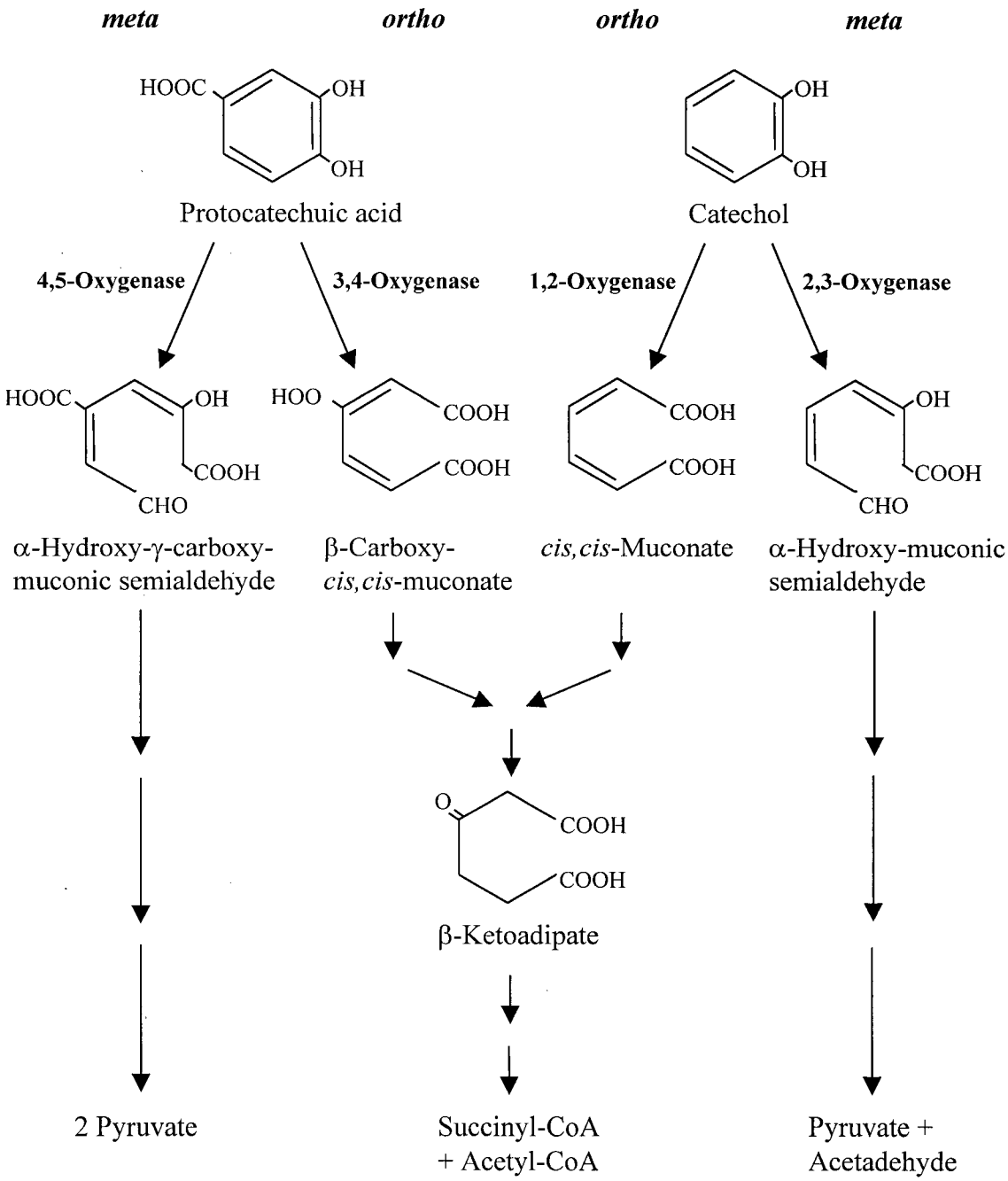

Figure 1 Oxidative aromatic ring-cleavage reactions. Different aromatic compounds are converted *via* various ring modification reactions to one of two central intermediates, which are ring-cleaved in order to be mineralized. Dioxygenases cleaving the central intermediate protocatechuic acid are on the left and those cleaving catechol are on the right. The intradiol or *ortho*-cleaving enzymes are protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenease while the extradiol or *meta*-cleaving enzymes are protocatechuate 4,5-dioxygenase or catechol 2,3-dioxygenase. The *ortho* ring-cleavage products are mineralized through the key intermediate in the pathway, β-ketoadipate, which is then converted to tricarboxylic acid cycle intermediates via a succinyl CoA:β-ketoadipate CoA transferase and a β-ketoadipate CoA thiolase.

of single ring aromatic compounds and a water soluble, acid precipitatable polymeric lignin (APPL). It has been suggested that lignolysis by actinomycetes such as *Streptomyces* spp. is a result of a coordinated multienzymic process (Giroux *et al.*, 1988) potentially involving many enzyme activities including peroxidases, esterases, oxygenases, demethylases, endogluconases, and cellulases. In the natural environment, the complete mineralization of lignin is presumably a complex process which involves a consortium of microorganisms. Fungi are thought to be most actively involved in depolymerizing lignin; the eventual breakdown products are simple aromatic compounds, which can be catabolized by bacteria through better characterized aromatic hydrocarbon degradation pathways (Wackett *et al.*, 1999). *Streptomyces* spp. are known to oxidatively catabolize these lignin breakdown products and are thought to play a significant role in the recycling of plant-derived aromatic compounds in soil (Godden *et al.*, 1992).

The oxidative metabolism of aromatic hydrocarbons such as lignin model compounds has been well studied in microorganisms, particularly in Gram-negative bacteria such as *Pseudomonas*, *Acinetobacter*, and *Ralstonia* (previously *Alcaligenes*) (Cain, 1980; Harayama & Timmis, 1992). Lignin model compounds and other aromatic compounds are catabolized through what are known as the upper pathways, in which they are converted through a series of ring modification reactions to one of two primary central intermediates, catechol or protocatechuic acid. The upper pathways meet the lower pathways as these central intermediates undergo aromatic ring fission by specific ring-cleaving dioxygenases. Catechol and protocatechuic acid can undergo two types of cleavage (Figure 1); either *ortho*, between the hydroxyl groups (intradiol cleavage), or *meta*, adjacent to one of the hydroxyls (extradiol cleavage). The subsequent reactions of the lower pathways convert the ring-cleaved product to tricarboxylic acid cycle intermediates. In *Streptomyces* the pathways for the mineralization of simple lignin model compounds such as

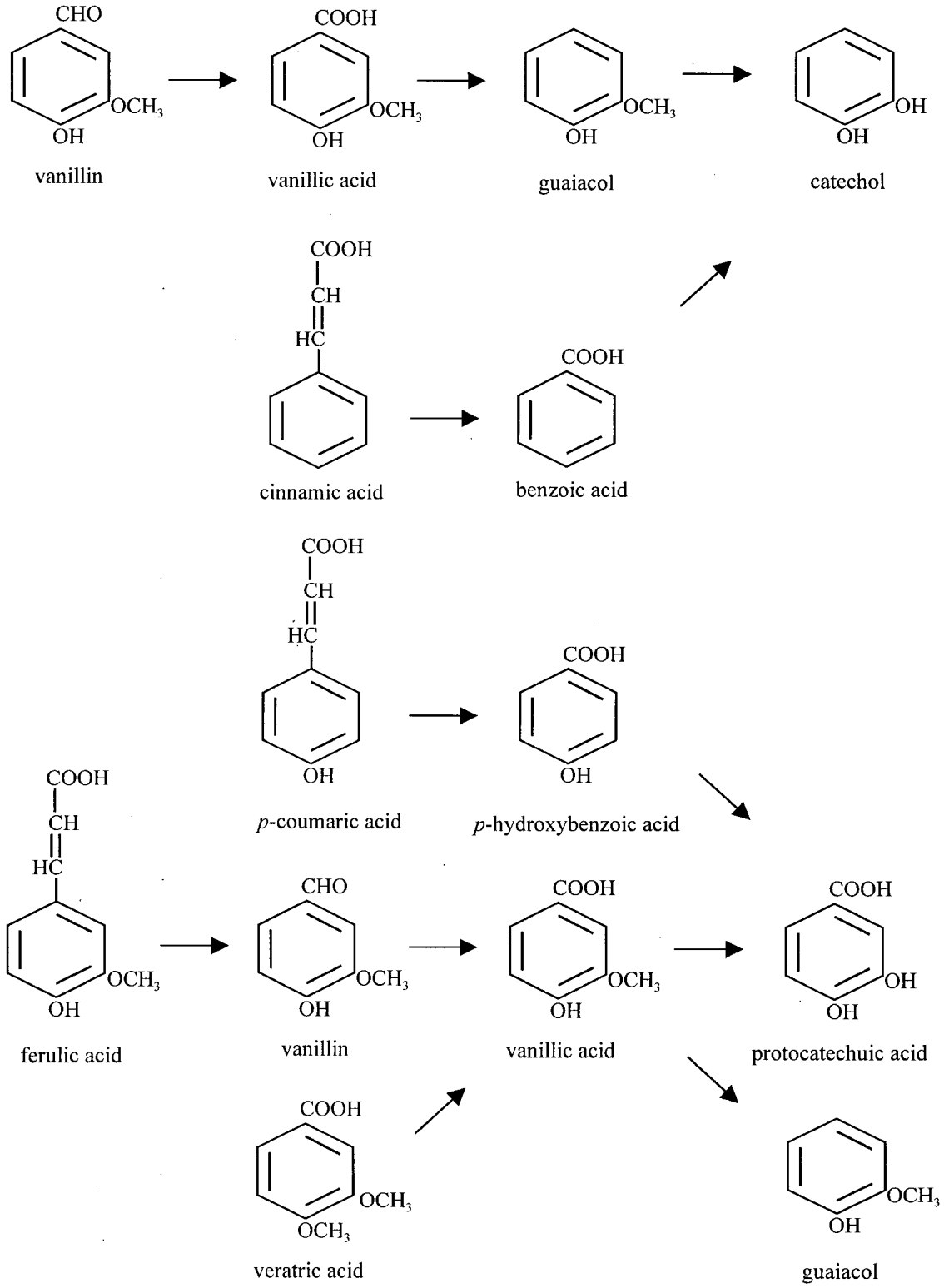

Figure 2 Aromatic hydrocarbon metabolism in *Streptomyces* spp. Each reaction shown has been found in one or more strains.

cinnamic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, vanillic acid, and veratric acid (Sutherland *et al.*, 1981; 1983) have been characterized biochemically, but little molecular biology is known about them (Figure 2). Benzoic acid has in all cases been seen to be metabolized *via* catechol and *p*-hydroxybenzoic acid *via* protocatechuate. *Streptomyces* sp. V7 metabolized veratric acid and *S. albus* metabolized vanillic acid *via* protocatechuic acid while *S. sioyanensis* and *Streptomyces* sp. strain 179 decarboxylated vanillic acid to guaiacol and did not metabolize it any further. *S. setonii* metabolized cinnamic acid and ferulic acid *via* catechol and *p*-coumaric acid *via* protocatechuic acid. *S. setonii* was observed to catabolize vanillic acid through a unique pathway *via* guaiacol and catechol when given vanillin as the starting substrate (Pometto *et al.*, 1981). In the degradation of these compounds induction of corresponding intradiol ring-cleavage enzymes was observed. Extradiol cleavage pathways have not been observed in *Streptomyces*, indicating that they may either not exist or are rare in this genus of bacteria (Grund *et al.*, 1990).

The intradiol or *ortho* ring-cleavage pathway is commonly known as the β-ketoadipate pathway, named after the key intermediate β-ketoadipate (3-oxoadipate) (Stanier & Ornston, 1973). This pathway is widely distributed among taxonomically diverse soil microorganisms, including both eubacteria and fungi, and is almost always chromosomally encoded. It is thought of as a "major utility pathway" because it plays a significant role in the processing and degradation of aromatic compounds derived from plant material and other sources found in soil (Harwood & Parales, 1996). The pathway consists of two branches, one starting at catechol (cat) and the other at protocatechuic acid (pca), these compounds are cleaved by catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, respectively (Figure 3). In bacteria the two branches of the pathway converge at the intermediate, β-ketoadipate enol-lactone. The β-ketoadipate pathway is

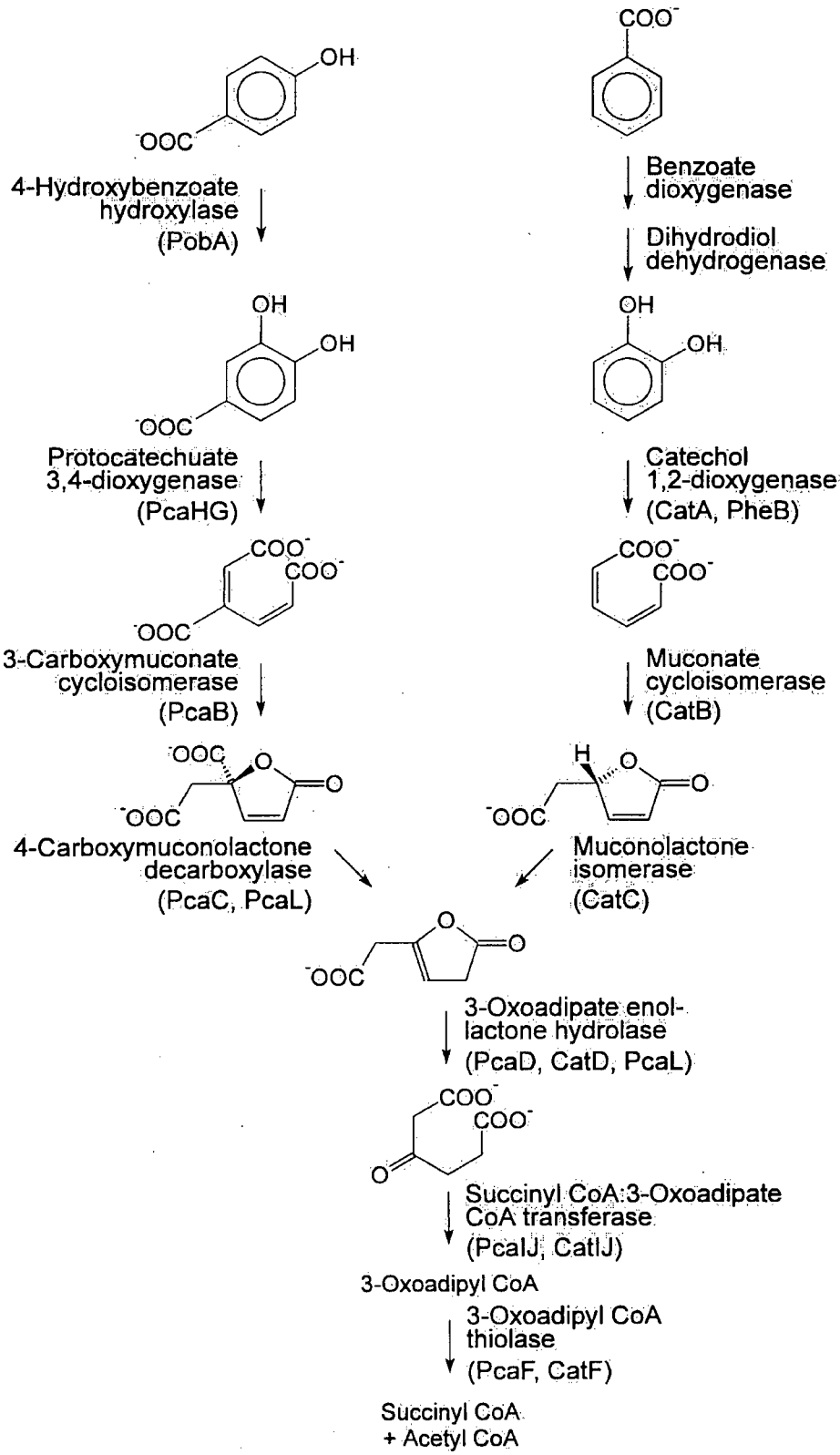

Figure 3 The protocatechuate and catechol branches of the β-ketoadipate pathway (Eulberg *et al.*, 1998). Gene products catalyzing reactions in the pathway are given in parentheses. For the reactions common to both branches, some bacteria possess only a single set of genes, while others have separate *pca* and *cat* genes for one or more of the steps.

biochemically conserved; the structural genes encoding enzymes of this pathway are homologous in widely differing bacterial species. Although the β-ketoadipate pathway has been found to coexist in bacteria with plasmid-encoded *meta* pathways, the presence or absence of the pathway has been used as a phenotypic trait to differentiate between bacterial species. For example, the solanacearum complex (rRNA group II) pseudomonads whose members tend to be plant and animal pathogens cleave protocatechuate by an *ortho* mechanism while the acidivorans complex (rRNA group III), which are mostly saprophytes, *meta* cleave this intermediate (Logan, 1994). All the genes of the β-ketoadipate pathway have been cloned and sequenced from *A. calcoaceticus* and *P. putida*, two organisms whose G+C contents differ by 20%, but amino acid sequence identities for isofunctional enzymes range from 45 to 68% (Harwood & Parales, 1996).

