TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS OF PROTEIN SYNTHESIS DURING AROMATIC ACID CATABOLISM BY STREPTOMYCES VIOLACEUSNIGER

by

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THE UNIVERSITY OF BRITISH COLUMBIA

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ABSTRACT

Streptomycetes were isolated from soil samples and screened for biodegradative activity against lignin-related aromatic compounds. One isolate, *Streptomyces violaceusniger,* efficiently utilized 4-hydroxy-3-methoxybenzoic acid (vanillate) and 4-hydroxybenzoic acid (*p*-hydroxybenzoate) as sole sources of carbon and energy. *S. violaceusniger* was able to biotransform indole to the dye indigo, indicative of aromatic dioxygenase activity. Colorimetric Rothera assays demonstrated the induction of aromatic dioxygenase activity in *S. violaceusniger* in the presence of vanillate and *p*-hydroxybenzoate. Two-dimensional gel electrophoresis of total cell proteins revealed the synthesis of specific groups of proteins upon growth in the presence of vanillate, *p*-hydroxybenzoate or 3,4-dihydroxybenzoic acid (protocatechuate). The amino-terminal sequence of a heavily expressed 52 kDa protein, induced by vanillate and *p*-hydroxybenzoate, was highly similar to the amino-terminus of a hypothetical 55 kDa protein discovered during the course of the *Escherichia coli* genome sequencing project. The *S. violaceusniger* sequence aligns with conserved regions adjacent to nucleotide binding domains of the GTP-binding α-subunit protein family and valyl-tRNA synthetases. Because biodegradative monooxygenases are known to possess nucleotide binding domains near the amino terminus and the protein is synthesized in such abundance, it is suggested that the 52 kDa protein is an enzyme involved in aromatic biodegradation. The results of this work are of interest to the study of lignin biodegradation, and also to the study of the catabolism of phenolic compounds in *Streptomyces*.

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This thesis is dedicated to the memory of Prof. Paul Steiner, Department of Wood Science, Faculty of Forestry, UBC, without whom this work would not have been possible.

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INTRODUCTION

Streptomycetes are gram-positive, filamentous eubacteria well known for their roles as producers of secondary metabolites, namely antibiotics. These prokaryotes also play a major role in global recycling of organic carbon (1). Secondary metabolite production by *Streptomyces* has been studied intensely for decades; however, biodegradation processes performed by *Streptomyces* have not been examined in detail.

Of the biodegradative pathways studied in *Streptomyces,* the biodegradation of lignin and lignin-related aromatic compounds has been scrutinized most thoroughly. The biodegradation of lignin is of interest because it presents an economical alternative to, or at least reduces the need for chemical delignification processes in the pulp and paper industry (2). Furthermore, lignin solublizing enzymes are sought after by industry because they may allow delignification by-products to be transformed into useful value-added chemicals (3). Biological activity against lignin-related methoxylated aromatic acids (Figure 1) has long been thought to be an indication of an organisms' potential to be effective against lignin itself, though this remains to be proven (4). Degradation of lignin-related aromatic compounds by prokaryotes has been studied thoroughly, and pathways leading to the catabolism of such compounds through the common intermediates 1,2-benzenediol (catechol), or 3,4-dihydroxybenzoic acid (protocatechuate) have been elucidated. The degradation of 4-hydroxy-3-methoxybenzoic acid (vanillate) by *Streptomyces setonii* 75Vi2 (Figure 2A) has been shown to proceed by decarboxylation of vanillate to yield 4-hydroxy-3-methoxybenzene (guaiacol) (5). Guaiacol is then demethoxylated to form catechol leading to mineralization via the *ortho* (3-oxoadipate) pathway. The degradation of vanillate by decarboxylation to guaiacol is unlike the degradation pathway elucidated in *Pseudomonas* (6), in which vanillate is demethoxylated to form protocatechuate, then mineralized (Figure 2B). While the *Pseudomonas* vanillate demethylase gene has been cloned and sequenced, information

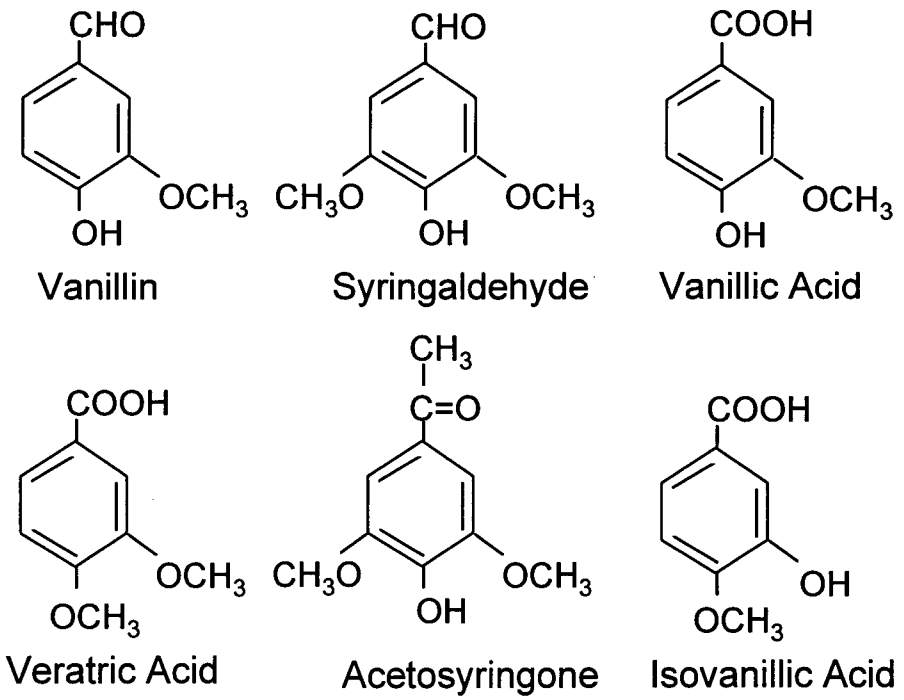


Figure 1 Some common low molecular weight products of partially decayed lignin.

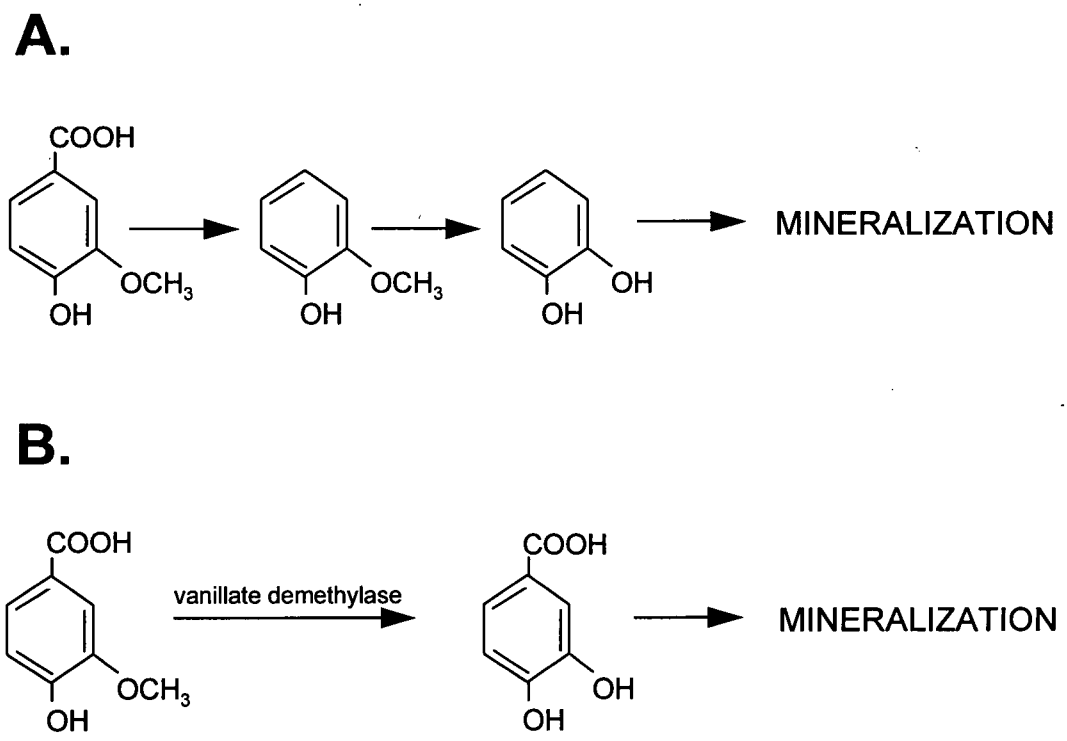


Figure 2 Two pathways of vanillate catabolism. (A) *Streptomyces* degradation of vanillate via a decarboxylase, leading to catechol formation and mineralization. (from Crawford *et al.,* 1978) (B) *Pseudomonas* biodegradation of vanillate via vanillate demethylase, leading to protocatechuate and mineralization. (from Brunel *et al.,* 1988)

regarding the molecular genetics of degradation of lignin-related aromatics by *Streptomyces* remains limited. The *Streptomyces* vanillate degradation pathway was deduced using spectrophotometric analysis of cell-free extracts and culture supernatant catabolic intermediates. The vanillate and *p*-hydroxybenzoate catabolic genes of *Streptomyces* have yet to be isolated, cloned and sequenced.

Not only is information regarding the genetics of aromatic acid degradation in *Streptomyces* limited, the regulation of streptomycete catabolic pathways is also little understood. Regulatory mechanisms of aromatic compound catabolic pathways in *Pseudomonas, Rhizobium, Agrobacterium, Alcaligenes, Acinetobacter, Nocardia,* and other prokaryotes have been shown to be as diverse as the organisms themselves. It is known that in the actinomycete *Nocardia opaca,* protocatechuate has no inductive function in its own degradation, β-ketoadipate being the specific inducer (7). *p*-Hydroxybenzoate hydroxylase, on the other hand, is substrate-induced in *N. opaca.* By comparison, in *Alcaligenes, Acinetobacter* and *Agrobacterium,* both the protocatechuate and *p*-hydroxybenzoate catabolic pathways are substrate-induced. Catabolic genes are usually organized into operons (8); such clustering allows an entire set of genes encoding biodegradative enzymes to fall under transcriptional control of a single regulatory step. A well characterized example is the *sal* operon of *Pseudomonas putida* (9), which consists of catabolic genes specifying the oxidative degradation of salicylic acid, the key intermediate in the pathway of naphthalene catabolism, as well as degradative genes for downstream intermediates such as catechol and *cis,cis*-muconic acid. The *sal* operon is positively regulated by a LysR-like regulatory protein, NahR, which binds a nucleotide motif upstream of the *sal* operon. LysR-like transcriptional regulators have been shown to be involved in activation of catabolism of a number of aromatic compounds (9). The work presented here examines, using protein two-dimensional gel electrophoresis, gene expression in response to catabolism of lignin-related aromatics by *Streptomyces*

violaceusniger. Proteins expressed during these catabolic processes may be involved in the regulation of associated catabolic pathways (such as the LysR family), or may be enzymes active in degradation of lignin-related aromatic acids.