Diversity in the β-ketoadipate pathways has evolved in pathway branching, inducing metabolites, genetic organization, operon clustering, and regulation. Some organisms carry only portions of the pathway and in others the genetic pathway convergence points are different. In *P. putida* the cat and pca branches converge at β-ketoadipate enol-lactone (Ornston, 1966), in *R. eutropha* the point of convergence is β-ketoadipate (Johnson & Stanier, 1971), while *A. calcoaceticus* has two independent sets of genes that encode the final three steps of the pathway (Canovas & Stanier, 1967). Different pathway metabolite induction patterns are seen in different bacteria. For example, in the pca branch of the β-ketoadipate pathway protocatechuate and/or β-ketoadipate are the key effectors in most bacteria, while in *Agrobacterium* β-carboxy-*cis,cis*-muconate is the important inducer (Parke, 1997). The *cat* and *pca* genes are generally clustered but no particular gene order seems to be maintained from species to species. Close linkage of groups of operons referred to as supraoperonic (Wheelis & Stanier, 1970) or superoperonic (Morgan & Dean, 1985) clustering has been observed with operons encoding enzymes for related catabolic activities

contiguous on the chromosome. For example, the *cat* and *ben* genes and the *pca* and *pob* genes, the former encoding enzymes for the conversion of benzoate to catechol and the latter for the conversion of *p*-hydroxybenzoic acid to protocatechuic acid, have been found to be closely associated in some bacteria (Harwood & Parales, 1996). In *P. putida* the *cat* genes are positively regulated by CatR, a LysR type of regulator, while PcaR, a member of the newly described PobR family of regulatory proteins, activates *pca* gene expression (Rothmel *et al.*, 1990; Romero-Steiner *et al.*, 1994). In *A. calcoaceticus* the CatM protein is, a CatR homologue that positively regulates the *cat* genes, while PcaU activates *pca* gene expression (Romero-Arroyo *et al.*, 1995; Harwood & Parales, 1996). In *A. tumefaciens,* PcaQ (a LysR type of regulator) and a PcaR homologue are found in *pca* transcription activation (Parke, 1997). In *B. cepacia* the induction of the genes encoding protocatechuate 3,4-dioxygenase were thought to be under negative control (Zylstra *et al.*, 1989). This was suggested by the pattern of constitutive expression of the cloned genes in a heterologous host which was subject to catabolite repression.

Enzymes of the β-ketoadipate pathway contribute to the degradation of environmental pollutants. The modified *ortho*-cleavage pathway is used to degrade chlorocatechols generated from the metabolism of chlorinated aromatic acids. The genes for the modified *ortho*-cleavage pathway are plasmid encoded and require the presence of the β-ketoadipate pathway in the host strain to completely mineralize chlorocatechols. The catechol branch of the β-ketoadipate pathway is thought to be the evolutionary precursor of some of the enzymes of the modified *ortho*-cleavage pathway. By comparing amino acid sequences, chlorocatechol 1,2-dioxygenases were found to share identity with catechol 1,2-dioxygenases as well as protocatechuate 3,4-dioxygenases (Neidle *et al.*, 1988). Although a 3,4-dihydroxychlorobenzoic acid *ortho*-cleaving enzyme has not been found, two protocatechuate 3,4-dioxygenase isozymes that oxidize 4-sulfocatechol were

identified recently from two members of a sulfanilic acid (4-aminobenezenesulfonate) degrading, mixed culture of *Agrobacterium radiobacter* strain S2 and *Hydrogenophaga palleronii* strain S1 (Hammer *et al.*, 1996). No gene sequence for this enzyme was reported, but the broader substrate specificity suggests that it is a modified type of the classical protocatechuate 3,4-dioxygenase.

Protocatechuate 3,4-dioxygenase (3,4-PCD [EC 1.13.11.3]) is an essential enzyme in the catabolism of aromatic compounds *via* the pca branch of the β-ketoadipate pathway. 3,4-PCD opens the aromatic ring and incorporates two atoms of oxygen from O_2 into protocatechuic acid to form β-carboxy-*cis*,*cis*-muconic acid. 3,4-PCD usually consists of a heterodimer of two subunits, α and β, arranged in a $(\alpha\beta Fe^{3+})_{3\text{-}12}$ quaternary structure (Frazee *et al.*, 1993), the exception is *Rhizobium trifolii* strain TA1 which was reported to have a $(\alpha_2\beta_2Fe^{3+})_2$ quaternary structure (Chen *et al.*, 1984) (Table 1). The αβ protomer of 3,4-PCD contains a catalytic non-heme Fe^{3+} iron located at the interface between the two subunits ligated by two histidyl and two tyrosyl side chains within the catalytic β-subunit. The α-subunit forms part of the active site and plays a role in substrate binding. It is regarded as the prototypical Fe^{3+} catecholic dioxygenase due to the fact that it is the most extensively studied intradiol-cleaving dioxygenase. The crystal structure for the uncomplexed wildtype enzyme from *Pseudomonas putida* has been solved, as well as structures for the enzyme complexed with its substrate, various inhibitors, and substrate analogs (Ohlendorf *et al.*, 1988; 1994; Orville *et al.*, 1997a; 1997b). The α and β subunits share a unique core tertiary structure, an eight-stranded β barrel of mixed parallel and antiparallel sheets folded in half to yield two layers. Amino acid sequence similarity and structural similarity of the α- and β-subunits suggest that the protomer may have diverged from an ancestral homodimer with two active sites.

Table 1 Characteristics of protocatechuate 3,4-dioxygenases from different bacteria.

Organism	Native size (kDa)	subunit size (kDa)	quaternary structure	Reference
Pseudomonas putida ATCC 23975	587	α = 22.3 β = 26.6	$(\alpha\beta Fe^{3+})_{12}$	Fujisawa & Hayaishi, 1968
Acinetobacter calcoaceticus Strain BD413	770[a]	α = 23.4[b] β = 27.1[b]	$(\alpha\beta Fe^{3+})_{12}$[c]	Hou *et al.*, 1976
Burkholderia cepacia Strain DB01	200	α = 23.0 β = 26.5	$(\alpha\beta Fe^{3+})_4$	Bull & Ballou, 1981
Azotobacter vinelandii Strain OP	480	α = 22.3 β = 26.6	$(\alpha\beta Fe^{3+})_{10}$	Durham *et al.*, 1980
Brevibacterium fuscum ATCC 15993	315	α = 22.5 β = 40.0	$(\alpha\beta Fe^{3+})_5$	Whittaker *et al.*, 1984
Moraxella sp. (PCase-P) Strain GU2	220	α = 29.5 β = 25.5	$(\alpha\beta Fe^{3+})_4$	Sterjiades & Pelmont, 1989
Moraxella sp. (Pcase-G) Strain GU2	158	α = 29.5 β = 25.5	$(\alpha\beta Fe^{3+})_3$	Sterjiades & Pelmont, 1989
Thiobacillus sp. Strain A2	660	α = 21.0 β = NR	NR	Durham *et al.*, 1980
Rhizobium trifolii Strain TA1	220	α = 26.5 β = 29.0	$(\alpha_2\beta_2Fe^{3+})_2$	Chen *et al.*, 1984
Nocardia erythropolis Strain S-1	150	α = NR β = NR	NR	Kurane *et al.*, 1984
Agrobacterium radiobacter Strain S2(P34OI)	435	α = 24.0 β = 28.0	$(\alpha\beta Fe^{3+})_8$[c]	Hammer *et al.*, 1996
Agrobacterium radiobacter Strain S2(P34OII)	97.4	α = 23.0 β = 28.5	$(\alpha\beta Fe^{3+})_2$[c]	Hammer *et al.*, 1996
Hydrogenophaga palleronii Strain S1(P34OII)	97.5	α = 22.0 β = 31.0	$(\alpha\beta Fe^{3+})_2$[c]	Hammer *et al.*, 1996
Rhodococcus opacus Strain 1CP	NR	α = 22.9[b] β = 26.8[b]	NR	Eulberg *et al.*, 1998
Streptomyces sp. Isolate 2065	158	α = 21.8[b] β = 29.3[b]	$(\alpha\beta Fe^{3+})_3$[c]	This study

[a]Holoenzyme molecular weight was determined from enzyme cloned and expressed in *E. coli.*
[b]Subunit molecular weights were deduced from gene sequence.
[c]Quaternary structure and Fe^{3+} stoichiometry inferred from subunit and native enzyme molecular weights.
NR, not reported.

3,4-PCD has been isolated from a variety of bacteria with different subunit sizes and quaternary structures (Lipscomb & Orville, 1992) (Table 1). The general physical properties of these enzymes have been shown to be similar except for the 3,4-PCD from *Brevibacterium fuscum* whose kinetic parameters (K_m, V_{max}, and TN) are 5-50 fold higher (Whittaker *et al.*, 1984). Sequence information for the *pcaGH* genes has been obtained from *Burkholderia cepacia* (Zylstra *et al.*, 1989), *Acinetobacter calcoaceticus* (Hartnett *et al.*, 1990), *Pseudomonas putida* (Frazee *et al.*, 1993), and *Rhodococcus opacus* (Eulberg *et al.*, 1998). Protein sequence alignments indicate that the α- and β-subunits of 3,4-PCDs from these widely divergent bacteria have 22% and 29% identity, respectively. The enzymes from *P. putida* and *A. calcoaceticus* are particularly similar, with α- and β-subunit amino acid identities of 53% and 56%.

In this study, a *Streptomyces* sp. isolate 2065 was found to mineralize vanillic acid and *p*-hydroxybenzoic acid and utilize these compounds as a sole carbon source. Induction of synthesis of one of the key enzymes in the β-ketoadipate pathway, protocatechuate 3,4-dioxygenase, was observed in the presence of both these aromatic acids. This intradiol ring-cleaving dioxygenase has been purified and the *pcaGH* genes encoding it have been cloned and sequenced from this *Streptomyces* sp. isolate. This is the first report of a β-ketoadipate pathway gene cloned from a streptomycete. It is also only the second streptomycete aromatic ring-cleavage dioxygenase that has been isolated. Zaborina *et al.* (1995) purified and characterized a 6-chlorohydroxyquinol 1,2-dioxygenase from *S. rochei* 303, but they have not yet reported any gene sequence information. The information presented in this thesis will allow for further characterization of aromatic hydrocarbon catabolism in *Streptomyces* and how it may relate to the degradation of lignin and its breakdown products. In addition it will provide some insight into the evolution of the β-ketoadipate pathway and how this pathway is seen to develop its own set of characteristics

in widely differing organisms while maintaining protein sequence conservation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Streptomyces sp. isolate 2065 was obtained from B.C. Research among the MacMillan Bloedel Jump Creek collection of actinomycetes isolated from the bark of coastal British Columbia trees. Other bacterial strains used in this study were *Escherichia coli* DH5α and *E. coli* XL-1 Blue (P2) from Stratagene (La Jolla, CA). DNA genomic libraries were created utilizing the λ DASH II bacteriophage vector from Stratagene. Other plasmids used were pBluescript KS+ and the TA cloning vector from Stratagene.

Growth Conditions

Streptomycetes were routinely grown on BACTO International *Streptomyces* Project (ISP) Medium 4, an inorganic salts starch agar, soy mannitol agar, and tryptic soy agar (TSA) plates (DIFCO Laboratories Inc., Detroit, Mich.). Spores were resuspended and stored in sterile 20% glycerol at -20°C. For total DNA isolation the streptomycetes were grown in liquid yeast extract-malt extract medium (YEME) cultures, containing (per liter) 3 g yeast extract, 5 g peptone, 3 g malt extract, 10 g dextrose, 340 g sucrose, $MgCl_2 \cdot 6H_2O$ (2.5 M), and supplemented with 20% glycine (Hopwood *et al.*, 1985). For enzyme assays and protein purification, cells were first grown in rich medium to early to mid-log phase then washed in isotonic medium before being transferred to mineral salts medium with yeast extract (MSMYE), pH 7.2, containing (per liter) 0.1 g $(NH_4)_2SO_4$, 0.1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 0.5g yeast extract, 1.0 g K_2HPO_4, 0.5 g KH_2PO_4, and supplemented with 0.3% *p*-hydroxybenzoic acid or vanillic acid (Sigma Chemicals, Oakville, ON, Canada). Streptomycetes were incubated at 30°C and liquid cultures were grown in baffled flasks or those containing steel springs in a rotary shaker at 260 r.p.m. *E. coli* strains were grown on Luria-Bertani (LB) medium containing

appropriate antibiotics at 37°C.

Characterization of Actinomycete Isolates

Actinomycete isolates were screened for activity against various aromatic acids on 24-well culture plates containing minimal medium agar, trace elements, bromothymol blue, and aromatic acid (1.5-3 g/l) which was phosphate buffered to pH 7.2 (Crawford & Olsen, 1978). Degradation of the aromatic acid was indicated by an increase in pH which resulted in the media turning from green (at pH 7.2) to blue (greater than pH 7.2). Aromatic acids tested were: benzoic acid, cinnamic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, *p*-hydroxybenzoic acid, vanillic acid, isovanillic acid, and veratric acid. Isolates which were positive by this bromothymol blue plate assay were grown in liquid minimal medium in the presence of the aromatic acid of interest as sole carbon source. Culture supernatants were sampled over time and analyzed by UV/Vis spectrophotometry. Removal of the aromatic acid from culture was detected as a decrease in absorbance at the λ_{max} for the particular aromatic acid tested. TerraGen Diversity Inc. (Vancouver, BC, Canada) provided cell wall fatty acid methyl ester (FAME) analysis and 16s rDNA sequence analysis services.

Enzyme Assays

Rothera Reaction. The presence of intradiol ring-cleavage dioxygenase activity was identified colourimetrically in crude cell free extracts by a Rothera reaction (Stanier *et al.*, 1966). For this reaction, the method of Ottow & Zolg (1974) was followed where 2 ml crude cell extracts were incubated with 2 mM catechol or protocatechuic acid as substrate. In the absence of the development of a yellow colour indicative of a muconic acid semi-aldehyde (the extradiol cleavage product) the samples were incubated for 18 hours at 28°C; solid ammonium sulfate (1

g), concentrated ammonium hydroxide (0.5 ml), and 1 % sodium nitroprusside (5 drops) were then added. Development of a deep purple colour determined visually is indicative of the *ortho* pathway intermediate β-ketoadipate.

Spectrophotometric Assays. Protocatechuate 3,4-dioxygenase activity was measured spectrophotometrically as described by Stanier & Ingraham (1954) with slight modifications. The assay mixture contained 50 mM Tris-HCl, pH 8.5, an appropriate amount of enzyme (for example, 20-50 μg of crude cell free extract), and 160 μM protocatechuic acid in a total volume of 300 μl. The reaction was initiated by addition of substrate, and a decrease in absorbance at 290 nm at 25°C was recorded on a Varian-Cary 1 Bio UV/Vis spectrophotometer (Varian Canada Inc., Edmonton, AB, Canada). One unit of enzyme activity is defined as the amount that oxidizes protocatechuic acid at an initial rate of 1 μmol per minute. Reaction rates were calculated using an extinction coefficient of 2.3 $mM^{-1} \cdot cm^{-1}$ for the conversion of protocatechuic acid to β-carboxy-*cis,cis*-muconic acid. Specific activity was expressed as units per milligram of protein. Protocatechuate 4,5-dioxygenase was monitored by an increase of absorbance at 410 nm (Wheelis *et al.*, 1967). Catechol 2,3-dioxygenase activity was measured by an increase in absorbance at 375 nm (Kojima *et al.*, 1961). Catechol 1,2-dioxygenase activity was measured by increase in absorbance at 260 nm (Cain, 1966) and gentisate 1,2-dioxygenase activity was measured by increase in absorbance at 334 nm (Crawford *et al.*, 1975).

Expression and Protein Purification

Harvesting of Cells, Cell Disruption, and Preparation of Cell-Free Extract. Cells were harvested by centrifugation and washed with 50 mM Tris-HCl, pH 8.5, which will be referred to hereon as Buffer A. The cell paste was frozen at -20°C until further use. The following steps

were performed at 4°C unless otherwise noted. For enzyme assays using crude cell-free extracts from small scale cultures, cells from 50 ml cultures were harvested and resuspended in 2 ml of Buffer A with 1 mg/ml lysozyme, 100 μg/ml DNase I, 100 μg/ml RNase A, and 1 mM PMSF. The cell suspension was incubated at 37°C for 1 hour and then lysed by homogenization on ice with a tissue grinder; the extract was centrifuged at 25,000 x g for 5 minutes to remove cellular debris. For protein purification, cells were resuspended in Buffer A containing 100 μ/ml DNase I and 100 μ/ml RNase A, and 1 mM PMSF. The cells were disrupted with a single passage through a French press operated at 20,000 p.s.i. The cellular debris was removed by centrifugation at 8,000 x g for about 30 minutes.

FPLC Purification of Protein. Protocatechuate 3,4-dioxygenase was purified at room temperature by fast protein liquid chromatography (FPLC) on a system from Pharmacia Biotech Inc. (Baie d'Urfé, QC, Canada). Proteins eluting from chromatographic columns were detected spectrophotometrically at 280 nm. The crude cell free extract was first precipitated with $(NH_4)_2SO_4$ on ice. Protein which precipitated between 40-60% $(NH_4)_2SO_4$ was resuspended and dialyzed in Buffer A at 4°C and then batch purified on a 3 cm x 6 cm (diameter x height) chromatography column packed with Q-Sepharose Fast Flow resin (Pharmacia Biotech Inc.) equilibrated in same buffer. Protein eluting between 350 and 450 mM NaCl was concentrated, exchanged into Buffer A and passed through a 0.45 μm filter before being purified by FPLC. This preparation was chromatographed on a Pharmacia Mono Q HR 5/5 equilibrated in Buffer A and eluted with a 250 mM to 550 mM NaCl gradient in the same buffer. The active fractions were pooled, concentrated, and exchanged into Buffer A with a 1.7 M $(NH_4)_2SO_4$. This preparation was chromatographed on a Pharmacia Phenyl Superose HR5/5 column equilibrated in Buffer A with 1.7 M $(NH_4)_2SO_4$ and the dioxygenase was eluted with a 700 mM to 200 mM

$(NH_4)_2SO_4$ gradient. The active fractions were again pooled, exchanged into Buffer A with 100 mM NaCl and chromatographed through a Pharmacia Superose 6 HR 10/30 column equilibrated in the same buffer. These final active fractions were pooled, concentrated and stored at 4°C.

Native Molecular Weight Determination. For determination of native molecular weight, a Superose 6 column (Pharmacia) was equilibrated in Buffer A with 100 mM NaCl. High molecular weight gel filtration standards were purchased from GIBCO BRL (Burlington, ON, Canada), which contained thyroglobulin, IgG, ovalbumin, myoglobin, and vitamin B-12 as molecular weight markers.

Manipulation of Protein and Electrophoresis

Protocatechuate 3,4-dioxygenase was concentrated and exchanged into appropriate buffers using Centricon and Centriprep concentrators (Amicon, Bedford, MA). The concentration of protein in cell free extracts and in stages throughout the enzyme purification was measured by the bicinchoninic acid method (Smith *et al.*, 1985) using a kit from Sigma Chemicals Ltd. Bovine serum albumin was used for a preparation of a standard curve. SDS-PAGE was performed on a Bio-Rad Miniprotein II apparatus (Mississauga, ON, Canada) using a modified procedure of Laemmli (Ausubel *et al.*, 1991) using 13% polyacrylamide gels. Samples were boiled with SDS for 5 minutes and separated on gels. For native PAGE gels 10% polyacrylamide was used and electrophoresis solutions contained no SDS or denaturing agents. Gels were silver stained to visualize proteins. Coomassie blue R-250 staining was used only for blotted protein for N-terminal sequencing. Pre-stained protein molecular weight markers were purchased from GIBCO BRL.

N-terminal Protein Sequencing

For protein sequencing the standard procedure for SDS-PAGE was used (Ausubel *et al.*, 1991) with the following exceptions. All gel solutions, excluding the running buffer, were filtered through a 0.45 μm filter. Samples were solubilized with sucrose-containing sample buffer instead of urea and heated to 37°C for 10-15 minutes prior to electrophoresis. The gel was blotted onto Immobilon-P^{SQ} PVDF membrane (Millipore, Bedford, MA) using a Milliblot semi-dry graphite electroblotter from Millipore. A three-buffer protocol was used in which ε-amino-n-caproic acid was substituted for glycine according to manufacturer's instructions. The membrane was stained with Coomassie Blue R-250 solution for several seconds, destained in 40% methanol, then washed with 18 $M\Omega \cdot cm^{-1}$ distilled water several times. The purified blotted band was excised from the membrane and stored in a 1.5 ml Eppendorf tube at 4°C. The protein sequencing was performed by the Edman degradation procedure, services were provided by the Nucleic Acid and Protein Sequencing (NAPS) Unit at the University of British Columbia (UBC) on a Applied Biosystems (ABI) Model 476A Protein Sequencer (Mississauga, ON, Canada) according to the manufacturer's recommendations.

Phage λ library. A *Streptomyces* sp. isolate 2065 λ phage total genomic library was prepared using a Lambda DASH II/Bam H1 Vector Kit from Stratagene. *Sau*3A I and *Mbo* I partially digested total genomic DNA was ligated to the vector DNA provided in the kit. Lawns of plaques were obtained by infecting *E. coli* XL-1 (P2) with recombinant λ phage library as described by the manufacturer. The library was amplified in the same bacterial host according to the manufacturer's instructions and the titer was determined before use.

Manipulation of DNA

General Handling of DNA. Restriction digests, ligation reactions, DNA analysis on agarose gels, and other procedures mentioned were performed according to standard protocols unless otherwise stated (Ausubel *et al.*, 1991; Sambrook *et al.*, 1989). Restriction enzymes and DNA modifying enzymes were purchased from GIBCO BRL, New England BioLabs Ltd. (Mississauga, ON, Canada), and Promega Corp. (Nepean, ON, Canada). Total genomic DNA from *Streptomyces* sp. isolate 2065 was purified by caesium chloride-ethidium bromide density gradient centrifugation. Bacteriophage DNA was purified by a standard protocol which included a PEG precipitation step. Plasmid DNA from *E .coli* strains was purified by QIAwell miniprep columns (Qiagen, Chatsworth, CA). *E. coli* strain DH5α was transformed by electroporation using MODEL 165-2076 Bio-Rad Gene Pulser Transfection Apparatus and Pulse Controller according to the instructions of the manufacturer.

Table 2 Sequences for PCR primers.

primer	Sequence (5'-3')
P34OAfor	CT$^{C}/_{G}$AC$^{C}/_{G}$CAGCACGACATCGACCT
βfor	GCCGAGCACGCGACGTACGAGAAGC
P34OArev	CTCGTGCG$^{C}/_{G}$TG$^{C}/_{G}$ATGCTCTTCGC
P34OBCrev	C$^{C}/_{G}$GG$^{C}/_{G}$CG$^{C}/_{G}$$^{C}/_{G}$$^{A}/_{T}$$^{C}/_{G}$TGTCGAT$^{C}/_{G}GT^{C}/_{G}$GT
αrev	ACGTGTCGATGGTCGTCATGGC
P34OBCfor	AC$^{C}/_{G}$AC$^{C}/_{G}$ATCGACAC$^{C}/_{G}$$^{T}/_{A}$$^{C}/_{G}$$^{C}/_{G}CG^{C}/_{G}CC^{C}/_{G}$G
S16S2	GTGGGGATTAGTGGCGAACGGGTG
S16S3	CACCAGGAATTCCGATCTCCCC

Polymerase Chain Reactions. PCR reactions were performed on a Model PTC-150 Minicycler (MJ Research, Inc., Watertown, MA). Primers used for PCR and/or DNA sequencing were prepared by DNA oligonucleotide synthesis services from TerraGen Diversity Inc. and the NAPS

Unit at UBC. Degenerate primers, P34OAfor and P34OBCrev, were designed from N-terminal protein sequences from the streptomycete 3,4-PCD subunits. After the 800-bp PCR product had been cloned and sequenced, new non-degenerate primers, βfor and αrev, were designed to amplify the *pcaH* gene. Streptomycete-specific 16s rDNA primers, S16S2 and S16S3, were used for PCR control reactions (Webb & Davies, 1993). Sequences for primers are listed in Table 2. PCR conditions using streptomycete DNA were was follows: reactions were performed in 20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, 100 μg/ml gelatin, 10% glycerol, 5% formamide, 250 mM dNTP, 2 μM primers (1 μm each) (Webb & Davies, 1993). Reactions were heated to 95°C for 2 minutes before 1.25U of Taq polymerase was added. The cycling parameters were as follows (Muth *et al.*, in press): initial denaturation for 4 minutes at 96°C, mixing of components at 72°C for 4 minutes then 35 cycles of denaturation (95°C, 1.5 minutes) and annealing-extensions (72°C, 1.5 minutes), and a final extension of 10 minutes at 72°C. DNA fragments produced by PCR reactions were cloned using the TA Cloning System (Invitrogen, San Diego, CA).

Southern Blot Hybridizations. Southern blot hybridizations (Southern, 1975) were performed using the DIG system (Boehringer Mannheim Biochemica, Laval, QC, Canada). Non-radioactive DNA labeling was performed by PCR incorporation of digoxygenin-11-dUTP (Lion & Haas, 1990). DNA was blotted onto positively charged nylon membranes (Boehringer Mannheim Biochemica) by the alkaline upward capillary transfer method and fixed by baking membranes at 80°C for 2 hours. For screening plaque lifts, Hybond N+ positively charged membranes from Amersham (Oakville, ON, Canada) were used. Hybridizations were performed in glass tubes in a Hybaid Micro-4 hybridization oven and rotisserie (Interscience, Inc., Markham, ON, Canada). Prehybridizations were performed at 68°C for one hour, while

hybridizations were performed at 68°C overnight. The membranes were washed twice for 5 minutes at room temperature in 2 x SSC with 0.1% SDS then twice for 15 minutes at 68°C in 0.5 x SSC with 0.1% SDS. DIG-labeled DNA was detected colourimetrically using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

DNA Sequencing and Analysis. TerraGen Diversity Inc. and the NAPS Unit at UBC provided DNA sequencing services. TA clones were sequenced using M13 Universal forward and reverse primers. Sequencing reactions were performed by *Taq* cycle sequencing using the DyeDeoxy terminator method (Applied Biosystems Inc.) according to the instructions of the manufacturer. Sequencing reaction products were analyzed on an ABI Model 373A DNA Sequencer. Computer-based sequence analysis were performed with PC/GENE (Intelligenetics Inc., Mountain View, CA); alignments were done using open and unit gap costs set to 10. Clustal W 1.7 was used for multiple sequence alignments and graphic output was generated by BOXSHADE 3.21. BLAST 2.0 was used to identify similarity to other sequences.

RESULTS AND DISCUSSION

Characterization of Streptomycete Isolates

Screening for Activity Against Aromatic Acid Compounds. A collection of soil actinomycetes were screened for the ability to degrade aromatic acids (Table 3). Among the 45 isolates screened, 11 were found to have activity against vanillic and/or *p*-hydroxybenzoic acid. No degradation of chlorobenzoic acids (including benzoic acids substituted at the 2, 3, or 4 positions), cinnamic acid, isovanillic, or veratric acid was observed by the blue plate assays. In addition none of the isolates screened were seen to degrade benzoic acid. Isolate 2065 degraded both vanillic acid and *p*-hydroxybenzoic acid and was selected for further analysis. When this isolate was grown in liquid culture with minimal salts media and vanillic acid or *p*-hydroxybenzoic acid added as sole carbon source, removal of the aromatic acid from the culture supernatants was observed when monitored spectrophotometrically. The rates of degradation were comparable to those previously observed with *Streptomyces* sp. isolate D7 (previously *S. violaceusniger*) (Chow, 1996).