We have isolated streptomycetes from soil which utilize several lignin-related aromatic acids as sole sources of carbon and energy. One isolate, identified by cell wall fatty acid methyl ester (FAME) analysis as *Streptomyces violaceusniger,* grew well on minimal medium supplemented with vanillate or *p*-hydroxybenzoate. Cultures of *S. violaceusniger* were pulse-labeled at several time points with ^{35}S-methionine following the addition of vanillate, *p*-hydroxybenzoate or protocatechuate to the growth medium. Total cellular proteins were visualized by two-dimensional gel electrophoresis (2D-PAGE) and autoradiography for analysis of the induction or repression of protein synthesis in response to these phenolic compounds. It is shown in this work that specific groups of proteins are induced or repressed in their synthesis in response to growth in the presence of lignin-related aromatic acids.

To our knowledge, this is one of the first reported uses of 2D-PAGE in the study of catabolic processes. A study by Lupi *et al.* highlighted the 2D-PAGE profiles of *Pseudomonas putida* KT2442 when exposed to 2-chlorophenol (10). *P. putida* KT2442 was shown to produce a number of proteins when grown in the presence of the aromatic compound; however, the organism was not capable of catabolizing 2-chlorophenol and was instead displaying a stress response. 2D-PAGE was also applied by Matin *et al.* to the study of xenobiotic-induced stress proteins in *Escherichia coli* (11). Similar to the work of Lupi *et al.*, this was a stress response study and did not involve an organism that catabolized the xenobiotics.

The information presented in this thesis should allow for the future characterization of the streptomycete genes responsible for vanillate and *p*-hydroxybenzoate catabolism. The data accumulated from these 2D-PAGE studies complements work being performed by Thompson *et al.* (12) on the *Streptomyces* "proteome" (a term used to describe an organism's entire protein complement) (13). Proteome studies such as the work described here, when combined with evolving knowledge of the *Streptomyces* genome, will provide a better understanding of this industrially significant organism.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Soil samples were taken from various sites around the University of British Columbia campus. In addition, borehole soil samples were obtained from sawmill sites in British Columbia that had been evaluated for soil contamination. Soil samples were air-dried for several days, and 1 gram of each sample was resuspended in 4 mL of sterile distilled water. Each sample was vortexed vigorously for ten seconds and then allowed to settle for about fifteen minutes to remove large particulate matter. Each sample was diluted 10^{-2}, and 0.1 mL of each diluted sample was spread on ISP4 agar plates containing 75 μ g/ml cycloheximide (Sigma) to suppress fungal growth. Plates were incubated overnight at 30°C lid-side up to allow liquid to soak into the agar. Subsequently plates were incubated lid-side down for 4-7 days. After incubation, sporulating colonies presumed to be streptomycetes were picked by sterile toothpicks and cultivated on ISP4 plates. *S. violaceusniger* was identified by both cell wall fatty acid methyl ester (FAME) analysis and 16S rRNA analysis by services provided at the West East Centre.

Screening of Isolates for Catabolism of Aromatic Acids

Screening for the catabolism of aromatic acids was carried out on agar minimal medium containing aromatic acid (0.5-3 g/L), trace elements, phosphate buffer and the pH indicator bromothymol blue, pH 7.2 (14). At pH values 7.2 and below, bromothymol blue is green to yellow. At pH values above 7.2, the indicator is blue. Degradation of the aromatic acid being tested would result in decreased acidity in the medium, thus causing a rise in the pH and a change of the medium colour from green (at pH 7.2) to blue (at pH > 7.2). In most cases, isolates which caused the medium to turn blue were also observed to grow well in the presence of the aromatic acid being tested (Figure 3). Isolates which were positive in the bromothymol blue plate assay were grown in liquid minimal medium in the presence of the aromatic acid for which catabolism was indicated by the plate

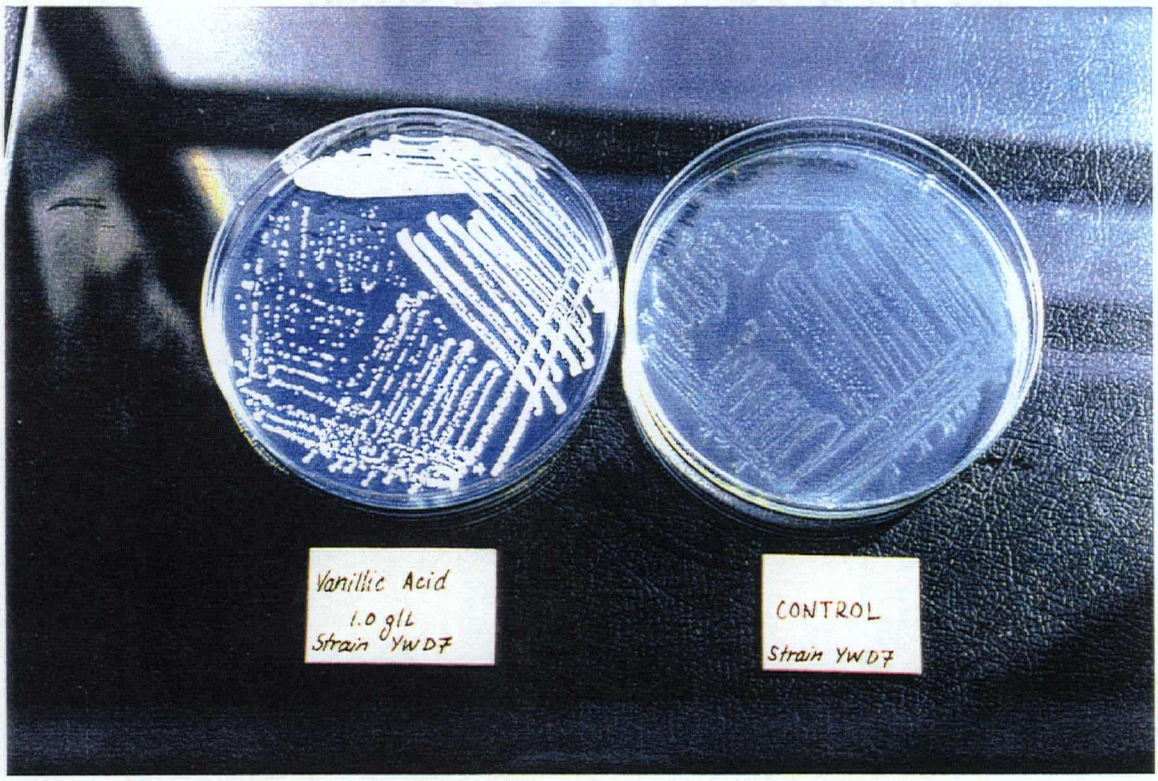


Figure 3 Bromothymol blue minimal medium agar plate assay. Plate on left contains 1 g/L vanillate, plate on right contains no aromatic carbon source. Both plates have been streaked with *Streptomyces violaceusniger* and incubated for one week at 30 degrees Celsius.

assay. Culture supernatants sampled at various times during time-course studies were analyzed by UV/Vis spectrophotometry. Decrease in absorbance at the λ_{max} for the particular aromatic being studied (for example, 250 nm λ_{max} for vanillate) was evidence that the aromatic was indeed being mineralized. In the case of vanillate catabolism by *S. violaceusniger,* culture supernatants were injected onto a HPLC C_{18} column, and degradation of the aromatic acid was readily observed when the results were correlated to known vanillate standards.

Assays for Oxygenase Activity

Bioconversion of Indole to Indigo. Conversion of colourless indole to the blue dye indigo is an indication of the presence of catabolic dioxygenase activity (15). *S. violaceusniger* was plated on both mineral salts medium with 0.5 g/L yeast extract (MSMYE) agar (5) and tryptic soy agar and incubated at 30°C for two days until mycelial growth was clearly visible. Two 0.25 inch filter paper discs were placed at opposite ends of the plate. 20 µL of 175 mM indole in 50% ethanol was added to one disc; an equivalent volume of 50% ethanol was added to the other disc. Observations were made after approximately four days incubation at 30°C. For a liquid media assay, 100 mL of MSMYE in a 250 mL baffled erlenmeyer flask was inoculated with one colony of *S. violaceusniger* from ISP4 agar and incubated for 36 hours. Indole was added to the flask to a final concentration of 2 mM from a stock solution of 175 mM in 50% ethanol. Upon observation of colour change in the medium, the culture was extracted with 10 mL chloroform and analyzed by UV/Vis spectrophotometry.

Colorimetric Rothera Assay. The colorimetric Rothera assay (16) determines if oxidative biodegradation of aromatics is occurring in cell-free extracts, and whether catabolism proceeds via the *ortho* (intradiol) or *meta* (extradiol) cleavage mechanisms. *S. violaceusniger* was grown in tryptic soy broth until the culture reached approximately

mid-logarithmic growth phase. Cells were harvested by centrifugation, washed twice in isotonic minimal medium, then resuspended in fresh minimal medium. Cells were incubated for nine hours under the following conditions: bromothymol blue liquid minimal medium + 0.3% vanillate, bromothymol blue medium + 0.3% *p*-hydroxybenzoate, bromothymol blue medium + 0.3% glucose and TSB + 0.3% glucose. Cells were harvested and washed twice in 50 mM Tris-HCl, pH 8.5, before being frozen at -20°C. Cells were thawed on ice, then resuspended in 2 mL of 50 mM Tris-HCl, pH 8.5, 1 mg/mL lysozyme, 100 μg/mL DNAse I, and 100 μg/mL RNAse A. Disruption of the cells was achieved by incubation at 37°C for one hour and homogenizing the suspension on ice. Unlysed cells and cell debris were removed by centrifugation. Assay reactions consisted of 100 μL cell-free extract, 100 μL of 100 mM sodium phosphate, pH 7.5, and 10 μM of either protocatechuate, catechol or gentisate as substrate. Reactions were observed at this time for the development of a yellow colour, characteristic of 2-hydroxy muconic semialdehyde, which is indicative of *meta* cleavage activity. Reaction tubes were then incubated for three hours at 37°C, after which solid ammonium sulfate was added to saturation, as well as one drop of 5N ammonium hydroxide and one drop of 25% sodium nitroprusside. Development of a purple colour indicated a positive reaction for *ortho* cleavage indicating the presence of β-ketoadipate, a key intermediate in *ortho* cleavage pathways.

Time Course Experiments for 2D-PAGE

One-Point Sampling. Preliminary time course experiments involved determining the rates of growth and vanillate catabolism, then harvesting vanillate-degrading cultures in mid-logarithmic phase of degradation for 2D-PAGE analysis. For these experiments, one colony of sporulating *S. violaceusniger* on ISP4 agar was used to inoculate a seed culture consisting of 10 mL of MSMYE and vanillate at 7.3 mM (1 g/L) in a 50 mL baffled flask (Bellco). The seed culture was incubated at 30°C, 260 rpm for approximately 21 hours,

after which the cells were centrifuged, washed twice in 10 mL sterile distilled water, and resuspended in 10 mL sterile, distilled water. 100 mL preparations of MSMYE with and without 7.3 mM vanillate in 250 mL baffled flasks were inoculated with 1 mL of the washed seed culture. Vanillate degradation was measured as the decrease in A_{250} and cell density was measured as the increase in A_{578}. In replicate experiments, cultures growing in the presence and absence of vanillate were harvested by centrifugation when vanillate degradation was most rapid, for cell lysis and analysis by 2D-PAGE.

Multi-Point Sampling with ^{35}S Labeling. For a more detailed study of the catabolism of aromatic acids by *S. violaceusniger,* vanillate, *p*-hydroxybenzoate or protocatechuate was added to a final concentration of 3.6 mM to separate 100 mL cultures of the organism in MSMYE in 250 mL baffled flasks during the early logarithmic phase of growth. A control culture contained no aromatic acids. Immediately prior to addition of the aromatic acids, 1 mL of the control culture was labeled with 100 μCi ^{35}S methionine/cysteine (DuPont-NEN) at 30°C, shaking at 260 rpm for 30 minutes in a 15 mL polypropylene tube. After incubation, the labeled sample was pelleted, frozen in a dry ice ethanol bath and stored at -70°C. 1 mL aliquots of each induced culture were labeled similarly 1, 2, 5, 12.5 and 18 hours after addition of the aromatic acids. The concentration of each aromatic in the culture medium was measured as absorbance at the λ_{max} for each compound in a UV/Vis spectrophotometer.

Preparation of Protein Extracts for Electrophoresis

Cell pellets were thawed on ice and washed twice in 2 mL lysis buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 100 μg/mL PMSF (Sigma), 1 μg/mL Pepstatin A), then resuspended in 0.5 mL (radiolabeled cells) or 1 mL (non-labeled cells) of the same buffer. Samples were sonicated on ice with a microtip in three bursts, 15 seconds each, with 15 second delays to allow for cooling. Immediately after sonication, samples were

centrifuged at 14,000 x g for 15 minutes. The pellet was discarded, and DNase/RNase solution (24 mM Tris base, 476 mM Tris HCl, 50 mM $MgCl_2$, 1 mg/mL DNAse I, 0.25 mg/mL RNAse A) was added to 1/10 the total volume of each supernatant, followed by incubation for 10 minutes on ice. For non-labeled samples from one-point experiments, the protein concentration of the extract was determined and volumes of extract containing 150 μg total cell protein were concentrated in 10,000 Da cut-off ultracentrifugation devices (Microcon-10, Amicon) and resuspended in 10 μL isoelectric focusing buffer (9.9M urea, 4% NP-40, 2.2% ampholytes pH 3-10, 100 mM DTT) for 2D-PAGE and silver staining. For labeled, multi-point experiment samples, total cell extracts (approximately 1 mL per time-point sample) were concentrated by ultrafiltration at a molecular weight cut-off of 10,000 Da, then resuspended in 20 μL isoelectric focusing sample buffer. In addition to concentrating the sample, the ultrafiltration step removed unincorporated ^{35}S-labeled amino acids.

Measurement of ^{35}S-Methionine/Cysteine Protein Labeling

To measure relative amounts of protein synthesis upon induction of cultures with aromatics, 1 μL of each sample protein cell extract was spotted on a Whatman GF/A glass fiber filter disc and placed in a scintillation vial with 5 mL of scintillation fluid. The radioactivity in each vial was counted for 1 minute. Background consisted of 1 μL of IEF sample buffer treated in the same manner.

Determination of Protein Concentration

Protein concentration in cell free extracts was measured by the bicinchoninic acid method (17) using a kit from Sigma Chemicals Ltd. Bovine serum albumin was used for preparation of a standard curve.

2D-PAGE Protein Electrophoresis

Two-dimensional gel electrophoresis was performed according to Garrels (18) using the protocols, chemicals, and equipment of the Investigator system (Oxford Glycosystems). First dimension isoelectric focusing (IEF) tube gels incorporated ampholytes in the pH range 3-10, optimized for analysis of total cell extracts. Second dimension high-tensile strength slab gels contained 12.5% acrylamide and an acrylamide to *N',N'*-methylene-bisacrylamide ratio of 30:0.65 (Duracryl, Oxford Glycosystems). Ten µL of each sample containing 1 x 10^6 CPM was applied to each IEF tube and focused for 18,000 $V \cdot h^{-1}$. After second dimension electrophoresis, slab gels were agitated in fix solution (40% methanol, 10% glacial acetic acid) for 1 hour, followed by treatment with a fluor solution (Enhance, DuPont-NEN) and dried. Proteins were visualized by exposure of dried gels to Kodak Bio-Max MR film for 7-10 days at -70°C.

Computer-Aided QUEST Analysis of Autoradiograms

Autoradiograms were scanned with a Howtek scanner controlled by PDQuest 2D analysis software version 5.0 (PDI) for 2D gels. Automated spot detection was performed using the PDQuest standard algorithms for ^{35}S-labeled gels. Spot quantitation was performed by computer generated two-dimensional Gaussian modeling. Gels were compared by a process of landmarking and matching spots among all gels in the experiment. A correction factor for each 2D gel protein spot was calculated as the total OD (optical density units) detected in the standard gel (a master reference gel created by merging all gels in a time course matchset) divided by the total OD detected in the particular gel to which each protein spot belonged. The PDQuest gel analysis software was run on a SparcStation 2 (Sun Microsystems).

Protein Sequencing

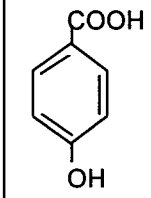
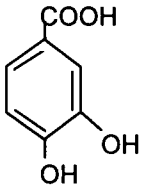
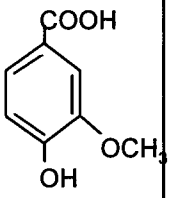
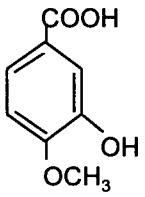
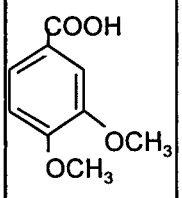
To obtain amino-terminal sequence from 2D gels, 150 μg of total cell protein from a non-radiolabeled, vanillate-induced sample was run on ten replicate gels and stained post-electrophoretically by the zinc-imidazole method (19). The zinc-imidazole stain results in a white background and clear protein spots that are not fixed in the gel matrix. The clear spots are visualized when the gel is viewed against a dark background or several centimeters from a lightbox. Protein spots of interest were cut from each gel and placed together in a single 1.5 mm thick well of a BioRad Protean II xi 12.5% acrylamide slab gel. Laemmli SDS-PAGE sample buffer (1x) was added on top of the gel pieces in the well, and the protein was electrophoresed into the slab gel. The slab gel was blotted to Immobilon-P^{SQ} PVDF membrane (Millipore) using the semi-dry graphite blotter supplied with the Investigator 2D system. A three buffer protocol was used, in which ε-amino-n-caproic acid was substituted for glycine (20). The membrane was stained with a 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 protein band was excised from the membrane, air-dried, then placed in a 1.5 mL eppendorf tube for delivery to the Nucleic Acid Protein Sequencing (NAPS) Unit at the University of British Columbia.

RESULTS AND DISCUSSION

Isolation of Streptomycetes from Soil

Drying of soil samples for several days prior to suspension and plating greatly enhanced the number of streptomycetes isolated. Soils consisting of sand and/or humic matter seemed to contain the greatest number of streptomycetes. Conversely, soil rich in wet clay contained few, if any, streptomycetes. The soil sample of most interest was taken from a wood-waste landfill at a sawmill in the British Columbia interior. Consisting of loose humic matter and wood debris (ie. sawdust), this sample produced an abundance of streptomycetes of varying morphologies. When screened for degradation of lignin-related phenolic compounds, many isolates were found to be active. However, each isolate displayed an unique "profile" of degradation capabilities (ie. they did not all degrade the same selection of compounds) (Figure 4). These overlapping catabolic profiles indicate that in a microcosm such as a wood-waste landfill, lignin degradation may not necessarily be attributed to one particular lignin degrading organism, but rather to a consortium of bacteria with varying degrees of catabolic activity against lignin.

For the purposes of this study, one isolate was chosen for its ability to degrade vanillate and *p*-hydroxybenzoate rapidly. This isolate, on ISP4 agar, had dark gray mycelia, light gray spores, and produced a brillant yellow pigment that later changed to a dull orange colour upon aging. Cell wall fatty acid methyl ester analysis performed by gas chromatography identified this isolate as *Streptomyces violaceusniger* (Figure 5). 16S rRNA analysis of the isolate confirmed its identity as a member of the genus *Streptomyces* (Figure 6). *S. violaceusniger* was routinely cultivated on ISP4 agar or tryptic soy agar (TSA).

Isolate	A 	B 	C 	D 	E 
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NL15-2D	++++	++	++	-	-
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NL15-2M	+++	++	++	-	-
NL15-2N	+++	-	+++	-	-
NL15-2P	+++	++	++	-	-
NL16-1A	+++	++	+++	-	-
S. violaceusniger	++++	+++	++++	-	-
S. rimosus	+++	-	-	-	-
S. lividans	+++	-	-	-	-

Figure 4 *Streptomyces* catabolism of lignin-related aromatic acids. Letter designations of aromatic acids are as follows: (A) *p*-hydroxy-benzoate (B) protocatechuate (C) vanillate (D) isovanillate (E) veratrate. NL isolates were obatined from a sawmill wood waste landfill in the interior of British Columbia. *Streptomyces rimosus* and *Streptomyces lividans* 1326, two commonly used laboratory organisms, are included for comparison purposes. (+) to (++++) indicate degree to which catabolism was qualitatively observed by the bromothymol blue plate assay. (-) indicates no observed catabolic activity.