Eleven isolates out of 45 tested were found to be able to increase the alkalinity of the medium which suggested that they may have activity against aromatic compounds. These actinomycetes were not isolated from an enrichment culture to select for those that preferentially catabolized aromatic compounds, but rather, were isolated from cultures grown on rich media. The fact that none of the isolates screened were seen to degrade chlorobenzoic, cinnamic, isovanillic, or veratric acid indicates that aromatic rings which are chlorinated or more heavily methoxylated tend to be recalcitrant to degradation. In fact, the growth of many of the isolates screened was inhibited by presence of chlorinated or methoxylated compounds when compared to controls in which no carbon source was added and the actinomycete was seen to grow, albeit poorly, on the

agar. It seems unusual that none of the isolates tested seemed to degrade benzoic acid since; along with *p*-hydroxybenzoic acid, it is one of the simplest and most commonly degraded aromatic hydrocarbons. In fact, benzoic acid has been seen as the catabolite of choice in the case where both compounds are present; *P. putida* which can metabolize both compounds, preferentially degrades benzoic acid by catabolite repression of the specific *p*-hydroxybenzoic transport protein, PcaK (Nichols & Harwood, 1995). The observation that those isolates, which were positive by blue plate assay for one or more aromatic acids, all degraded *p*-hydroxybenzoic acid is consistent with the fact that this compound is considered to be one of the most prominent aromatic compounds in soil produced by plants. It also may suggest that in their catabolism of such compounds actinomycetes commonly convert more structurally complex aromatic compounds to *p*-hydroxybenzoic acid. This has been seen for other soil-dwelling bacteria since it is known that quinate which has been estimated to account for 10% by weight of leaf litter (Harwood & Parales, 1996) is degraded by *A. calcoaceticus via* protocatechuic acid.

Table 3 Bromothymol blue plate screening of actinomycetes. A sample of the 45 actinomycete isolates tested for activity against aromatic acids. Growth in the presence of the aromatic acid tested is indicated by +, slight growth is indicated by "+", and no growth is indicated by -. Development of a blue color indicative of degradation of the compound is indicated by shading.

actinomycete	**none**	**BA**	**HBA**	**CBA**	**CA**	**IVA**	**VA**	**VRA**
Streptomyces sp. D7	+	+	++	+[a]	"+"	"+"	++	+++
Streptomyces sp. 2017	+	+	+++	+[b]	+	++	+	++
Streptomyces sp. 2065	+	+	+++	+	+	+	+	+
Isolate 5634	+	nd	++	+	+	+	"+"	+
Isolate 2456	+	-	+	-	-	-	-	-
Isolate 2102	+	+	-	-	+	+	+	-

Abbreviations are as follows: BA, benzoic acid; HBA, *p*-hydroxybenzoic acid; CBA, chlorobenzoic acid (includes 2, 3, and 4-chlorobenzoic acid); CA, cinnamic acid; IVA, isovanillic acid; VA, vanillic acid; and VRA, veratric acid.
[a]slight growth observed with 3-chlorobenzoic acid, no growth with 4-chlorobenzoic acid.
[b]no growth observed on 4-chlorobenzoic acid.
nd, not determined.

Identification of Isolate 2065. When grown on ISP4 medium agar, *Streptomyces* sp. isolate 2065 had light gray mycelia, lighter gray spores, and produced a blue, diffusable pigment. On soy mannitol agar it formed beige colonies and white spores. The 16S rDNA sequence analysis of this isolate showed 97% identity to *Streptomyces* sp. strain 254. Cell wall fatty acid methyl ester (FAME) analysis performed by gas chromatography confirmed its identity as a member of the genus *Streptomyces.* It was weakly related to *S. halstedii* with a fatty acid profile similarity of 44.8%, which indicated that the species group to which this isolate belongs has not been previously characterized in the FAME database.

Investigation of Enzyme Activity. To determine the pathway (*ortho* or *meta*) and the ring-cleavage intermediate (catechol or protocatechuic acid) through which vanillic acid and *p*-hydroxybenzoic acid were degraded, enzyme assays were performed on crude cell-free extracts of isolate 2065 grown in the presence of these compounds. When extracts from cells grown in minimal medium with vanillic acid or *p*-hydroxybenzoic acid were incubated with protocatechuic acid or catechol, there was no development of a yellow *meta*-cleavage product. By the subsequent Rothera reaction, a positive reaction was observed only with protocatechuate as the substrate indicating that the mode of cleavage was *ortho via* the intermediate protocatechuic acid. This is consistent with what was previously observed for vanillic acid degradation by other *Streptomyces* spp. (Sutherland *et al.*, 1981; 1983; Pometto *et al.*, 1981). Spectrophotometric enzyme assays confirmed protocatechuate 3,4-dioxygenase activity. Presumably the same enzyme was induced by either compound, as opposed to two separate isozymes. The highest total activity for the vanillic acid induced cultures was detected at 4 hours after addition of substrate while highest total activity for the *p*-hydroxybenzoic acid induced

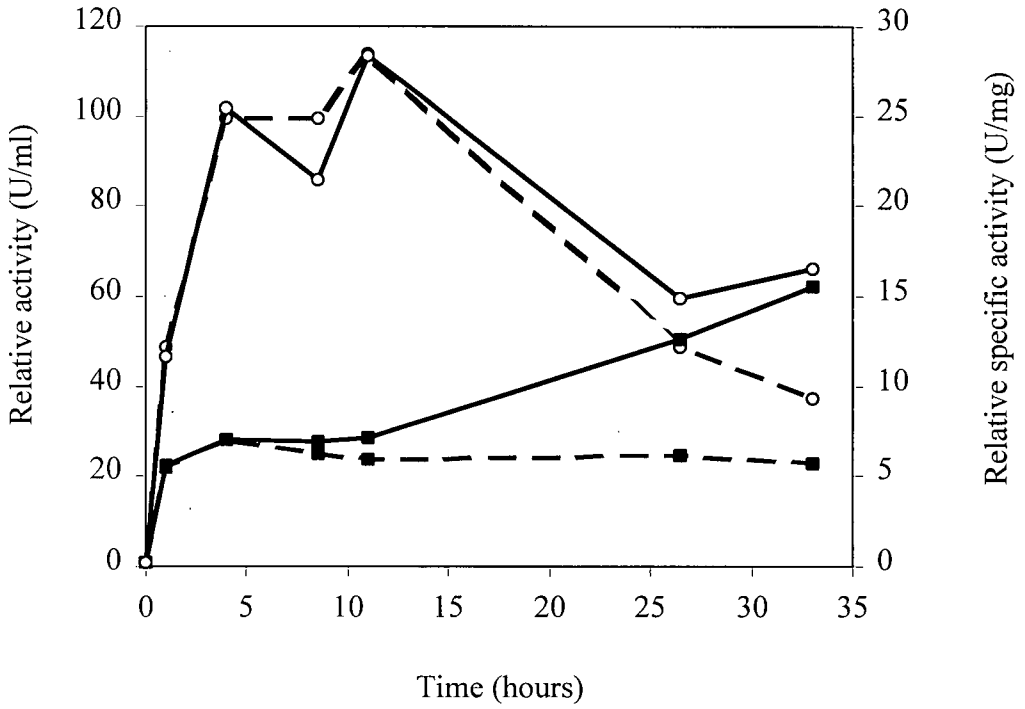

Figure 4 Induction of protocatechuate 3,4-dioxygenase from *Streptomyces* sp. isolate 2065 with vanillic acid and *p*-hydroxybenzoic acid. Vanillic acid induced cultures are represented with closed squares and the *p*-hydroxybenzoic acid induced cultures are represented by open circles. Relative specific activity is represented by dotted lines while relative total activity is represented by solid lines.

cultures was at 11 hours. The highest specific activity for the vanillic acid induced cultures detected at 33 hours while the highest specific activity for the *p*-hydroxybenzoic acid induced cultures was at 11 hours. At these times about 4-times higher total enzyme activity was observed in cell-free extracts of the *p*-hydroxybenzoic acid grown cells while 1.8-times higher maximum specific activity was observed in extracts from cells grown in MSMYE with *p*-hydroxybenzoic acid than with vanillic acid (Figure 4). For this reason, the dioxygenase was chosen to be purified from cells harvested 11 hours after addition of *p*-hydroxybenzoic acid. Although induction of protocatechuate 3,4-dioxygenase was also observed by the Rothera reaction in *Streptomyces* sp. isolate D7, accumulation of guaiacol was also observed; this may indicate that vanillate is also incompletely metabolized to this compound (Chow, personal communication). *S. setonii* has been shown to convert vanillic acid to catechol when provided with vanillin as initial substrate, or to protocatechuate when ferulic acid was provided (Sutherland *et al.*, 1981). Although the presence of guaiacol was not tested in cultures of isolate 2065 grown on vanillic acid, no catechol 1,2-dioxygenase activity was detected by spectrophotometric assays of cell-free extracts from these cultures. In addition, no protocatechuate 4,5-dioxygenase, catechol 2,3-dioxygenase, catechol 1,2-dioxygenase, or gentisate 1,2-dioxygenase activity was detected by spectrophotometric assays of cell-free extracts.

Vanillic acid and *p*-hydroxybenzoic acid have been previously shown to be degraded *via* protocatechuic acid [and in one species, via catechol (Figure 2)] while benzoic acid was degraded *via* catechol in *Streptomyces* spp. The catechol branch of the β-ketoadipate pathway was not observed in *Streptomyces* sp. isolate 2065. This is consistent with the observation that isolate 2065, as with the other isolates tested, did not degrade benzoic acid. Some members of the rhizobial/agrobacterial phylogenetic groups and *Azotobacter* spp. have also been reported to

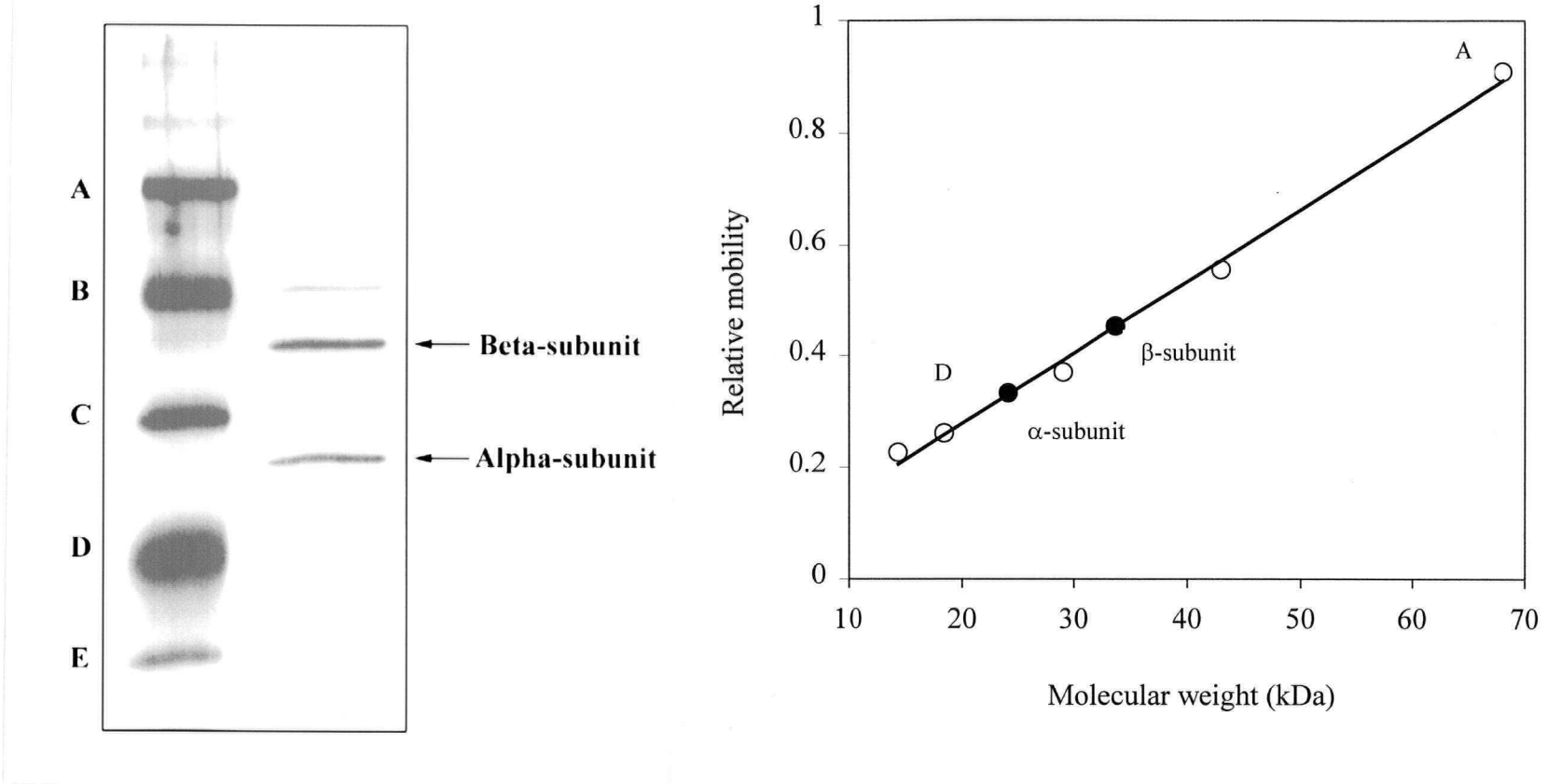

Figure 5 SDS-PAGE electrophoresis and subunit molecular weight determination of protocatechuate 3,4-dioxygenase. SDS-PAGE was performed as described under Materials and Methods. The α- and β-subunits of the streptomycete dioxygenase are represented as closed circles while the molecular weight markers are represented by open circles. A, bovine serum albumin (molecular weight, 68 kDa); B, ovalbumin (43); C, carbonic anhydrase (29); β-lactoglobulin (18.4); E, lysozyme (14.3); α- and β-subunits of the streptomycete dioxygenase (24.2 and 33.7, respectively).

possess only the protocatechuate branch of the β-ketoadipate pathway (Chen *et al.*,1984; Parke & Ornston, 1976; Hardisson *et al.*, 1969). It is interesting to note that *Azotobacter* spp. have been found to metabolize catechol through a *meta*-cleavage route. No *meta*-cleavage activity was observed in my analysis, as was observed with previous aromatic hydrocarbon catabolic studies in other *Streptomyces* spp. (Grund *et al.*, 1990).

Table 4 Summary of the purification of protocatechuate 3,4-dioxygenase from *Streptomyces* sp. isolate 2065.

Purification step	**total activity (U)**	**specific activity (U/mg)**	**total protein (mg)**	**recovery (%)**	**purification (fold)**
Crude cell-free extract	14, 710	14.2	604	100	1
40-60% $(NH_4)_2SO_4$ fractionation precipitate	nd	20	nd	nd	1.4
Q-Sepharose	nd	59	nd	nd	4.2
Mono-Q	nd	70	nd	nd	4.9
Phenyl-Superose	381	106	3.6	2.6	7.5

nd, not determined.