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6.722	666	0.039	. . .	13.752	. . . . .	. . .		
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8.002	53796	0.035	0.977	14.622	15:0 ISO	10.46	ECL deviates 0.001	Reference 0.000
8.140	142716	0.035	0.975	14.713	15:0 ANTEISO	27.70	ECL deviates 0.002	Reference 0.001
8.576	12036	0.036	0.968	15.000	15:0	2.32	ECL deviates 0.000	Reference -0.001
9.330	6234	0.038	0.959	15.458	16:1 ISO H	1.19	ECL deviates -0.003	
9.608	122376	0.039	0.956	15.627	16:0 ISO	23.28	ECL deviates 0.001	Reference -0.000
9.919	2520	0.038	0.952	15.816	16:1 CIS 9	0.48	ECL deviates -0.001	
10.218	32172	0.040	0.949	15.998	16:0	6.08	ECL deviates -0.002	Reference -0.004
10.934	5796	0.042	0.942	16.416	16:0 9? METHYL . . .	1.09	ECL deviates 0.000	
11.119	5136	0.041	0.940	16.524	17:1 ANTEISO C . . .	0.96	ECL deviates -0.001	
11.298	24930	0.040	0.939	16.629	17:0 ISO	4.66	ECL deviates 0.000	Reference -0.002
11.458	53718	0.041	0.937	16.722	17:0 ANTEISO	10.02	ECL deviates 0.000	Reference -0.002
11.577	1452	0.044	0.936	16.792	17:1 CIS 9	0.27	ECL deviates -0.000	
11.741	2058	0.050	0.935	16.888	17:0 CYCLO	0.38	ECL deviates -0.000	Reference -0.002
11.932	4968	0.044	0.934	16.999	17:0	0.92	ECL deviates -0.001	Reference -0.003
13.038	1326	0.044	0.926	17.633	18:0 ISO	0.24	ECL deviates 0.001	Reference -0.001
13.677	618	0.038	0.922	17.999	18:0	0.11	ECL deviates -0.001	Reference -0.003
16.127	2880	0.111	. . .	19.415	. . . . .	. . .	> max ar/ht	

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
326791800	525678	520866	99.08	502423	17	0.001	0.002

ACTIN1 [Rev 3.80] Streptomyces 0.647
S. violaceusniger 0.647
S. v. hygroscopicus 0.647
S. v. h. hygroscopicus 0.647
S. v. violaceusniger 0.636
S. exfoliatus 0.594
S. halstedii 0.436
S. h. scabies 0.436
S. h. olivaceus . . . . . 0.356 (incl. S. scabies ISP5078)

Figure 5 Cell wall fatty acid methyl ester (FAME) identification of *Streptomyces violaceusniger.*

```
                                                                    Smallest
                                                                      Sum
                                                          High      Probability
Sequences producing High-scoring Segment Pairs:           Score     P(N)        N

gb|U22972|SSU22972    Streptomyces sp., strain GP 772 16S...  1170  8.4e-139  3
gb|U22973|SSU22973    Streptomyces sp., strain GP 773 16S...  1170  8.4e-139  3
emb|X60514|SC16SRNA   S.coelicolor rRNA 16S gene              1170  5.9e-138  3
gb|U22974|SSU22974    Streptomyces sp., strain GP 770 16S...  1156  1.3e-137  3
emb|X79852|SG16SRR    S.galbus gene for 16S ribosomal RNA     1152  3.2e-137  3
emb|X79325|SG16SRRN   S.galbus (DSM 40480) 16S rRNA gene.    1152  3.2e-137  3
emb|X79853|SH16SRR    S.hygroscopicus gene for 16S riboso...  1152  3.2e-137  3
emb|X80824|SC16SRDN   S.caelestris 16S rRNA gene              1152  3.2e-137  3
emb|X79854|SL16SRR    S.lincolnensis gene for 16S ribosom...  1152  3.2e-137  3
gb|M27245|STMRRND     Streptomyces ambifaciens rrnD gene ...  1170  1.1e-136  3
emb|X79851|SB16SRRN   S.bikiniensis gene for 16S ribosoma...  1152  1.8e-136  3
emb|X79323|SM16SRN    S.mashuense (DSM 40221) 16S rRNA gene.  1143  1.8e-136  3
emb|X79326|SO16SRN    S.ornatus (DSM 40307) 16S rRNA gene.    1143  1.8e-136  3
emb|Y00484|SLRRNB     Streptomyces lividans rrnB gene for...  1170  3.3e-136  4
emb|X80825|SSRDNA16S  S.subrutilus 16S rRNA gene              1134  1.0e-135  3
emb|X81574|SSPRNA16S  Streptomyces sp. 16S rRNA gene           855  1.8e-135  4


>gb|U22972|SSU22972 Streptomyces sp., strain GP 772 16S ribosomal RNA, partial
           sequence.
           Length = 1481

 Minus Strand HSPs:

 Score = 1170 (327.2 bits), Expect = 8.4e-139, Sum P(3) = 8.4e-139
 Identities = 238/243 (97%), Positives = 238/243 (97%), Strand = Minus / Plus

Query:   248 GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGGCGGTACCTGCAGAA 189
            ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct:   382 GCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAA 441

Query:   188 GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCC 129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   442 GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCC 501

Query:   128 GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGG 69
            |||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||
Sbjct:   502 GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTTGCGTCGGTTGTGAAAGCCCGGG 561

Query:    68 GCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAT 9
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   562 GCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAA 621

Query:     8 TCC 6
             | |
Sbjct:   622 TTC 624
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Figure 6 16S rRNA sequence confirmation of *Streptomyces violaceusniger* as a member of the genus *Streptomyces.* BLAST database search results are shown. Although *Streptomyces coelicolor* is a good match, the result is likely a consequence of limited data in the streptomycete 16S rRNA database. The isolate did not show any of the morphological characteristics of *S. coelicolor.*

Biotransformation of Indole to Indigo

Pseudomonas dioxygenases active in the degradation of naphthalene have been shown to oxidize indole, a product of tryptophan catabolism in *Escherichia coli* (15). The resulting oxidized compound undergoes dimerization via an abiotic process to form indigo, a commercial blue dye used in the colourization of blue jeans. Because enzymatic oxidation is the initial step in the bioconversion process, the transformation of indole to indigo is an indicator of dioxygenase activity. When *S. violaceusniger* was grown on MSMYE agar plates in the presence of indole, a dark blue, water-insoluble pigment formed in the medium following several days' growth. Pigment formation was also observed when the organism was grown on tryptic soy agar in the presence of indole, but at a reduced level. *S. violaceusniger* was grown in MSMYE to high culture density when indole was added to a final concentration of 2 mM. After overnight incubation, the culture medium appeared blue, and blue pigment specks were observed in the foam covering the medium. Upon extracting the culture with chloroform, the organic phase appeared a rich blue colour while the aqueous phase retained the cell mass and appeared clear, the original colour of MSMYE. UV/Vis spectrophotometric analysis of the blue chloroform extract confirmed the presence of indigo when compared to a spectrum of commercially produced indigo (Sigma) (data not shown). Ensley *et al.* reported that indigo formation is a property of dioxygenase systems that form *cis*-dihydrodiols from aromatic hydrocarbons (15). While the work performed by that group focused on naphthalene catabolism by *Pseudomonas putida* PpG7, the production of indigo by *S. violaceusniger* is indicative of the organism's healthy aromatic catabolic processes. Furthermore, it is possible that the enzymes involved in the production of indigo may be linked to catabolism of the aromatics studied in this thesis.

Evidence of Protocatechuate-3,4-Dioxygenase Activity

Results for the colorimetric Rothera assay are listed in Table 1. *Ortho* cleavage activity, indicative of the presence of protocatechuate 3,4-dioxygenase, was observed in the cell-free extracts from 0.3% *p*-hydroxybenzoate, 0.3% vanillate and TSB + 0.3% vanillate grown cells. No other *ortho* cleavage, gentisate 1,2-dioxygenase or *meta* cleavage activity was detected with this assay. The strongest enzyme activity was observed in the cell-free extract from *p*-hydroxybenzoate-grown cells. The results of this assay suggest that both *p*-hydroxybenzoate and vanillate are degraded via the intermediate protocatechuate and not catechol as was observed for *Streptomyces setonii* 75Vi2 (21). Therefore, it is probable that an enzyme with functions similar to the vanillate demethylase found in *Pseudomonas* (6) is involved in vanillate catabolism in *S. violaceusniger*.

Table 1 Colorimetric Rothera assay results. Purple (and shades of purple) color development indicates positive reaction for *ortho* cleavage indicating presence of beta-ketoadipate. NT = not tested

Growth Substrate(s)	Protocat.	Catechol	Gentisate	Extract Alone