Protein Purification

Summary of Purification of Protocatechuate 3,4-Dioxygenase. The purification is summarized in Table 4. Enzyme from 6 litres of cells induced with *p*-hydroxybenzoic acid was purified 7.5-fold. The final purified enzyme had a specific activity of 106 U/mg. This compares with a final specific activity for the *Agrobacterium radiobacter* 3,4-PCD of 105 U/mg (Hammer *et al.*, 1996). Lower specific activities (ranging from 7-47 U/mg) have been observed for most purified 3,4-PCDs from other bacteria. For my purposes there was sufficient enzyme available only for protein sequencing and a few preliminary characterizations. The final purified streptomycete 3,4-PCD on a native PAGE gel was observed as a single protein band (data not shown), on a corresponding SDS-PAGE gel two proteins of approximate masses of 24.2 kDa and

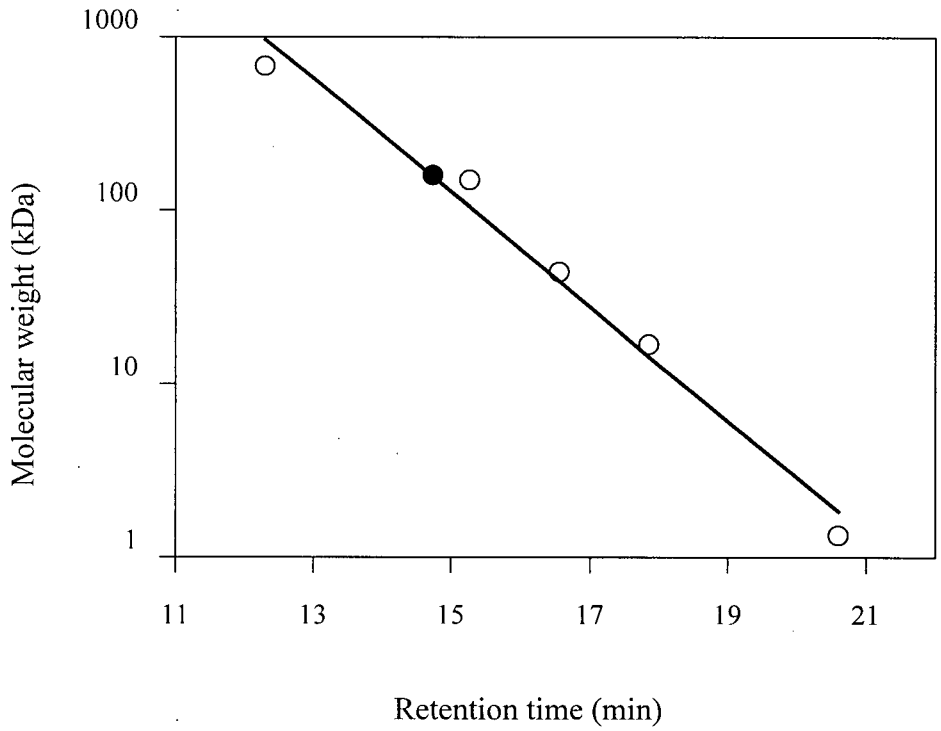

Figure 6 Native molecular weight determination of protocatechuate 3,4-dioxygenase by Superose 6 column chromatography. Gel filtration was performed as described under Materials and Methods. A, thyroglobulin (molecular weight, 670 kDa); B, IgG (150); C, ovalbumin (44); D, myoglobin (17); E, vitamin B-12 (1.35); the streptomycete dioxygenase is represented as a closed circle (158).

33.7 kDa were observed (Figure 5). These proteins were present in apparently equimolar amounts, indicating that the enzyme is composed of two protein subunits. A fainter 22.6 kDa protein band was seen migrating just below the smaller protein subunit, suggesting the presence of a degradation product of this protein. The larger protein migrating at approximately 43 kDa was a contaminant, its presence was not consistent in different protein preparations and did not correlate with protocatechaute 3,4-dioxygenase activity.

Native Molecular Weight Determination. Streptomycete 3,4-PCD activity was observed in the retentate of a Centriprep concentrator with a molecular weight cut-off of 100 kDa but not from one with a 300 kDa cut-off, which indicated its native molecular weight to be between these two values. When chromatographed over a calibrated Superose 6 column and its retention time compared with those for high molecular weight standards, the native size of the protein was calculated to be approximately 158 kDa (Figure 6). This falls in the lower end of the range for native molecular weights of previously purified 3,4-PCDs, 150-700 kDa (Table 1). Only the recently identified type II 3,4-PCDs have been found to be smaller, about 97.5 kDa (Hammer *et al.*, 1996). The streptomycete enzyme is the same size to one isolated from *Moraxella* sp. Strain GU2 (Sterjiades & Pelmont, 1989) and similar in size to one isolated from *Nocardia erythropolis*; in this Gram-positive bacterium it was found to be 150 kDa (Kurane *et al.*, 1984). Based on a α-subunit size of 21.8 kDa and a β-subunit size of 29.3 kDa, as determined by the predicted gene products below, the quaternary structure for this 3,4-PCD may be $(\alpha\beta Fe^{3+})_3$.

N-terminal Sequencing of Protocatechuate 3,4-Dioxygenase α and β Subunits

The 33.7, 24.2 and trailing 22.6 kDa proteins observed on SDS-PAGE and described above were blotted onto PVDF membranes and N-terminal protein sequence information was obtained, the

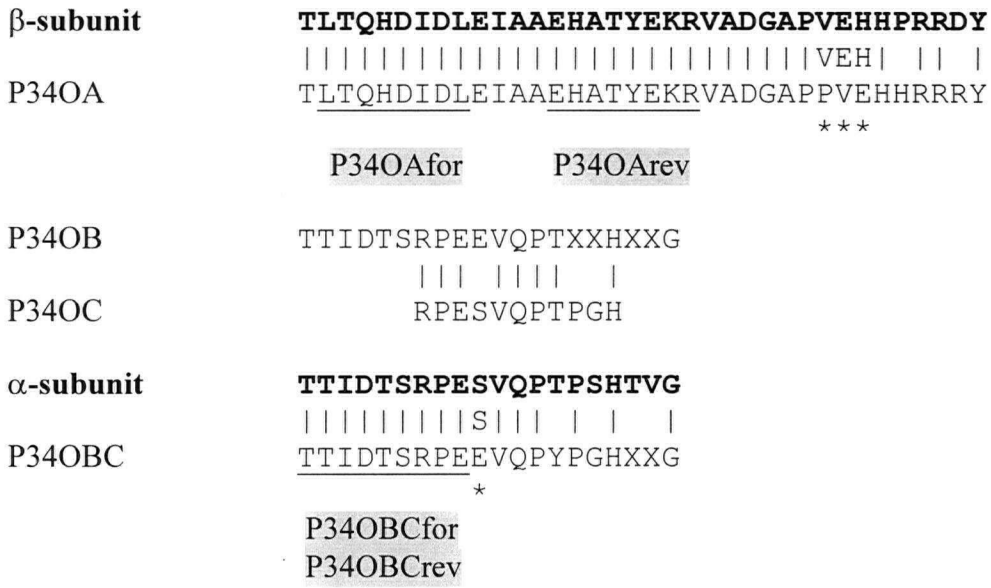

Figure 7 N-terminal protein sequences for protocatechuate 3,4-dioxygenase subunits from *Streptomyces* sp. isolate 2065. Positions where the identity of the amino acid was ambiguous are shown with an asterisk. Corresponding predicted amino acid sequence as determined by protein translation of *pcaH* and *pcaG* are in bold. Regions where primers for PCR amplification studies were designed are underlined and labeled below.

results are shown in Figure 7. The 24.2 kDa (P34OB) and 22.6 kDa (P34OC) protein sequences lined up to give a combined sequence (P34OBC), confirming that the smaller protein was a degradation product of the larger protein. The N-terminal methionine of both subunits were absent. In 3,4-PCDs previously studied in other bacteria the α- and β-subunits are similar in size, the β-subunit being slightly larger (Table 1). This streptomycete enzyme has a β-subunit significantly larger than the α-subunit. This has been seen for 3,4-PCD II, a novel 3,4-PCD which oxidizes 4-sulfocatechol, from *Hydrogenophaga palleronii* (Family *Pseudomonadaceae*), whose α/β-subunit sizes were found to be 22 kDa/31 kDa (Hammer *et al.*, 1996). The extreme example is the enzyme from *Brevibacterium fuscum* (Coryneform Group) with a α-subunit of 22.5 kDa and a β-subunit of 40 kDa (Whittaker *et al.*, 1984).

Table 5 Activation and inhibition of protocatechuate 3,4-dioxygenase by different compounds.

Compound	concentration (mM)	% activity	
$FeSO_4$	2.6	72	reductants
$FeCl_3$	2.6	117	
L-ascorbic acid	10	90	oxidant
DTT	8	106	divalent cation chelator
H_2O_2	8.8	105	
EDTA	10	106	ferric ion chelator
EDTA	20	102	
Tiron	10	53	ferrous ion chelator
Tiron	20	10	
2,2-dipyridyl	1	100	
2,2-dipyridyl	2	89	
2,2-dipyridyl	5	58	
2,2-dipyridyl	10	0	

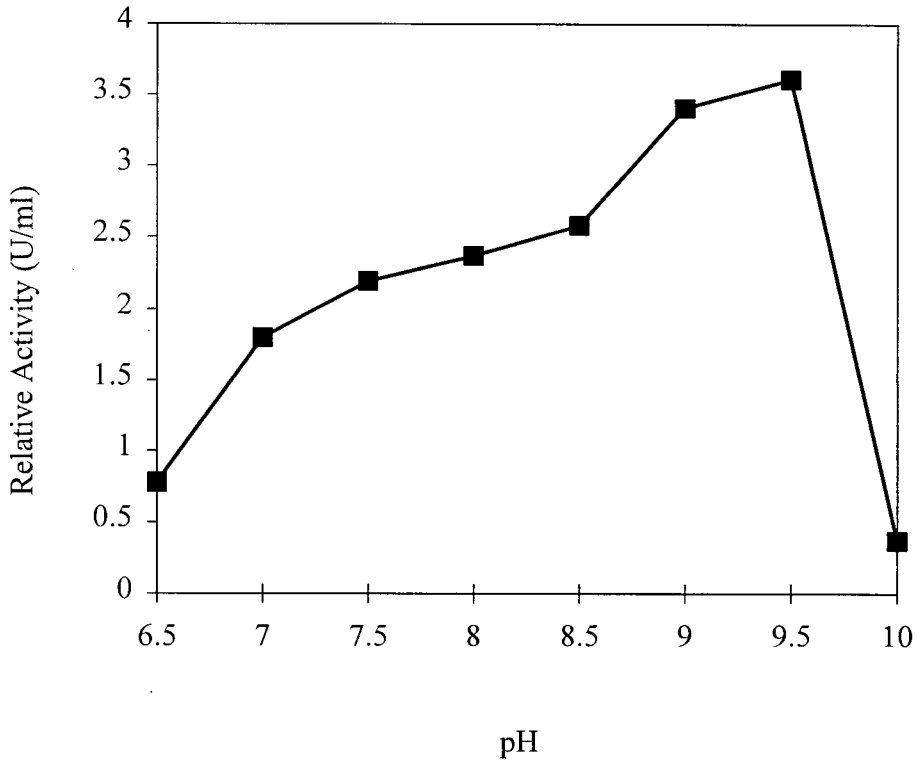

Figure 8 Effect of pH on the activity of protocatechuate 3,4-dioxygenase. The following buffers were used in each pH region by addition of protocatechuate 3,4-dioxygenase to the cuvette in the presence of the usual Tris-HCl, pH 8.5 buffer: pH 6.5-7.5, phosphate; pH 8.0-9.0 Tris-HCl, pH 9.5-10.0 carbonate-bicarbonate.

Preliminary Characterization of Protocatechuate 3,4-Dioxygenase

Sufficient amounts of protein were not obtained for purposes of detailed enzyme characterization, but a few preliminary characterization assays were performed on a preparation of semi-purified 3,4-PCD which was not purified by gel filtration chromatography (Table 5). The enzyme was stable at room temperature for several days and at 4°C for several weeks with only a slight reduction in activity. The streptomycete 3,4-PCD was tested for activation or inactivation after a brief incubation with various compounds. Fe^{2+} was seen to inhibit activity slightly and Fe^{3+} was seen to increase it, which suggests that the streptomycete dioxygenase contains Fe^{3+} and is consistent with what has been found with other 3,4-PCDs. The fact that Fe^{3+} did not greatly increase enzyme activity suggests that the purified enzyme had a close to optimal iron content. Enzyme activity was slightly reduced by ascorbate but no reduction in activity was observed with dithiothreitol (DTT) and no change in activity was observed with H_2O_2. The slight decrease in activity due to ascorbate, a reductant, is a property consistent with a prosthetic Fe^{3+} responsible for catalysis. Fe^{2+} containing enzymes are sensitive to oxidizing agents while Fe^{3+} containing enzymes are often activated by them. DTT was seen to inactivate the *B. cepacia* dioxygenase slowly over time (Bull & Ballou, 1981); while for the *Rhizobium trifolii* enzyme, inactivation by an iron chelator was accelerated by addition of DTT (Chen *et al.*, 1984). The *Azotobacter vinelandii* 3,4-PCD was not affected by short incubation with a low concentration of DTT but was partially inactivated by H_2O_2 (Durham *et al*, 1980).

Reduction of activity was observed with the following iron chelators: 10 mM Tiron (4,5-dihydroxy-*m*-benzenedisulfonic acid), a Fe^{3+} chelator, and 2 mM 2,2-dipyridyl, a Fe^{2+} chelator; no effect was seen with up to 20 mM EDTA, a non-specific iron chelator. Only the iron specific chelators, particularly the Fe^{2+} chelator 2,2-dipyridyl, were seen to inhibit enzyme activity. The

3,4-PCDs from *A. vinelandii*, *R. trifolii*, and *B. cepacia* were similarly not effected by EDTA. *R. trifolii* enzyme was inactivated by incubation with another Fe^{3+} chelator, 1,10-phenanthroline (Durham *et al.*, 1980). *A. vinelandii* enzyme was slightly inhibited by low concentrations of 2,2-dipyridyl and 1,10-phenanthroline but significantly inhibited by a one hour preincubation with 1.7 mM Tiron. In comparison 3,4-PCD from *B. cepacia* had an unchanged iron content after incubation with Tiron at room temperature for several days (Bull & Ballou, 1981). Tiron and 2,2-dipyridyl are both used as reagents in general iron determination, therefore they may not be entirely Fe^{3+}/Fe^{2+} specific. In addition the two compounds are different structurally, 2,2-dipyridyl consists of two pyridine rings while Tiron is a single-ring catecholic structure with sulfonate substituents at positions 2 and 4. The reason why I saw stronger inhibition of enzyme activity with 2,2-dipyridyl than with Tiron may be because the two-ring structure of 2,2-dipyridyl would allow it to chelate more iron. Since the pyridine ring of 2,2-dipyridyl has a substituent at only one position making it less bulky than Tiron, the compound may fit more easily into the substrate binding pocket of the enzyme to extract the iron. Although it is difficult to compare inactivation of different 3,4-PCDs by iron chelators due to the fact that different concentrations and incubation times were used, the streptomycete dioxygenase seems to have a tightly held Fe^{3+} which is only effected by high concentrations of iron-specific chelators.