0.3% *p*-hydroxybenzoate	purple	brown/orange	yellow/orange	yellow/orange
0.3% vanillate	pink/orange	brown	orange	orange
TSB + 0.3% vanillate	pink/orange	brown	orange	orange
0.3% glucose	orange/brown	brown	brown/yellow	brown/yellow
no extract	orange	orange	orange	NT

Rates of Catabolism of Aromatic Acids

As shown in Figure 7, vanillate, *p*-hydroxybenzoate and protocatechuate were not catabolized at the same rates. Vanillate degradation began approximately two hours sooner than *p*-hydroxybenzoate degradation, accelerated rapidly then gradually slowed down by approximately eight hours after induction. *p*-Hydroxybenzoate, in contrast, began disappearing from the medium about five hours post-induction, and degradation accelerated rapidly until the compound was almost depleted from the medium at 20 hours post-induction. Protocatechuate did not appear to induce its own degradation, and its concentration remained relatively constant in the medium even at 20 hours post-induction, when vanillate and *p*-hydroxybenzoate concentrations had fallen by at least 75% of their original levels. However, by 20 hours post-induction the concentration of protocatechuate had gradually fallen, and assays of the culture 35 hours post-induction indicated that the majority of protocatechuate had been depleted. The differences in degradation rates for the aromatic acids is not unexpected. Aromatic catabolism in bacteria is known to consist of several layers of regulatory control (22), and transcriptional activation systems respond differently depending on which catabolite is present in the environment. For example, Nichols *et al.* demonstrated that *Pseudomonas putida* degrades benzoate in preference to *p*-hydroxybenzoate (23). The observation that protocatechuate degradation displayed such a long lag phase may be explained by the fact that protocatechuate, although an intermediate of many aromatic catabolic pathways, was shown not to induce any *pca* (protocatechuate) gene products in *Rhizobium leguminosarum* as well as in *Agrobacterium tumefaciens* (22, 24). In these prokaryotes, β -carboxy-*cis,cis*-muconate, a degradation intermediate of protocatechuate and other aromatic acids, was shown to induce the *pca* gene products. It has been demonstrated

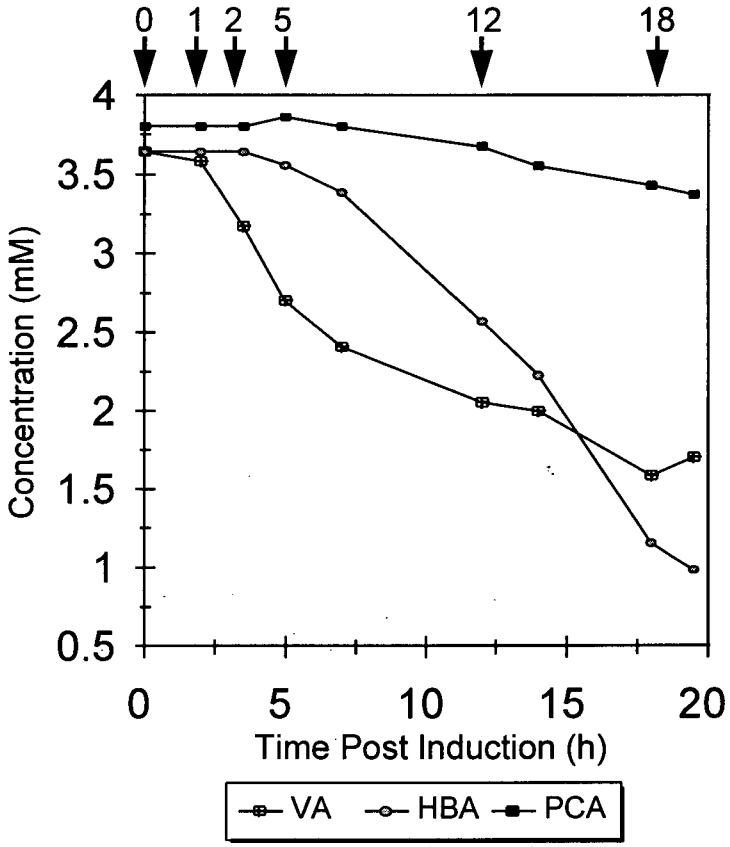

Figure 7 Time course of catabolism of aromatic acids by *Streptomyces violaceusniger*. Time zero is the time point at which 3.6 mM of each aromatic was added to separate cultures in early log phase. VA = vanillate, HBA = *p*-hydroxybenzoate, PCA = protocatechuate. Time points at which cell aliquots were radiolabeled are indicated by the arrows at the top of the graph.

that in these organisms, the protocatechuate degradation enzymes are expressed at very low constitutive levels (25, 26). Therefore, a slow accumulation of β-carboxy-*cis,cis*-muconate would occur until the concentration of the intermediate would be high enough to induce amplified expression of the *pca* genes, resulting in rapid protocatechuate catabolism. Such a response could explain the phenomenon observed in *S. violaceusniger.*

Gene Expression Induced by Aromatic Acids

Addition of 3.6 mM vanillate, *p*-hydroxybenzoate or protocatechuate to *S. violaceusniger* cultures in early exponential phase resulted in a dramatic increase in incorporation of ^{35}S-methionine/cysteine into cell protein versus the uninduced culture, as shown in Figure 8. This period of increased protein synthesis peaked at about two hours post-induction and fell back to the uninduced level at approximately five hours post-induction. The increase in protein synthesis is possibly due to expression of a sensory response elicited by the organism when significant concentrations of aromatics are present in the environment. Interestingly, 2D-PAGE indicated there was no dramatic difference in gene expression patterns of induced cells in comparison to uninduced cells, with the exception of several proteins (as will be outlined below). This observation suggests that the response of the organism to the aromatic acids used in this study is not a global stress response (as seen in the study of *Pseudomonas putida* KT2442 exposure to 2-chlorophenol (10), when dozens of proteins were induced), but rather a highly specific response characteristic of an organism genetically endowed to catabolize lignin-related aromatic acids.

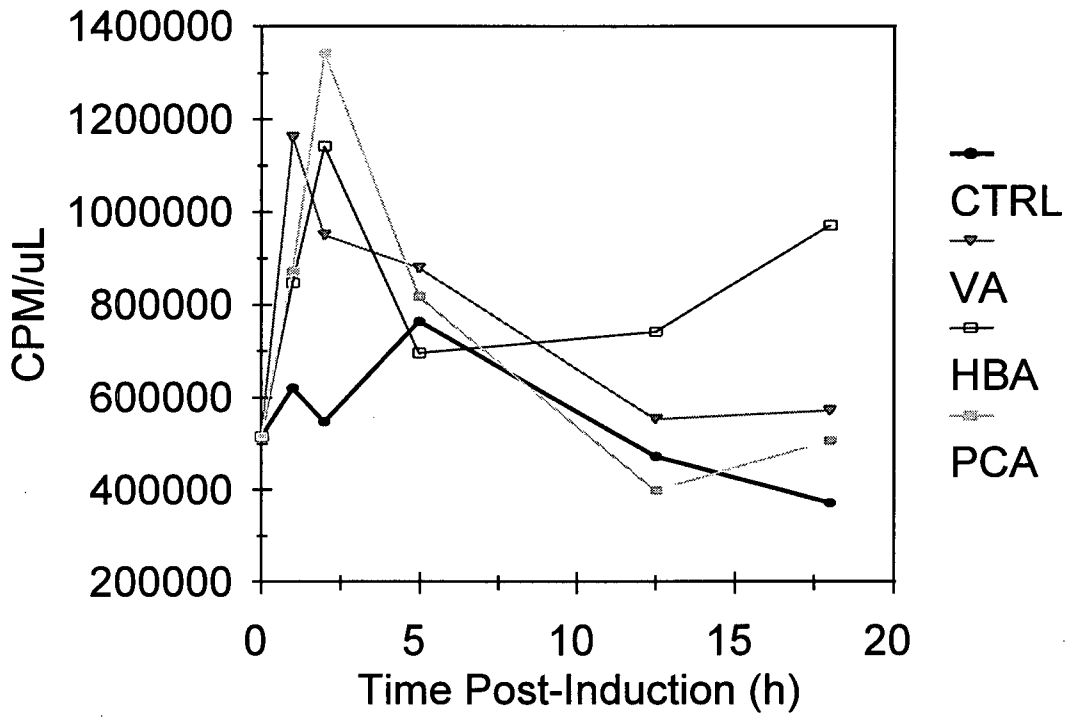

Figure 8 Radioactivity incorporation into cell protein during induction with aromatic acids. Time points are the same as in Figure 5. 1 uL of labeled, crude cell extract for samples at each time point was counted for one minute. Heavy, solid line represents CPM data for the uninduced, control culture.

PDQuest 2D-PAGE analysis of radiolabeled time course samples revealed the induction of a specific group of proteins in response to vanillate, *p*-hydroxybenzoate and protocatechuate. Eight proteins demonstrated significant increases in synthesis versus uninduced cell extracts, as shown in Figure 9 and listed in Table 2. Expression patterns for the proteins are displayed in Figures 10A and 10B. All eight proteins were present in *p*-hydroxybenzoate-induced cell extracts; two of the eight were unique to *p*-hydroxybenzoate induction. Synthesis of six proteins was detected in response to vanillate induction. Only three of the eight proteins responded significantly in protocatechuate-induced cell extracts; this may be due to the low rate of protocatechuate degradation (compared to the other two aromatic acids) during the course of the experiment. In contrast to other 2D-PAGE xenobiotic stress studies with *Pseudomonas* and *E. coli* mentioned in the Introduction, in which many proteins were observed to be repressed upon exposure to xenobiotics, in this study only three proteins were observed to be significantly repressed compared to uninduced cell extracts (Figures 11A, 11B). The seemingly small number of repressed proteins may be indicative of *S. violaceusniger*'s selective adaptation to catabolize the aromatic acids used in this study. By comparison, when in the presence of 3.6 mM catechol, a toxic aromatic diphenol, 2D-PAGE protein patterns of *S. violaceusniger* appeared dramatically different from those typical of induction by vanillate, *p*-hydroxybenzoate or protocatechuate (data not shown). Catechol-induced cells displayed numerous repressed and induced proteins, some of the latter were likely to be due to a stress response.

The identities of the specific aromatic-induced polypeptides will not be known until they are sequenced and the corresponding genes characterized. One of the eight proteins (52 kDa) was submitted for amino-terminal amino acid sequence analysis as is discussed in the next section. The other proteins have been characterized by molecular mass and charge only. Three of the eight proteins were approximately 30 kDa in mass. It is

Table 2 Proteins showing significant variation on growth of *Streptomyces violaceusniger* in aromatic acids. SSP = sample spot number; SSPs are also shown in Figure 9B.

	SSP	m.w. (kDa)	pI
Induced	1821	57.4	4.7
	3717	51.6	5.0
	7622	44.8	5.5
	7623	42.0	5.5
	4408	35.0	5.1
	1309	29.7	4.7
	3210	28.7	5.0
	7205	28.3	5.5
Repressed	7619	41.8	5.6
	1405	34.0	4.7
	4208	27.5	5.2

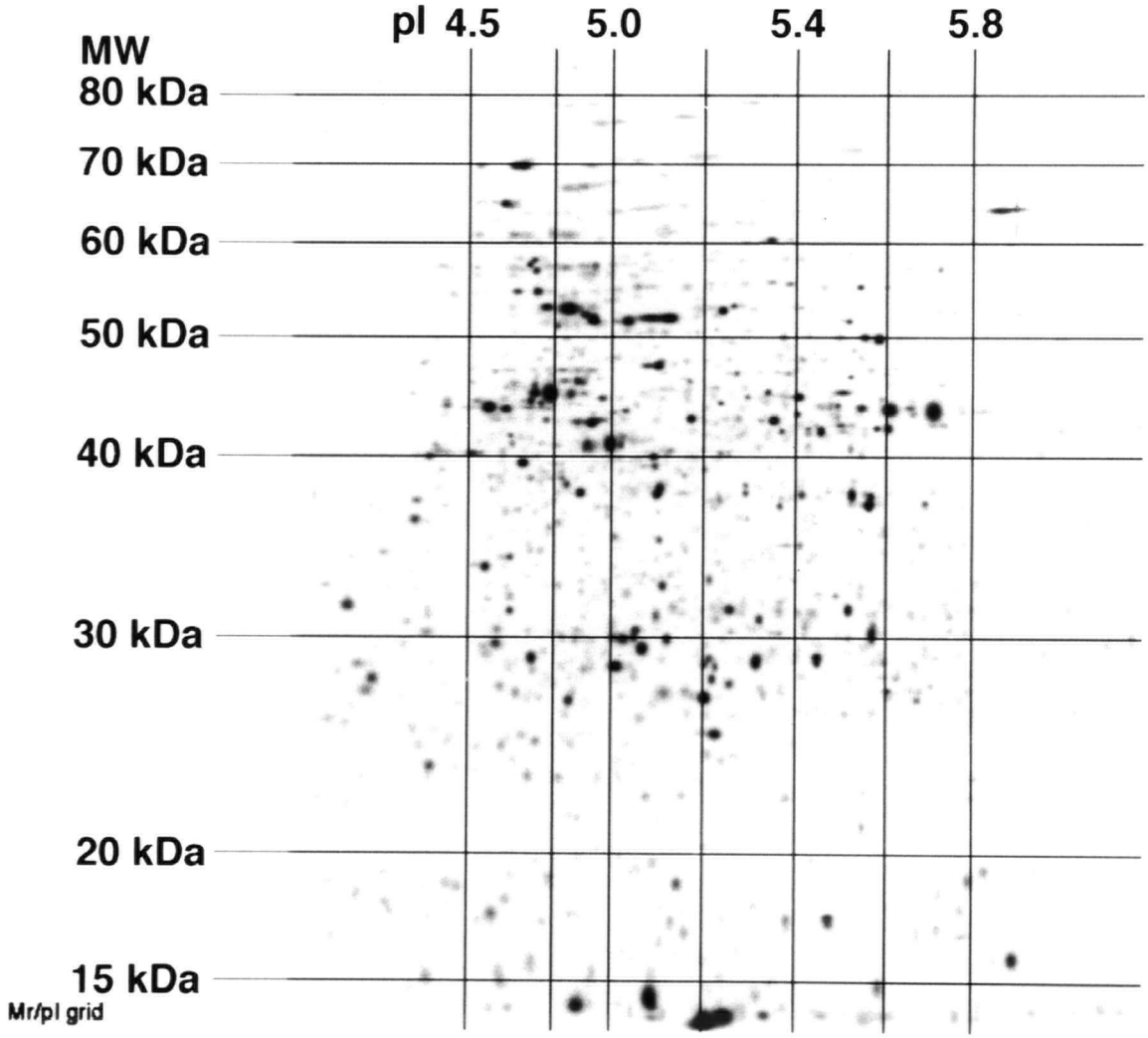

Figure 9 2D-PAGE of aromatic acid induced *Streptomyces violaceusniger* cell extracts. Shown here is the standard reference gel image used to create the PDQuest database. (**A**) molecular weight and pI grid. (**B**, next page) proteins showing induced or repressed synthesis with associated spot database numbers. Induced proteins are marked with squares; proteins showing repressed synthesis are marked with triangles.

B.

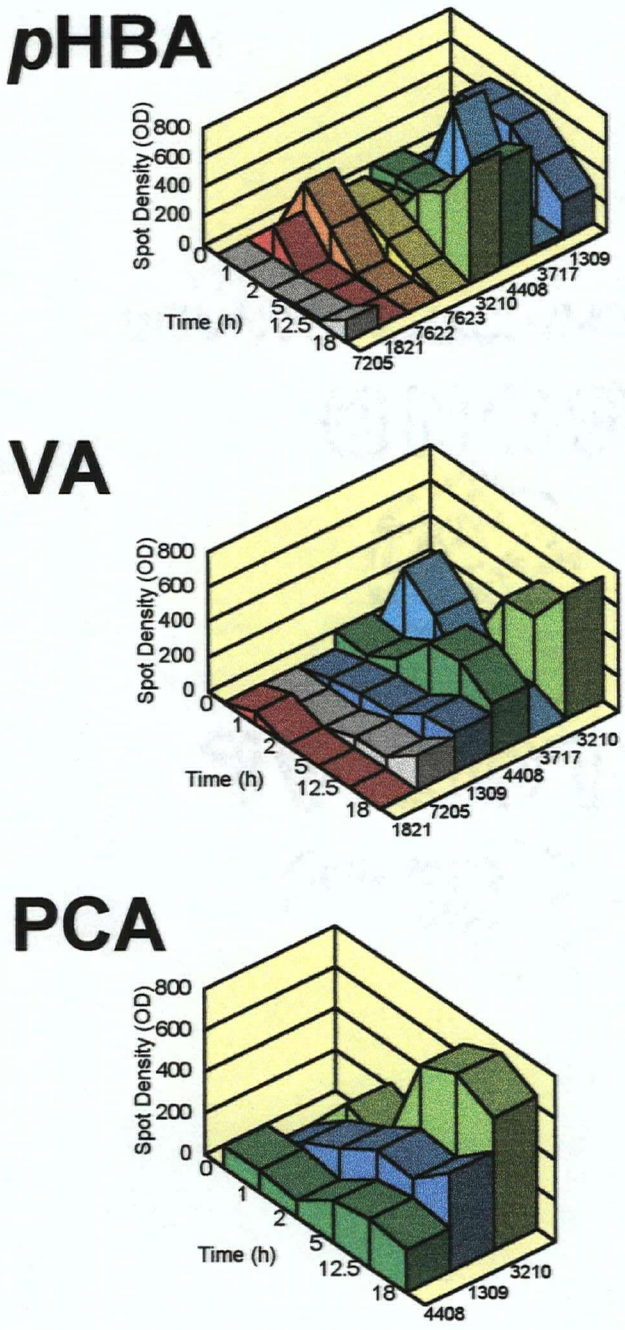

Figure 10A Expression patterns for the eight major aromatic acid induced proteins. (*p*HBA) *p*-hydroxybenzoate induction, (VA) vanillate induction, (PCA) protocatechuate induction. Axes: (x) = time post-induction, (y) = protein spot density, (z) = protein spot number in PDQuest database. (Note: z-axis spot numbers are not in the same order for each graph to make data most visible in 3D format)

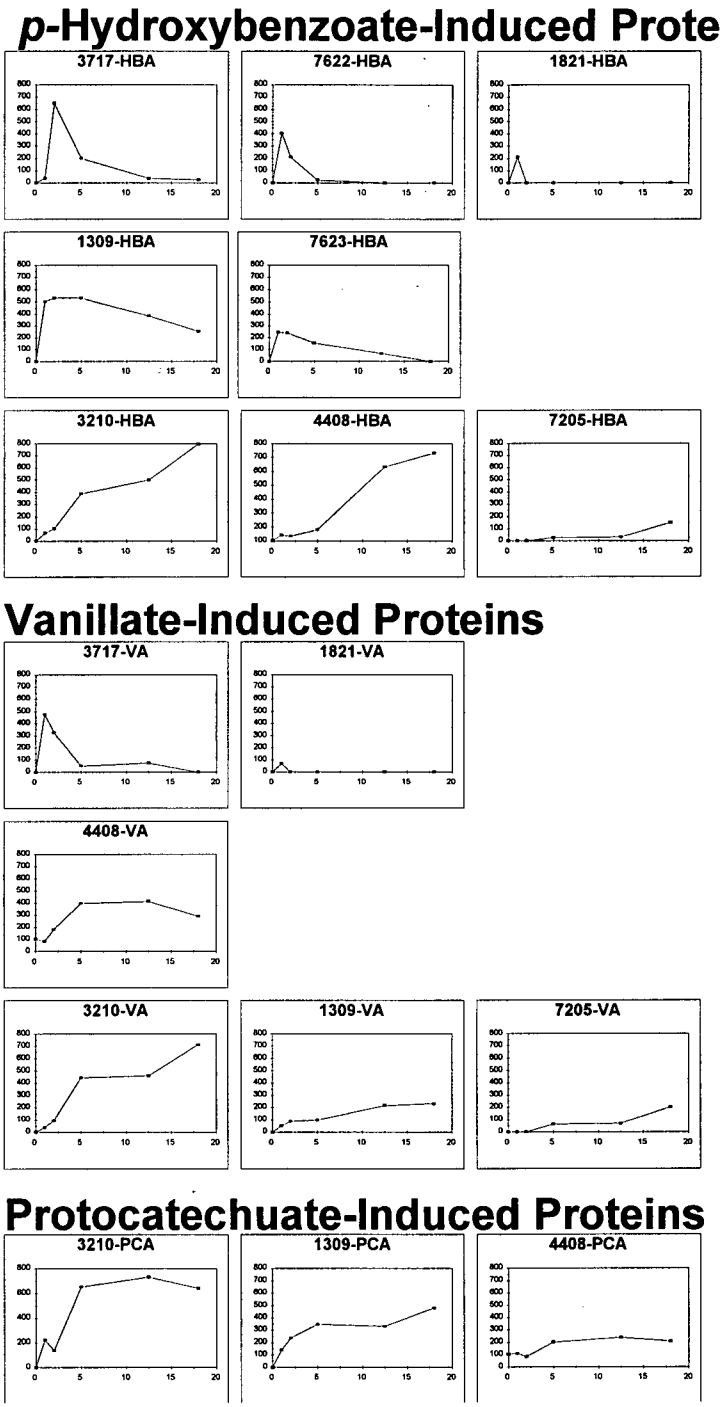

Figure 10B Individual graphs for proteins showing induced synthesis in the presence of aromatic acids. Axes: (x) time post-induction in hours; (y) protein spot density in optical density units.

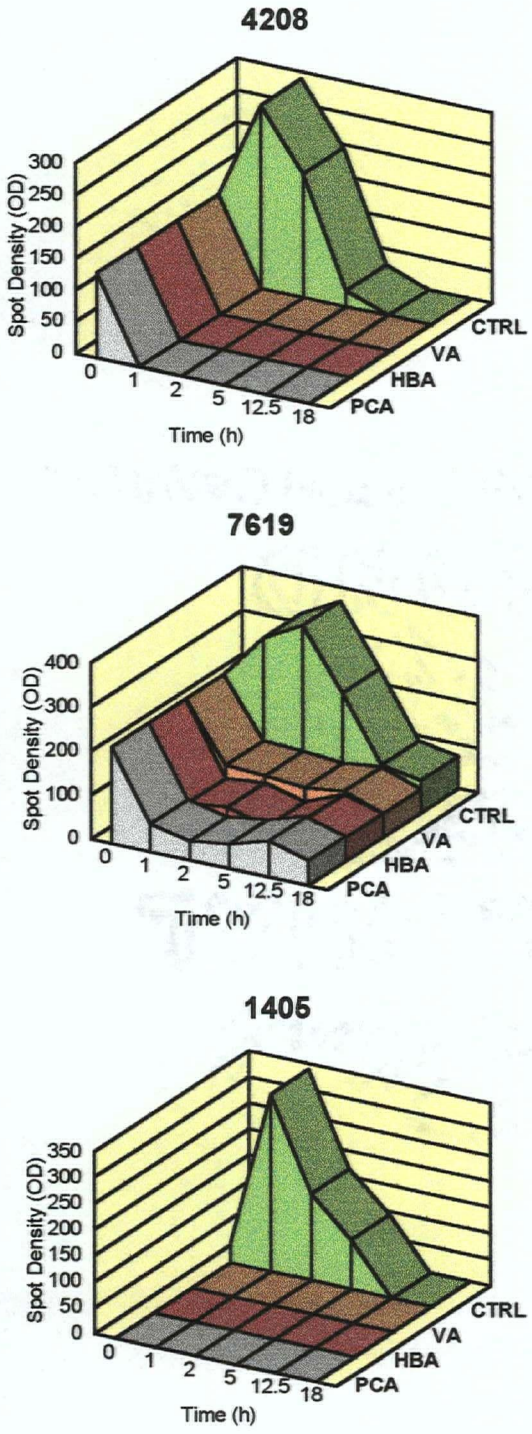

Figure 11A Proteins whose synthesis is repressed on growth of *Streptomyces violaceusniger* in aromatic acids. Each graph represents one of the proteins with repressed synthesis. Database spot numbers are at the top of each graph. Axes: (x) = time post induction, (y) = protein spot density, (z) = treatment (aromatic acid inducer). Note the change in the z-axis of the graphs from Figure 10A.

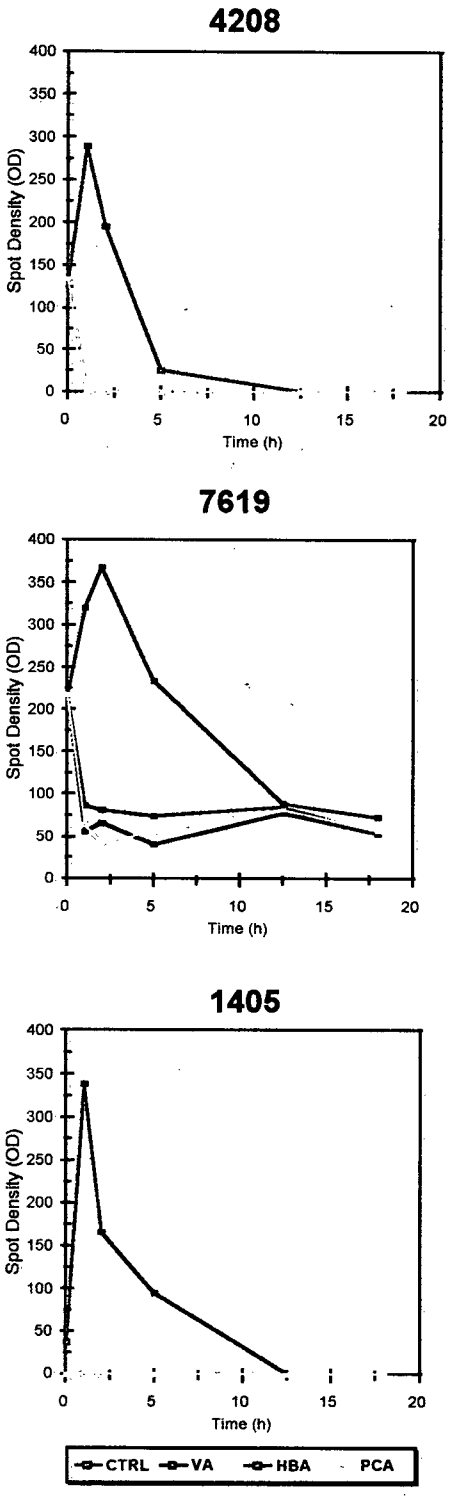

Figure 11B Proteins showing repressed synthesis in the presence of aromatic acids (same data as Figure 11A).

possible that at least one of these polypeptides could be a subunit of protocatechuate 3,4-dioxygenase (P34O). P34O has been purified from *Pseudomonas putida,* and consists of two subunits, α and β, with molecular masses of 22.3 kDa and 26.6 kDa respectively (27). Protein 3210 has a mass of 28.7 kDa. The protein is present at very low levels at one and two hours post-induction, but is produced for the remainder of the time course experiment (Figure 12). This observation could be explained if one considers that in the early stages of degradation, vanillate must first be demethylated and *p*-hydroxybenzoate must be hydroxylated to form the protocatechuate intermediate before the aromatics can be mineralized. It is also notable that the increase in synthesis of protein 3210 corresponds with the onset of disappearance of protocatechuate after the lengthy lag period (discussed previously).

Not all proteins of interest were simply the result of gene expression being completely activated ("turned on") by aromatic acid induction. Instead, some proteins were upregulated in their synthesis versus low-level synthesis in the uninduced state. An example is protein 4408, which showed increased synthesis under induction conditions, particularly induction by *p*-hydroxybenzoate (Figure 13). Certain proteins, discussed above, had their synthesis repressed non-selectively by all three aromatics. An example of a synthesis-repressed protein, protein 1405, is shown in Figure 14. Repressed proteins could possibly be enzymes needed for catabolic processes in MSMYE medium deemed unnecessary when "preferred" carbon sources such as lignin-related aromatic acids, requiring other enzymes, are introduced into the culture medium.

Detection and Sequencing of a Putative Aromatic Degradative Enzyme

Cells harvested during the catabolism of vanillate reproducibly expressed a 52 kD protein with a pI of 4.9, as revealed by silver-staining on one-point analysis 2D-PAGE gels. This protein, designated 3717 in the PDQuest database, was completely absent in cells

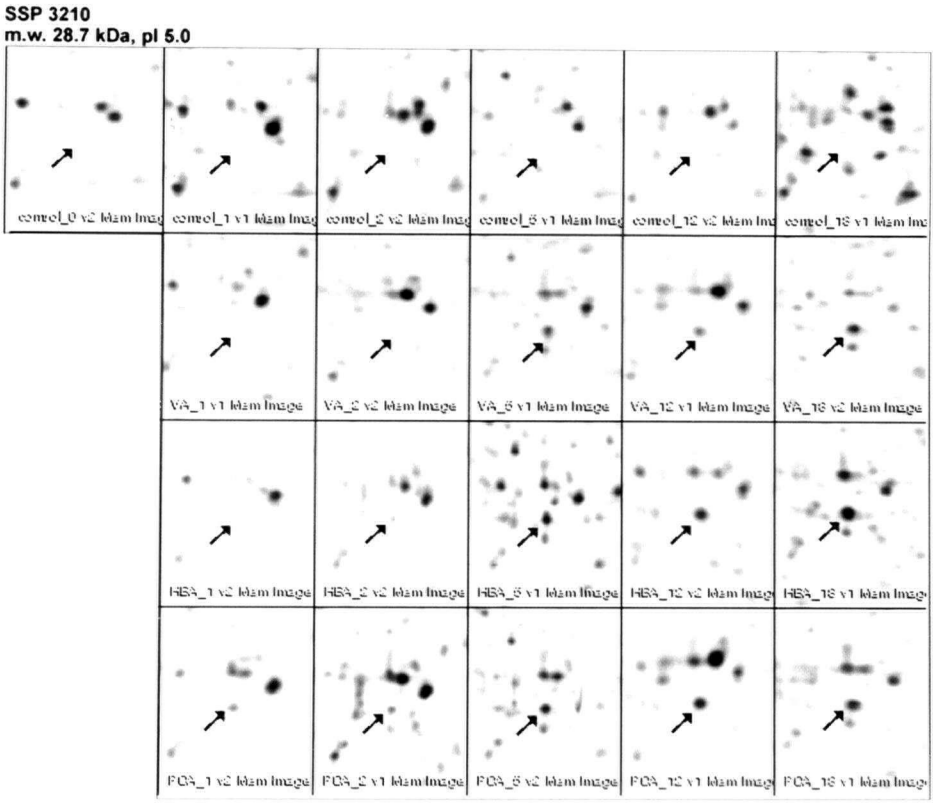

Figure 12 Expression profiles of SSP 3210. Rows (top to bottom): Control (uninduced), vanillate-induced, *p*-hydroxybenzoate-induced, protocatechuate-induced. Columns (left to right): pre-induction, +1h, +2h, +5h, +12h, +18h.

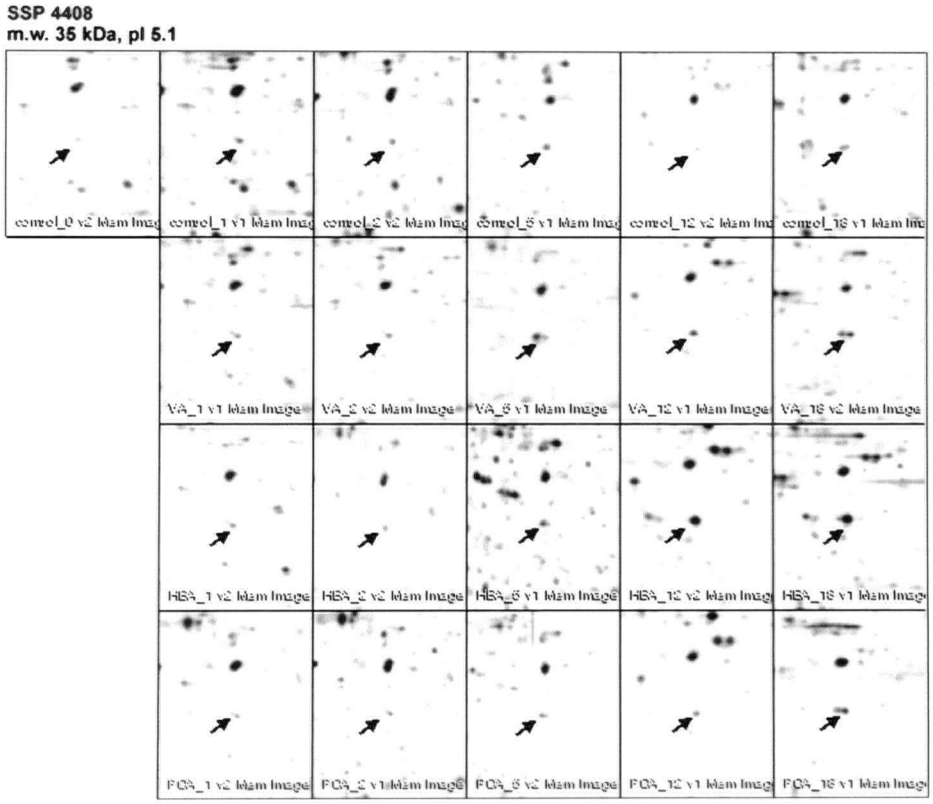

Figure 13 Expression profiles of SSP 4408. See Figure 12 for legend.

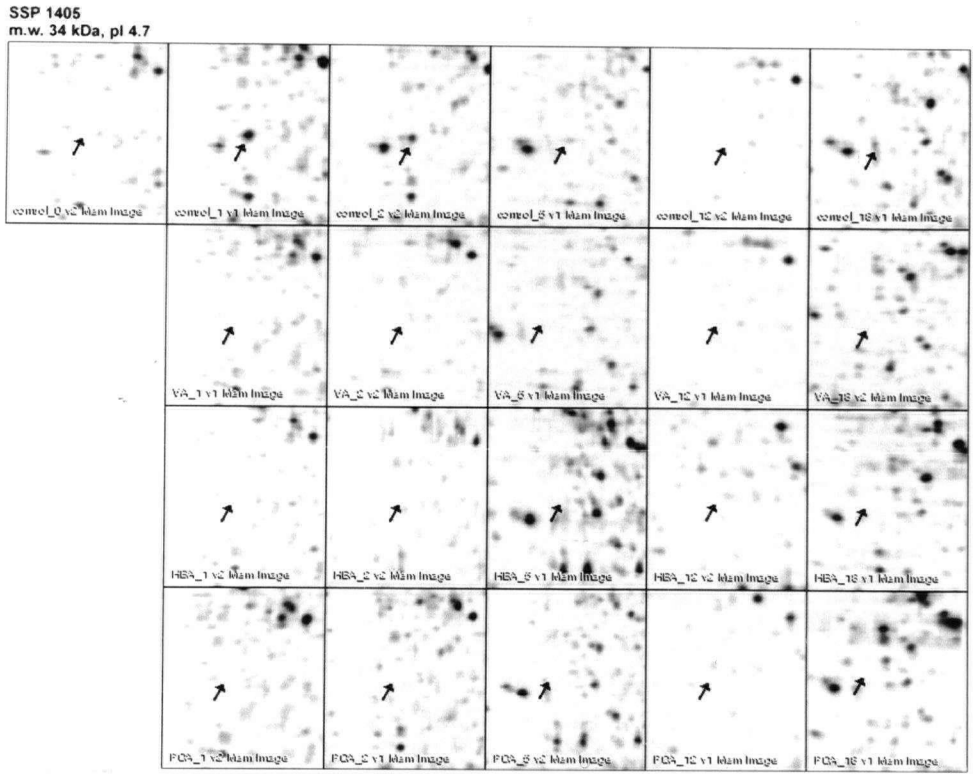

Figure 14 Expression profiles of SSP 1405. See Figure 12 for legend.

growing without vanillate. Radiolabeled samples analyzed by 2D-PAGE demonstrated that protein 3717 was induced by both vanillate and *p*-hydroxybenzoate during the early stages of the time course experiment (Figure 15). Sequencing of the protein revealed the 22 amino-terminal amino acids, Ala-Tyr-Asp-Asp-Leu-Arg-Tyr-Phe-Leu-Asp-Thr-Leu-Glu-Lys-Glu-Gly-Gln-Leu-Leu-Arg-Ile-Thr (AYDDLRYFLDTLEKEGQLLRIT). Using the BLAST-P protein alignment search utility (28), this protein sequence was found to be most similar to the amino terminus of a hypothetical *Escherichia coli* 55.3 kDa protein (Figure 16), the function of which is as yet unknown. The nucleotide sequence encoding the 55.3 kDa hypothetical protein lies in the *rfa*H-*rfe* intergenic region of the *E. coli* chromosome. This region is adjacent to the *fre* gene encoding a flavin reductase and the *fad*AB operon which encodes a fatty acid-oxidizing multienzyme complex (29). When the streptomycete sequence was input into a database search using BEAUTY (BLAST Enhanced Alignment Utility), which searches for similarity to highly conserved regions, the amino-terminal sequence aligned with the beginning of a highly conserved nucleotide binding region found in 26 guanine nucleotide (GTP) binding alpha subunit (G_α-subunit) proteins (Figure 17). Much of the N-terminal sequence also aligned with a portion of the ATP nucleotide binding domain found in valyl tRNA synthetases (Figure 18). Many flavoprotein monooxygenases involved in biodegradation of aromatics characteristically possess nucleotide (FAD) binding motifs near the amino terminus (30). The 52 kD protein amino terminal sequence obtained in this study, though not possessing a nucleotide binding motif, was similar to regions nearby nucleotide binding motifs in G-proteins and tRNA synthetases, suggesting the possibility that the 52 kD protein is indeed a biodegradative enzyme. It is also encouraging that the sequence of the *S. violaceusniger* 52 kDa protein aligned with an *E. coli* protein (albeit hypothetical), which was encoded by an open reading frame in the vicinity of other biodegradative genes, though it is difficult to make parallels between gene organization in *Streptomyces* and *E. coli*. It must be noted that several frameshift mutations had to be introduced in the *E. coli*