The relative activity of 3,4-PCD was seen to increase over 4.5-fold as pH was increased from 6.5 to 9.5 at increments of 0.5, and maximum relative activity was detected at pH 9.5 (Figure 8). The pH optimum may be higher than what could be tested since at above pH 9.0 protocatechuic acid undergoes non-enzymatic oxidation (Stanier & Ingraham, 1954). In this respect the streptomycete 3,4-PCD behaves the same way as the enzymes from *B. cepacia* (Bull & Ballou, 1981), *A. calcoaceticus* (Hou *et al.*, 1976) and *R. trifolii* (Chen *et al.*, 1984), and has a higher pH

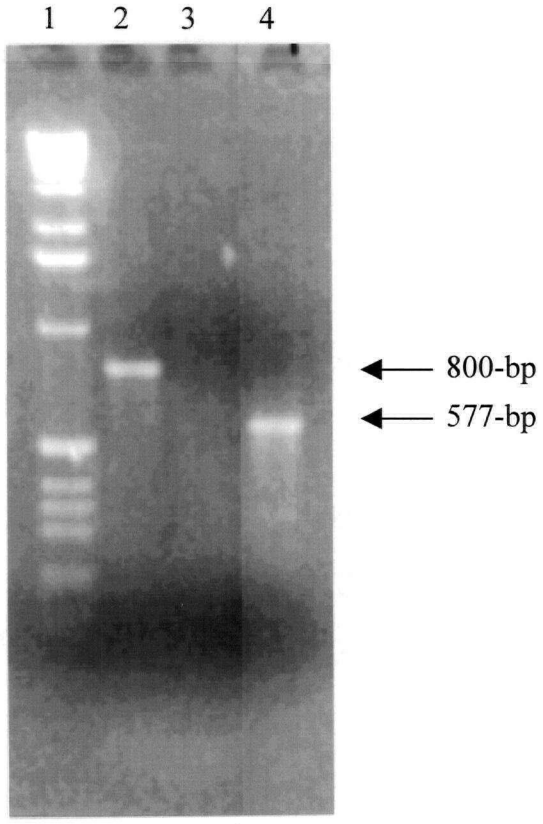

Figure 9 PCR amplification of the *pcaH* gene. *Streptomyces* sp. isolate 2065 genomic DNA was PCR amplified with the following primer combinations. 1, 1-kb ladder; 2, P34Ofor and P34OBCrev; 3, P34OBCfor and P34OArev; and 4, S16S2 and S16S3. The 800-bp PCR amplification product corresponding to the *pcaH* gene and the 577-bp product from the control PCR reaction are labeled.

optimum than the enzymes from *A. vinelandii* (Durham *et al.*, 1980) or *P. putida* (Fujisawa & Hayaishi, 1968).

PCR Amplification of the *pcaH* Gene

The order of the *pcaG* and *pcaH* genes (encoding the α- and β-subunits for 3,4-PCD, respectively) have been found to be conserved in those organisms for which sequence information is available, the two genes being transcribed on the same transcriptional unit as *pcaHG*. To determine the order of the streptomycete 3,4-PCD genes, forward and reverse degenerate DNA oligonucleotides were designed from the N-terminal amino acid sequences obtained above (Table 7). These oligonucleotides were designed to match the codon bias of the high G+C streptomycete genome (69-78%). They were then used as PCR primers and paired in different combinations with isolate 2065 chromosomal DNA as a template. An 800-bp product was obtained when the primer combination of P34OAfor and P34OBCrev was used, confirming the order of the genes as *pcaHG* (Figure 9). In the control PCR reactions the streptomycete-specific 16s rDNA PCR primers amplified the expected 577-bp product from isolate 2065 genomic DNA. The 800-bp DNA fragment was cloned into a TA cloning vector and sequenced using M13 universal and reverse sequencing primers. The protein translation of the sequence from the 800-bp insert had greatest similarity to the PcaH from *B. cepacia*. This DNA fragment was labeled non-radioactively with Dig-11-dUTP by PCR incorporation and then used as gene probe in DNA hybridization studies described below.

Cloning and Identification of *pcaHG* Genes

The *pcaHG* genes were isolated from a bacteriophage λ genomic library of *Streptomyces* sp. isolate 2065 prepared using *Sau*3AI partially digested total DNA. The titer of the library was

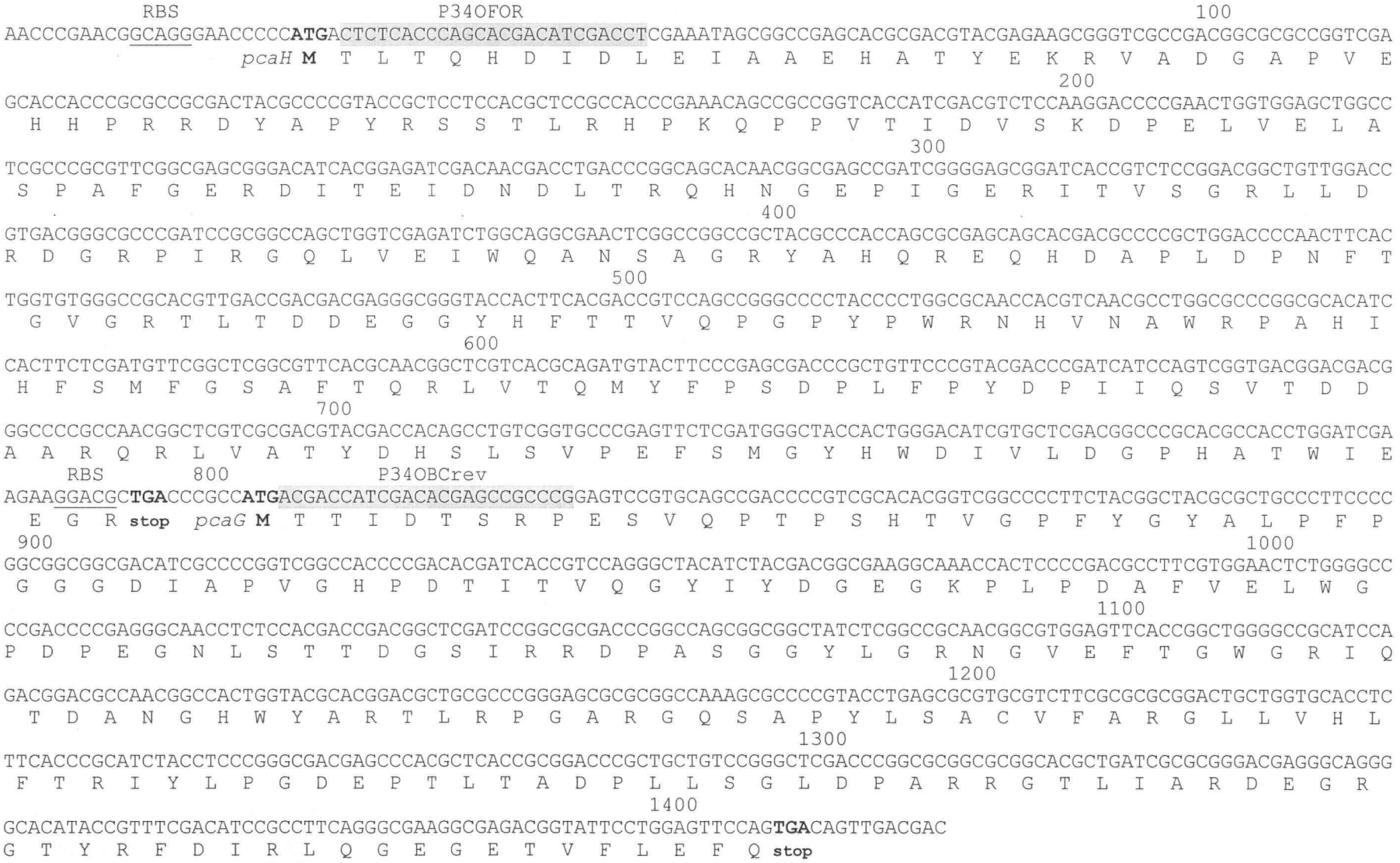

Figure 10 Nucleotide sequence of the *pcaHG* genes and deduced amino acid sequence from *Streptomyces* sp. isolate 2065. Start and stop codons are in bold and putative ribosome binding sites are underlined. Sequences where PCR primers annealed are labeled and shaded.

determined to be 6.3 x 10^5 recombinant plaques/μg. The genes were cloned by plaque hybridization with a non-radioactively labeled DNA probe for the *pcaH* gene which was obtained as described above. Nylon lifts of plaques that arose from infection of *E. coli* with the genomic library were screened by hybridization with the 800-bp *pcaH* gene probe. In a primary screen of 30,000 plaques, 30 plaques were chosen that hybridized to the probe. From the secondary screen 9 plaques were plated from which 24 single plaques were isolated from each. Ten isolates were plated in the tertiary screen and shown by hybridization to be homogeneous. Bacteriophage DNA was isolated from these 10 clones and digested with *Sal* I; three clones had a 4.5-kb *Sal* I fragment that hybridized to the *pcaH* probe. When total genomic DNA was digested with *Sal* I the gene probe was seen to hybridize to a DNA fragment of the same size (data not shown); the 4.5-kb *Sal* I fragment was subcloned from the bacteriophage λ clone into pBluescript KS+. The resulting subclone was confirmed to contain the *pcaH* gene by observation of a 800-bp product when its DNA was PCR amplified using the βfor and αrev primers. The βfor and αrev primers were used in sequencing reactions to verify that this subclone contained at least part of the *pcaG* gene, all of the *pcaH* gene and to confirm the sequence obtained from the 800-bp PCR product (data not shown).

Nucleotide Sequence of *pcaGH* Genes

Two consecutive open reading frames were sequenced on both strands of the 4.5-kb *Sal* I subclone with overlapping oligonucleotides. The *pcaG* gene is 606 bases long while the *pcaH* gene is 774 bases long. The entire sequence for the *pcaHG* genes is shown in Figure 10; putative ribosome binding sites are underlined and there are 6 bases separating the two genes. The order of the genes encoding 3,4-PCD has been conserved. The gene encoding the α-subunit, *pcaG*, has been reported to be located downstream from *pcaH*, the gene for the β-subunit, for

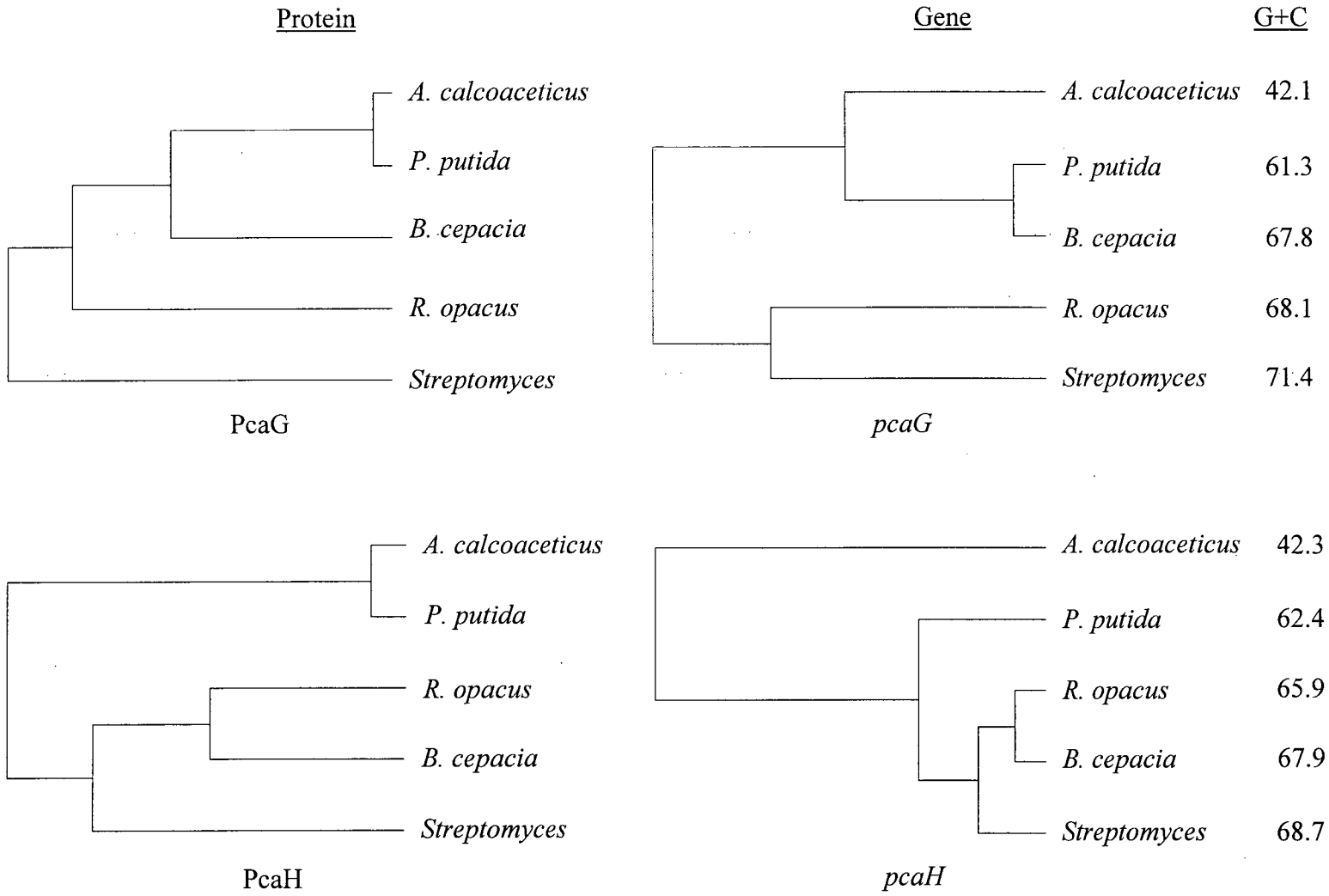

Figure 11 Comparative similarities of the protocatechuate 3,4-dioxygenase from *Streptomyces* sp. isolate 2065 at the gene and protein level to those from widely differing bacteria. Dendrograms on the left panel show relatedness of the α- and β- subunit proteins, PcaG and PcaH. Dendrograms on the right panel show relatedness of *pcaG* and *pcaH* genes encoding the α- and β-subunits. The column to the right lists the G+C content for the respective genes.

Agrobacterium tumefaciens, *B. cepacia*, *A. calcoaceticus*, and *P. putida*, representatives of the α, β, and γ-proteobacteria as well as for *R. opacus*, a Gram-positive nocardioform actinomycete (Frazee *et al.*, 1993; Hartnett *et al.*, 1990; Parke, 1997; Zylstra *et al.*, 1989; Eulberg *et al.*, 1998). Although the *pca* and *cat* genes are generally clustered on bacterial chromosomes, the order of the β-ketoadipate pathway genes vary from species to species. The only exceptions to this are the *pcaHG* and the *pcaIJ* genes (encoding a succinyl CoA:β-ketoadipate CoA transferase) which are always transcribed and regulated together, which is not surprising as they encode two-subunit enzymes and might be expected to evolve as a transcriptional unit (Harwood & Parales, 1996).

The DNA sequences for the streptomycete 3,4-PCD genes were aligned with those previously sequenced from other bacteria. The *pcaG* genes share 16.4% identity while the *pcaH* genes share 25.4% identity. Since the β-subunit contains the ligands to the catalytic iron, this may reflect the fact that it is under stronger evolutionary constraints in order to maintain enzyme function. The streptomycete *pcaG* shared greatest similarity to the gene from *R. opacus* (33.3%), *B. cepacia* (31.8%), and *P. putida* (30.7%), and the least similarity to one from *A. calcoaceticus* (22.7%). The streptomycete *pcaH* shared greatest similarity to same gene from *B. cepacia* (47.4%), *R. opacus* (46.1%), and *P. putida* (43.8%), and least similarity to one from *A. calcoaceticus* (30.2%). Regions of highest DNA sequence identity are those encoding iron-binding ligands, substrate binding residues, and conserved amino acid residues that form the binding pocket (data not shown).

The coding regions of the *pcaG* and *pcaH* sequences contained 71.4% and 68.7% G+C, the highest of all the *pcaGH* genes sequenced so far. This is due primarily to a preference for guanine and cytosine in the third codon position; the percentage of codons ending in G or C was

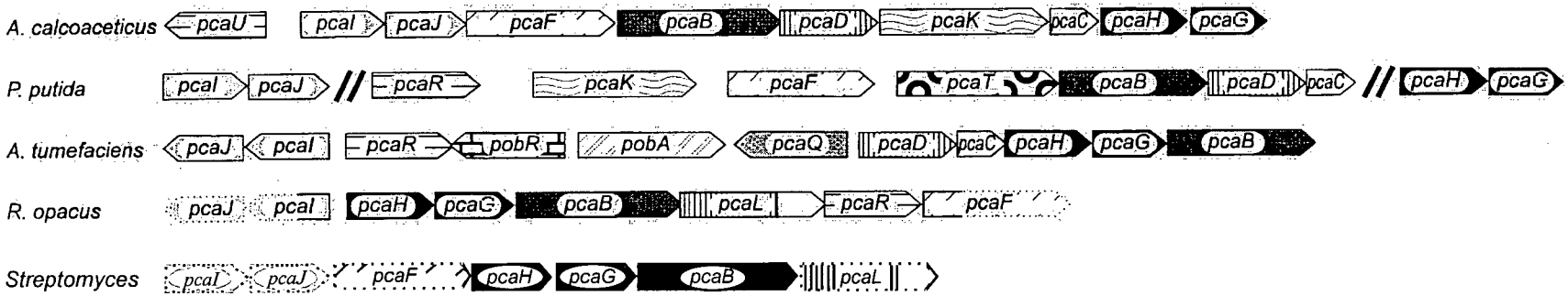

Figure 12 Gene organization of the *pca* gene clusters from different bacteria. Homologous genes are shaded the same way. Dotted borderlines represent genes for which partial sequence is available. Figure modified from Eulberg *et al.* (1998).

93.2%. Where there was a choice for G or C in the third codon position the distribution was more or less even except for arginine, proline, and glycine. Out of 37 arginine codons 23 were CGC while 11 were CGG, out of 41 proline codons 26 were CCG while 14 were CCC, and out of the 46 glycine codons 37 were GGC and 4 were GGG. In the cases where there was an A/T or G/C choice for the first position in the codon, the preference was for G or C. In *Streptomyces* sp. TTA codons for leucine are rare, they are absent in the *pcaGH* gene sequences from isolate 2065. Out of 34 leucine codons 32 started with C rather than T and 36/37 arginine codons started with C rather than A. The streptomycete *pcaGH* genes were more similar to the same genes from other bacteria with high G+C content, the more similar the genes were the higher the G+C content was. This is presumably related to codon preference and G+C content of the host organism and reflects evolutionary relatedness between the genes. Although my DNA sequence and codon analysis are not presented, they are summarized in Figure 11.

It appears that in *Streptomyces* sp. isolate 2065 the *pcaGH* genes are part of a larger cluster (Yang, personal communication) (Figure 12). Partial preliminary gene sequence upstream from *pcaGH* has been obtained for 3 open reading frames (ORFs) whose predicted protein sequence shows homology to two enzymes. The first two ORFs show similarity to succinyl-, acetate-, and butyrate-CoA transferases from different organisms, including the PcaIJ, a β-ketoadipate succinyl-CoA transferase, from *A. calcoaceticus* and *P. putida*. The third shows homology to the PcaF, a β-ketoadipyl CoA thiolase, from *P. putida*. Downstream of the *pcaGH* there are two consecutive open reading frames that have predicted gene products which show homology to PcaB, β- carboxymuconate cycloisomerase, and PcaL, a fused β-ketoadipate enol-lactone-hydrolase and γ-carboxymuconolactone-decarboxylase enzyme, from *R. opacus* (Eulberg *et al.*, 1998). All five genes are in the same orientation as *pcaHG* on the 4.5-kb genomic insert (Figure

8). In *P. putida* the *pcaGH* genes have not been found to cluster with other *pca* genes. No other *pca* genes other than sequences for *pcaGH* have been reported for *B. cepacia*. In *A. calcoaceticus* the genes are contiguous in a *pcaIJFBDKCHG* cluster while in *A. tumefaciens* they are in a *pcaDCHGB* cluster. Preliminary sequence data indicates that the streptomycete *pca* gene cluster seems to closely resemble that of *R. opacus*, in which the 3,4-PCD genes are in a contiguous *pcaHGBLRF* cluster which is similar to the *pcaIJFHGBL* gene order, the *pcaF* gene being transposed in the streptomycete cluster. The gene order *pcaIJF* is conserved within the *A. calcoaceticus* and the streptomycete *pca* gene clusters. It is interesting to find a *pcaL* homologue in the streptomycete *pca* gene cluster because the β-ketoadipate enol-lactone-hydrolase and γ-carboxymuconolactone-decarboxylase enzymes are encoded by separate genes, *pcaD* and *pcaC*, in *A. calcoaceticus*, *P. putida*, *A. tumefaciens* and *B. japonicum*. This is the second report of the presence of a *pcaL* gene; the first resulted from identification of the dual enzyme activity in the protein from *R. opacus* after which the gene was cloned (Eulberg *et al.*, 1998). Eulberg *et al.* (1998) hypothesized that the separate γ-carboxymuconolactone-decarboxylase/β-ketoadipate enol-lactone-hydrolase enzyme arrangement of proteobacteria may be the more ancient one and that the presence of a fused enzyme was a Gram-positive trait, since the general trend of protein evolution goes from simple to more complex.

Protein Sequence of Protocatechuate 3,4-Dioxygenase α- and β-Subunits

Translation of the two open reading frames *pcaHG*, revealed polypeptide sequences that confirmed the N-terminal sequence obtained for the α- (first 13 amino acids) and β-subunits (first 31 amino acids), except for a few discrepancies when the protein sequencing signal was

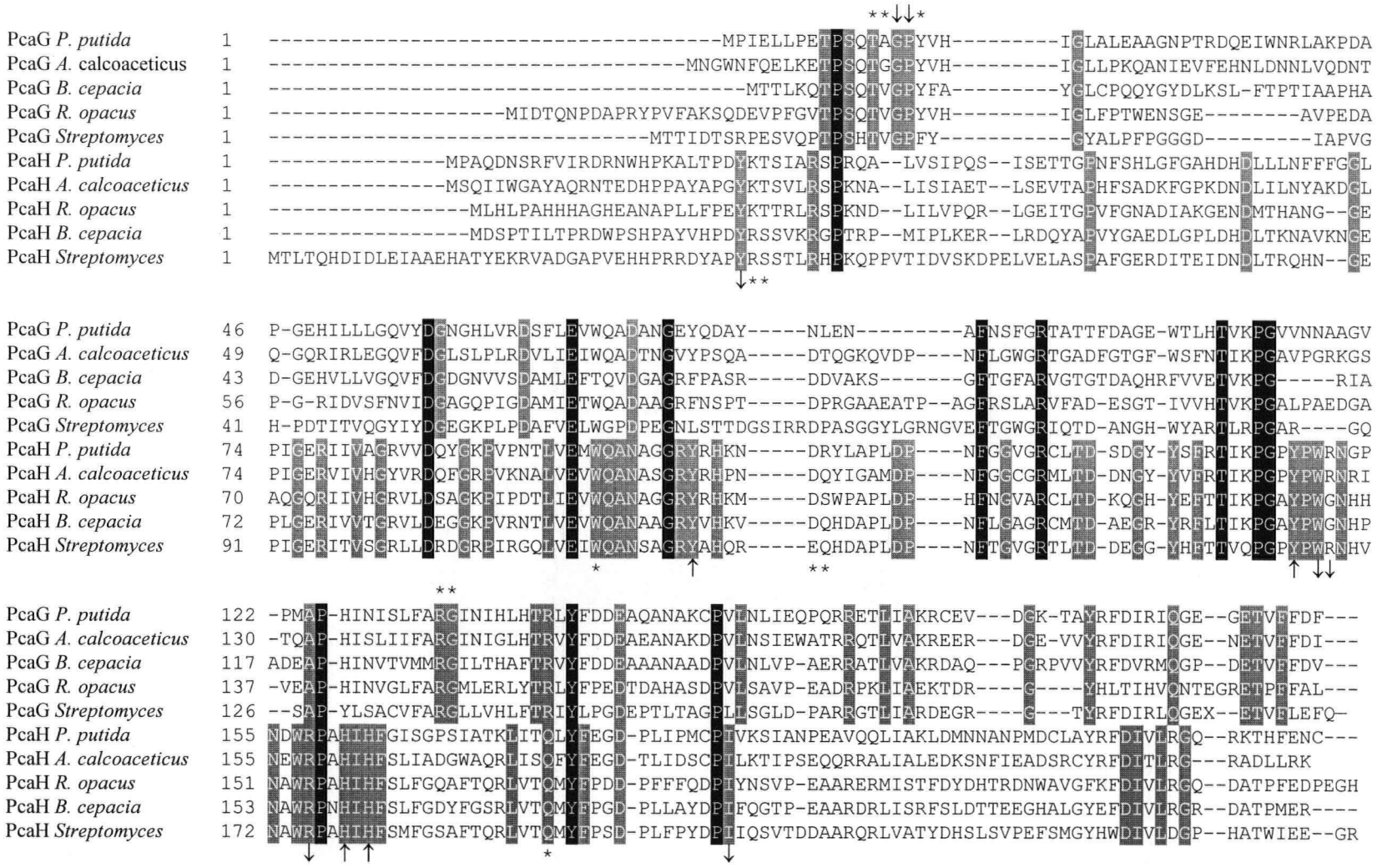

Figure 13 Sequence alignment of the subunits of protocatechuate 3,4-dioxygenases. Positions identical in both α- and β-subunits of all five aligned enzymes are highlighted by black boxes; those identical in only one subunit of all five enzymes are shaded. Numbers refer to positions in the individual sequences. Iron ligands are indicated by upward pointing arrows. Residues involved in active site are indicated by downward pointing arrows. Asterisks indicate other active site residues. Accession numbers for the published sequences are: M33798 (*A. calcoaceticus*), M30791 (*B. cepacia*), L14836 (*P. putida*), and AF003947 (*R. opacus*)

subunit	active site perimeter								mid-site α			mid-site β		near Fe^{3+}				Fe^{3+} ligands			
														*		*	*	*	*	*	*
β *Str.*	**Y_{40}**	**R_{41}**	**S_{42}**	S_{186}	A_{187}	**E_{131}**	**Q_{132}**	**R_{168}**	L_{66}	A_{67}	S_{68}	**W_{167}**	**I_{209}**	P_{69}	A_{70}	**W_{118}**	**R_{175}**	**Q_{195}**	**Y_{126}**	**Y_{165}**	**H_{178}**	**H_{180}**
β *R. o*	**Y_{23}**	**K_{24}**	**T_{25}**	Q_{165}	A_{166}	**D_{110}**	**S_{111}**	**G_{147}**	I_{45}	T_{46}	G_{47}	**W_{146}**	**I_{188}**	P_{48}	V_{49}	**W_{97}**	**R_{154}**	**Q_{174}**	**Y_{105}**	**Y_{144}**	**H_{157}**	**H_{159}**
β *B.c.*	**Y_{23}**	**R_{24}**	**S_{25}**	D_{167}	Y_{168}	**D_{112}**	**Q_{113}**	**G_{149}**	Q_{45}	Y_{46}	A_{47}	**W_{148}**	**I_{190}**	P_{48}	V_{49}	**W_{99}**	**R_{156}**	**Q_{176}**	**Y_{107}**	**Y_{146}**	**H_{159}**	**H_{161}**
β *A.c.*	**Y_{25}**	**K_{26}**	**T_{27}**	D_{169}	G_{170}	**D_{114}**	**Q_{115}**	**R_{151}**	V_{47}	T_{48}	A_{49}	**W_{150}**	**I_{192}**	P_{50}	H_{51}	**W_{101}**	**R_{158}**	**Q_{178}**	**Y_{109}**	**Y_{148}**	**H_{161}**	**H_{163}**
β *P p.*	**Y_{24}**	**K_{25}**	**T_{26}**	P_{167}	S_{168}	**D_{113}**	**R_{114}**	**R_{150}**	T_{46}	T_{47}	G_{48}	**W_{149}**	**I_{191}**	P_{49}	N_{50}	**W_{100}**	**R_{157}**	**Q_{177}**	**Y_{108}**	**Y_{147}**	**H_{160}**	**H_{162}**
α *P. p.*	-	-	-	**R_{133}**	**G_{134}**	D_{81}	-	A_{117}	**T_{12}**	**A_{13}**	**G_{14}**	N_{116}	V_{157}	**P_{15}**	**Y_{16}**	W_{71}	M_{122}	R_{142}	Y_{79}	V_{114}	H_{125}	N_{127}
α *A. c.*	-	-	-	**R_{142}**	**G_{143}**	D_{88}	T_{89}	R_{126}	**T_{16}**	**G_{17}**	**G_{18}**	G_{125}	V_{166}	**P_{19}**	**Y_{20}**	W_{75}	Q_{131}	R_{151}	Y_{83}	V_{123}	H_{134}	S_{136}
α *B. c.*	-	-	-	**R_{130}**	**G_{131}**	D_{82}	D_{83}	-	**T_{11}**	**V_{12}**	**G_{13}**	-	V_{154}	**P_{14}**	**Y_{15}**	T_{69}	E_{119}	R_{139}	F_{77}	I_{115}	H_{122}	N_{124}
α *R. o.*	-	-	-	**R_{149}**	**G_{150}**	D_{94}	P_{95}	E_{133}	**T_{31}**	**V_{32}**	**G_{33}**	A_{132}	V_{173}	**P_{34}**	**Y_{35}**	W_{81}	E_{138}	R_{158}	F_{189}	L_{110}	H_{141}	N_{143}
α *Str.*	-	-	-	**R_{137}**	**G_{138}**	D_{85}	P_{86}	-	**T_{19}**	**V_{20}**	**G_{21}**	-	L_{161}	**P_{22}**	**F_{23}**	W_{67}	S_{126}	R_{146}	L_{175}	R_{123}	Y_{129}	S_{131}
				*							*			*								
function	aromatic substrate specificity													catalysis and F^{3+} stabilization							

Figure 14 Alignment of active site residues in protocatechuate 3,4-dioxygenases based on the structure of the *P. putida* enzyme complexed with protocatechuic acid. The shaded residues are within approximately 8 angstroms of protocatechuic acid. Primary sequences were aligned and adjusted slightly to reinforce the three-dimensional structural homology between the α- and β-subunits (Orville *et al.*, 1997b). Asterisks indicate residues conserved among catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenaeses. The abbreviations are as follows: *Str.*, *Streptomyces* sp. isolate 2065; *R. o.*, *Rhodococcus opacus*; *B. c.*, *Burkholderia cepacia*; *A. c.*, *Acinetobacter calcoaceticus*; and *P. p.*, *Pseudomonas putida*

more ambiguous (Figure 7). The *pcaG* and *pcaH* genes encode 201 and 257 amino acid polypeptides with predicted sizes of 21,768 Da and 29,262 Da. These sizes are smaller than those determined for the original proteins on SDS-PAGE, 24.2 kDa and 33.7 kDa, but this estimation of molecular weight was only approximate because pre-stained protein markers were used routinely throughout the purification. It has been found for previously studied 3,4-PCDs that the predicted and actual sizes of the subunits were identical.