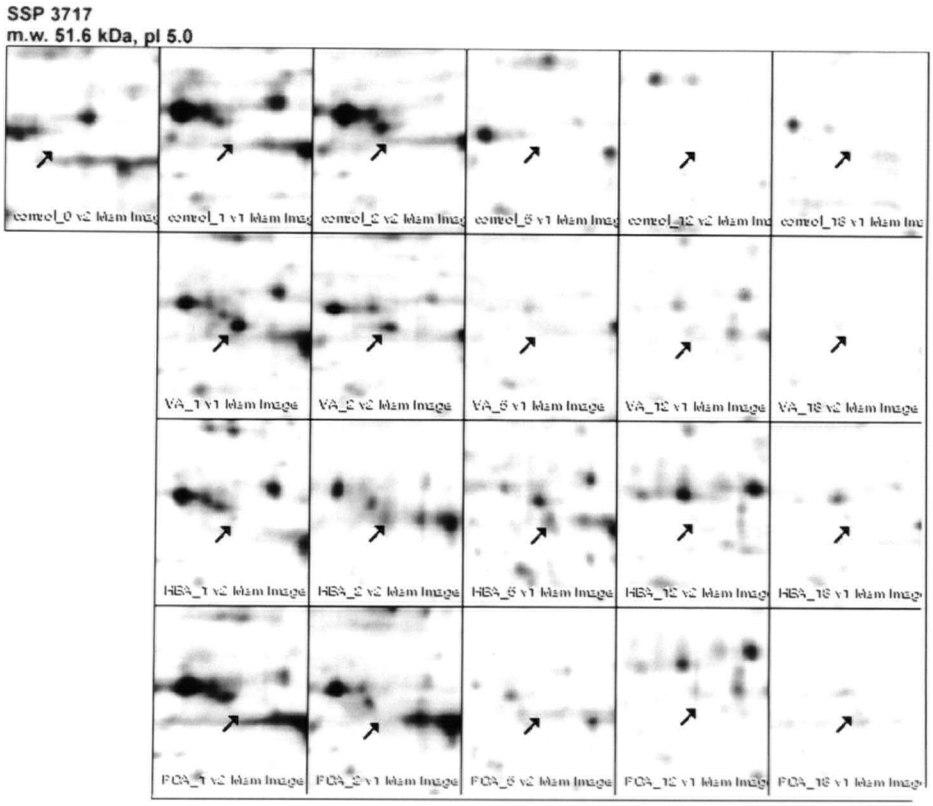

Figure 15 Expression profiles of SSP 3717. See Figure 12 for legend.

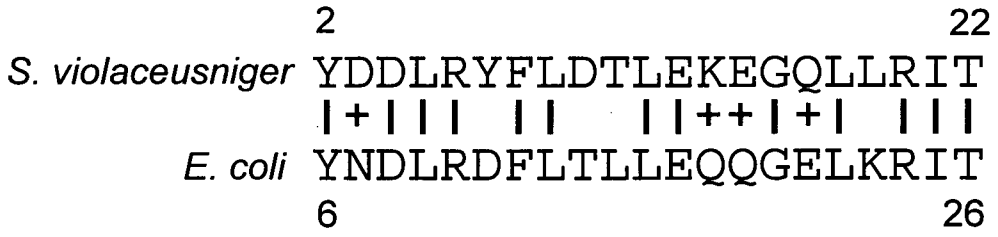

Figure 16 Alignment of *Streptomyces violaceusniger* 52 kDa amino terminal sequence with amino terminal sequence of *Escherichia coli* hypothetical 55.3 kDa protein. (|) indicates exact amino acid match, (+) indicates similar amino acid

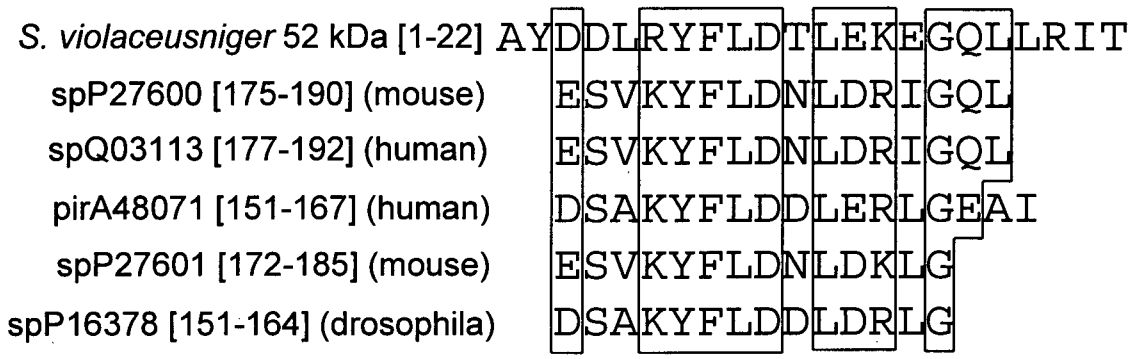

Figure 17 Alignment of *Streptomyces violaceusniger* 52 kDa amino terminal sequence with a conserved domain in several GTP-binding alpha subunit proteins. The amino acids of each sequence shown, relative to the entire sequence of each protein, are listed in square brackets to the right of each accession number. Boxed regions indicate matches between identical or similar amino acids.

Figure 18 Alignment of *Streptomyces violaceusniger* 52 kDa amino terminal sequence with *Bacillus subtilis* region adjacent to valyl-tRNA synthetase ATP binding site. The ATP binding site begins at amino acid 528 of the *Bacillus* sequence. (|) indicates exact amino acid match, (+) indicates similar amino acid.

nucleotide sequence to reveal the hypothetical 55 kDa protein (29), and therefore may not be produced in *E. coli.* If *S. violaceusniger* protein 3717 is a biodegradative enzyme synthesized early in the time course, it could be involved in demethoxylation and hydroxylation of vanillate, or *meta* hydroxylation of *p*-hydroxybenzoate, since these steps occur early in the catabolic pathways of the aromatics.

The similarity between the 52 kDa amino-terminal sequence and the highly conserved G-protein region may suggest that the product identified is a sensory protein involved in activation of biodegradative processes. However, this is unlikely, since the protein was produced in abundance (visible by silver-staining, and slightly by Coomassie R-250 staining) during biodegradation, which would not be expected for a signal transduction protein in unfractionated cell extracts (31).

The suggestion that bacteria as diverse as *S. violaceusniger* and *E. coli* share a similar catabolic enzyme is not unfounded. Indeed, the similarity of catabolic enzymes amongst *Pseudomonas, Alcaligenes, Azotobacter, Acinetobacter* and *Rhizobium* has been documented (32). These studies indicate that while the amino acid sequences of catabolic enzymes are not very similar between different species of prokaryotes, there are enough similarities to suggest common ancestors for some catabolic oxidative enzymes. Furthermore, it should also be noted that the first six amino acids in the amino-terminal sequence of the *S. violaceusniger* 52 kDa protein (AYDDLR) are only found in one other protein in the database -- salicylate-1-monooxygenase of *Pseudomonas putida* (30). This observation is of interest in light of the fact that salicylate is an aromatic acid similar in structure to vanillate and *p*-hydroxybenzoate. However, in the *Pseudomonas* enzyme, the sequence is not at the amino terminus but rather towards the carboxy terminus. Nakatsu *et al.* have shown evidence for rearrangement of catabolic enzyme domains throughout molecular evolution (33). The connection between the streptomycete 52 kDa protein and

salicylate-1-monooxygenase is speculative, but will become more clear when the gene encoding the 52 kDa protein is sequenced in its entirety.

Catabolic oxidoreductases do not share high overall sequence homology. Rather, sequence homologies are localized in motifs such as FAD binding sites (30). For example, the overall similarity between salicylate hydroxylase and *p*-hydroxybenzoate hydroxylase is 25%, and the overall similarity between salicylate hydroxylase and phenol hydroxylase is only 20%. Therefore, the observation that the amino-terminal sequence of the 52 kDa protein does not align with any known catabolic enzymes does not eliminate it from this class. The complete gene sequence will indicate if the protein is, in fact, a biodegradative enzyme and will reveal whether or not it is a homolog to the 55 kDa *E. coli* hypothetical protein and/or related to salicylate-1-monooxygenase. The cloned gene will be used to perform gene disruption experiments, which will permit the function of the protein to be determined.

Streptomyces, though exploited extensively over the years for its antibiotic production capabilities, has shown potential for use in biotransformation processes such as chloroaromatic bioremediation. In 1966, the dechlorination of polychlorinated aromatic pesticides by several *Streptomyces* strains was reported (34). The dechlorination activity observed during that study was likely mediated by catabolic oxidoreductases similar to those involved in degradation of lignin-related aromatic acids. This study has shown 2D-PAGE to be a powerful tool in the analysis of catabolic processes, and demonstrates that gene expression changes during catabolism of aromatic chemicals can be effectively monitored by this method. By expanding our knowledge of the proteomes of *Streptomyces* and other organisms, future studies using 2D-PAGE should reveal more enzymes involved in biodegradation of aromatic chemicals, including man-made xenobiotic compounds. Continued studies will permit the characterization of pathways

for the degradation of xenobiotic and lignin model compounds, particularly the cloning and sequencing of *Streptomyces* genes responsible for vanillate and *p*-hydroxybenzoate catabolism. The results of such work may ultimately lead to the isolation and development of novel microorganisms, ideally suited for use in biotransformation and bioremediation applications.

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