The predicted protein sequences were found to be homologous to PcaG and PcaH of other known 3,4-PCDs from *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Pseudomonas putida*, and *Rhodococcus opacus* (Figure 13). The β-subunits lined up with 23.8% identity and 29.1% similarity while the α-subunits lined up with 15.1% identity and 21.4% similarity. The streptomycete β-subunit showed greatest similarity with those from *B. cepacia* (41.8%) and *R. opacus* (41.0%), and least similarity to those from *P. putida* (37.2%) and *A. calcoaceticus* (35.6%). The streptomycete α-subunit was more or less equally similar to those from other bacteria, 21.4% for *B. cepacia* and *P. putida* and 20.5 % for *R. opacus* and *A. calcoaceticus*. At the protein level the similarities between different β-subunits generally reflect those observed for the sequences of the *pcaH* genes encoding them, except that the *P. putida* and *A. calcoaceticus* β-subunits were more similar to each other at the protein level than at the nucleotide level (Figure 11). The streptomycete β-subunit had higher similarity at the nucleotide level to the sequences from *B. cepacia*, *R. opacus* and *P. putida* but not for *A. calcoaceticus*. For the α-subunit very different similarities were observed, at the nucleotide level the streptomycete *pcaG* was most similar to the same gene from *R. opacus* and least similar to the gene from *A. calcoaceticus* but on the amino acid level these sequences were equally similar to the streptomycete protein.

Higher sequence similarities were observed at the nucleotide level for all the *pcaG* genes.

From the *P. putida* 3,4-PCD crystal structure, of the 22 residues found in the active site (Ohlendorf *et al.*, 1994; Orville *et al.*, 1997), 16 are conserved in the enzyme from *Streptomyces* sp. isolate 2065 (Figure 14). The iron ligands which are absolutely conserved among all known intradiol cleaving dioxygenases, Tyr108(β), Tyr147(β), His160(β), and His162(β) (*P. putida* numbering) are conserved as Tyr126, Tyr165, His178 and His180 in the streptomycete enzyme. In the *P. putida* crystal structure Arg157(β) is positioned to align the substrate and is held in place by a hydrogen bond with Gln177(β) and both these residues are important for catalysis. In my streptomycete enzyme these residues are conserved as Arg175 and Gln195. In the *P. putida* crystal structure Gly14(α), Pro15(α), Tyr16(α), and Trp100(β) are among the residues around the putative O_2 binding cavity and, except for Tyr16, they are also absolutely conserved in all intradiol cleaving enzymes. Tyr16 is conserved in all 3,4-PCDs, and in other intradiol cleaving enzymes it is either conserved or is a Leu (Orville *et al.*, 1997); in the streptomycete enzyme the residue in this position is a Phe, whose side chain is more nonpolar than Tyr but retains the bulky aromatic group and may serve an analogous structural role. The *P. putida* Tyr24(β) is conserved as Tyr40 in my protein; in *P. putida* this residue forms a hydrogen bond with the carboxylate group of protocatechuic acid. Around the active site perimeter the basic character of *P. putida* residues Arg133(α), Lys25(β), and Arg150(β) are conserved in my protein as Arg137, Arg41, and Arg168; as is the side chain property of Thr26(β) in *P. putida* as Ser42 in my protein. Ala13(α) is a mid-site residue whose main chain atoms form part of the active site and side chain points away from it; this residue differs in the *P. putida* and *A. calcoaceticus* 3,4-PCD but is a Val20 in *B. cepacia*, *R. opacus*, and my *Streptomyces* sp. isolate. These residues around the

active site perimeter and Ala13(α) are thought to be involved in substrate specificity, favoring the relatively more negatively charged aromatic substrate protocatechuic acid (Ohlendorf *et al.*, 1994). The Trp149(β) and Ile191(β) of the *P. putida* enzyme are conserved as Trp167 and Ile209 in my protein; in *P. putida* they are thought to form sides of the active site and influence the substrate binding orientation (Lipscomb & Orville, 1992).

The N- and C-termini of the α- and β-subunits of 3,4-PCD are divergent by primary sequence and structure. From the crystal structure of the enzyme from *P. putida* it was determined that the β-subunit was seen to be involved in forming the main contacts for protomer and aggregate assembly while the N-terminus of the α-subunit is involved in substrate binding in the active site. In addition the interface of the αβ protomer included residues throughout both subunits, particularly in their N-terminal regions and especially in the N-terminal region of the β-subunit (Ohlendorf *et al.*, 1988). The observation that the α- and β-subunit N-termini are divergent reflects their different functional and structural roles. Much of the differences in sequence alignments within the different β-subunits are in the regions attributable to protomer assembly, and so enzymes with different quaternary structures would be expected to be divergent in these regions. The stoichiometries for the known 3,4-PCDs, and therefore their protomer assemblies, are different: *P. putida*, *A. calcoaceticus* [both $(\alpha\beta Fe^{3+})_{12}$], *B. cepacia* $(\alpha\beta Fe^{3+})_4$, and *Streptomyces* sp. isolate 2065 [which may be $(\alpha\beta Fe^{3+})_3$]. The streptomycete β-subunit is 18 amino acids longer than the β-subunit of the *P. putida* enzyme. When the sequences are aligned almost all the extra amino acids are placed in the N-terminal portion of the protein. The core region of the α-subunit is less conserved and when aligned with the subunit from *P. putida* there is a 9 amino acid gap from Arg31 to Leu40 in a loop region, as well as two insertions of 5 and 9 amino acids in an 18 amino acid loop from Ala73 to Asn90. Despite the sequence divergence in

the termini of the α- and β-subunits, this suggests that the streptomycete αβ unit protomer of 3,4-PCD is structurally similar to the enzyme from *P. putida*, as none of the regions of secondary structure seem to have been significantly disrupted, whereas the assembly of protomers in the greater quaternary structure is more likely to be different.

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

The protocatechuate 3,4-dioxygenase purified from *Streptomyces* sp. isolate 2065 seems to be similar in subunit and native enzyme structure to those characterized from other bacteria. Preliminary characterization studies indicate, as with other intradiol cleaving dioxygenases, that the enzyme contains a tightly held Fe^{3+} required for catalysis and is active over a wide pH range (pH 6.5 to >9.5). I cloned the streptomycete *pcaGH* genes and sequenced them to show similarity to homologous genes in other bacteria, particularly *Rhodococcus opacus* and *Burkholderia cepacia*, both high G+C bacteria (the former being Gram-positive and the latter Gram-negative). The *pcaGH* genes appear to be part of a greater *pca* gene cluster which includes at least 5 other genes. The sequences of the protein products of the *pcaGH* genes reflect the similarities observed at the nucleotide level. Amino acid alignments against known 3,4-PCD protein sequences, including the enzyme from *P. putida* for which the crystal structure has been solved, demonstrate that residues involved in the active site have been conserved or changed to similar residues. The residues specifically involved in iron binding, substrate-binding and catalysis have been absolutely conserved in all 3,4-PCDs and for some residues, including the iron binding ligands, they are also conserved in all intradiol-cleaving dioxygenases.

Further enzyme characterization including kinetic analysis and determination of substrate range will be useful in comparing this *Streptomyces* dioxygenase to those purified from other bacteria. With the genes cloned and sequenced, the streptomycete 3,4-PCD may be expressed in a heterologous host using an expression vector, and isolated following the purification procedure developed in this study. Detailed characterization studies have been done on the enzyme from *P. putida* and, based on the structural information obtained, 3,4-PCD is a good candidate for protein engineering studies. Recently the crystal structure of a site-directed mutant (Y447H) of the *P.*

putida 3,4-PCD was solved (Frazee *et al.*, 1998). If the properties of this streptomycete enzyme are favorable for industrial processes its specificity, activity or stability may be further enhanced by protein engineering based on structural information obtained from the similar *P. putida* dioxygenase. Additionally the streptomycete enzyme could be used for X-ray structural analysis to compare with the *P. putida* enzyme. This engineered enzyme may then be exploited for use in controlled biotransformation reactions. For example in the production of *cis,cis*-muconic acid, which is a potentially useful raw material for production of resins, pharmaceuticals and agrichemicals, and can be converted into adipic acid for the synthesis of the synthetic polymer nylon (Yoshikawa *et al.*, 1990). *Streptomyces* have already been shown to be commercially viable hosts for the production of a variety of active biopharmaceutical proteins and have been shown to secrete heterologous proteins (Binnie *et al.*, 1997; Brawner *et al.*, 1991). A streptomycete could be engineered to secrete a biodegradative enzyme of interest to aid in large scale purification. In addition, since *Streptomyces* are saprophytes that grow on decaying plant matter in soil, lignin and derived aromatic compounds from lignin are their normal substrates which make them natural candidates for use in lignin biodegradation and/or biotransformation.

Other targets for study in *Streptomyces* sp. isolate 2065 would be enzymes for the upper pathway ring-modification reactions whose genes may not necessarily be clustered or linked to the β-ketoadipate pathway operons. However such clustering has been observed for closely related catabolic activities; for example the *pob* genes encoding enzymes for the conversion of *p*-hydroxybenzoic acid to protocatechuic acid have been found to be linked to the *pca* operon in *A. calcoaceticus* and *A. tumefaciens* (Harwood & Parales, 1996). A vanillate demethylase would also be expected to be present in *Streptomyces* sp. isolate 2065 because there was induction of 3,4-PCD when cells were grown on vanillic acid as a sole carbon source which suggests

mineralization of vanillate *via* protocatechuic acid (Figure 2). Specifically, the position 3 methoxyl group of vanillic acid would be demethylated to a hydroxyl group producing protocatechuic acid, allowing the ring then to be cleaved via 3,4-PCD. An aromatic ring demethylating enzyme would be of great interest because many of the lignin derived aromatic compounds are heavily methoxylated making them more recalcitrant to degradation (Turner & Allison, 1995).

The 3,4-PCD from *Streptomyces* is most closely related to the same enzyme from high GC bacteria, especially from high GC Gram-positive bacteria. The streptomycete enzyme was most similar to the dioxygenase from *R. opacus* and *B. cepacia*, a little less similar to the same enzyme from *P. putida* and the least similar to the one from *A. calcoaceticus* (Figure 11). This may suggest that *Burkholderia* is more related to high GC Gram-positive bacteria such as *Streptomyces* and *Rhodococcus* than *Pseudomonas* is related to them. The rest of this streptomycete *pca* gene cluster will be more fully characterized when the 4.5-kb genomic insert is completely sequenced. In addition the regulation of the pathway including inducing metabolites and regulatory proteins will be investigated. This is the second *pca* operon characterized from a Gram-positive bacterium, the first being that of *Rhodococcus opacus* (Eulberg *et al.*, 1998). The 3,4-PCD enzyme has been purified from *Brevibacterium* but no gene sequence information has been reported (Whittaker *et al.*, 1984). Preliminary data indicates that the *pcaL* gene may be a Gram-positive characteristic, evolved from the fusion of the *pcaD* and *pcaC* genes. Also the gene order *pcaHGBL* is shared by *Rhodococcus* and *Streptomyces*.

The recent *S. coelicolor* genome sequencing project has revealed that the chromosome of this streptomycete also contains *pca* genes (Redenbach *et al.*, 1996). When *Streptomyces* sp. isolate

2065 *pcaGH* genes were BLAST searched against the *S. coelicolor* genome database it was revealed that homologous genes were present on cosmid St4C6 (Redenbach *et al.*, 1996). Although sequencing of this cosmid clone at this point is incomplete, analysis of the DNA sequence obtained thus far identifies the same *pcaIJFGHBL* operon structure seen with isolate 2065. The *pcaGH* genes from these two streptomycetes had an identity of 85%. It will interesting to compare the rest of *pca* gene sequences from these two closely related bacteria in detail as any genetic changes may reflect recent evolutionary changes between members of the same genera. Cosmid St4C6 contains 33.7 kb of DNA located less then 1200 kb from the right telomere on the *S. coelicolor* chromosome but before the *S. lividans* AUD1 (amplifiable unit of DNA) homologue. The ends of the *Streptomyces* chromosome undergo frequent large deletions and AUDs are thought to act as buffers against progressive deletions and to protect chromosomal regions carrying essential genes (Redenbach *et al.*, 1994). Sequences which lie between the AUD and the chromosome end have been found to mainly contain genes not essential for cell viability (Redenbach *et al.*, 1996). The discovery that the *pca* genes are located outside of the genetically unstable regions of the *S. coelicolor* chromosome indicates the underlying importance of the β-ketoadipate pathway in the metabolic activities of this soil bacteria.

Once the complete *pca* operon has been fully sequenced from isolate 2065 as well as from *S. coelicolor*, the gene organization and operon structure may be examined more closely and the individual genes and their protein products analyzed in more detail. Characterization of the inducing metabolites and the mechanisms of regulation of the β-ketoadipate pathway in *Streptomyces,* and detailed examination of the genetic structure of the pathway will allow comparison to such pathways from other bacteria. This will provide useful information about the biology of the β-ketoadipate pathway and the selective pressures that have shaped the diversity

seen among widely divergent bacteria.

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