Bacterial Proteomes and Metabolism of Polycyclic Aromatic Hydrocarbons

서 종 수

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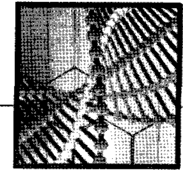
Introduction

Bioremediation utilizes the metabolic versatility of microorganisms to clean up hazardous pollutants. Its ultimate aim is to completely degrade organic contaminants to harmless constituents such as carbon dioxide and water. It can be a feasible remedial technology if the microorganisms are capable of acquiring new metabolic pathways and synthesizing appropriate enzymes to achieve the desired degradation within a reasonable period of time.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. Theirpersistence and toxicity raise environmental concerns. The decontamination of PAHs polluted sites is of major importance because many of PAHs are either known or suspected carcinogens and mutagens. Bioremediation of PAHs occurs through biodegradation or natural microbial processes in the soil environment to achieve transformation, degradation and ultimate detoxification of the hazardous PAHs. The microbial degradation of PAHs is thought to be the major process involved in effective site bioremediation and the number of PAHs known to be biodegraded has increased (Cerniglia, 1992). Therefore, the isolation of microorganisms capable of degrading the target compounds, as well as biochemical and enzymatic information about the degradation, may lead to the development of new bioremediation technologies for contaminated sites. Also, understanding metabolic pathways is a crucial step in the development of effective bioremediation technologies for environmental cleanup.In addition to the metabolism of aromatic hydrocarbons, identification of proteins involved in the biodegradation is also an important step to understand bacterial degradation mechanisms because the use of proteomic toolsallows a global and dynamic view of proteins that are expressed by bacteria (Manso et al., 2005). In addition, proteomic studies can offer insight to the evolutionary difference between the pathogenic and saprophytic species which may give further understanding to the prevention of infection and environmental remediation.

This reportincludes 1) isolation and identification of



PAHs degrading bacteria from petroleum contaminated soil, 2) screening test for PAHs and other aromatic compounds degradation of the isolated bacteria, 3) elucidation of metabolic pathways for various aromatic compounds, and 4) proteomic analysis of aromatic compound-induced proteins.

Isolation and identification of PAHs degrading bacteria

Bacterial strains in the soil were grown in minimal medium (MM) (1 g soil per 25 ml MM) to which solid fluorene, phenanthrene, fluoranthene, pyrene, or benzo[a]pyrene was added at a level of 1 mg ml^{-1}. Each suspension of 2 g of soil, 50 mg of a PAH, and 50 ml of MM was incubated at 28 ℃ while shaking at 150 rpm in the dark. After 3 months of incubation, the cultures were shaken vigorously for 3 h to loosen bacteria from the soil particles. The aqueous phase was used to inoculate liquid enrichment cultures in 50 ml of MM fortified with fluorene, phenanthrene, fluoranthene, pyrene, or benzo[a]pyrene as the sole carbon and energy source. The cultures were incubated at 28 ℃ while shaking at 150 rpm in the dark. When turbidity was observed, an aliquot of the culture was serially diluted and was transferred onto nutrient agar plates for determination of colonial numbers and strain isolation. Genomic DNA was extracted with a mixture of phenol and chloroform.The 16S rRNA gene was amplified from the genomic DNA (100-200 ng l^{-1}) by polymerase chain reaction (PCR) with TaqDNA polymerase and primers 27F and 1492R. Amplification was performed with a Techne thermal cycler at 95 ℃ for 4 min. followed by 30 cycles of 95 ℃ for 1 min, 55 ℃ for 50 sec. and 72 ℃ for 1.5 min., and a final elongation step at 72 ℃ for 7 min. The PCR product was purified using the Ultraclean PCR purification kit, and sequenced in both directions using an Applied Biosystems 377XL DNA sequencer. 16S rDNA sequences were manually edited and assembled in Sequencher and Seqman (DNASTAR). Assembled sequences each in excess of 1300 bases were compared with those in the public domain through a BLASTn search.

Screening test for PAHs degradation of the isolated bacteria

The isolated pure strains were tested with two different spray-plated methods and a liquid medium turbidity test for five individual PAHs (fluorene, phenanthrene, fluoranthene, pyrene and benzo[a]pyrene) to determine the PAH-degradation potential of the strains. The first spray-plated method was that isolates were pre-grown on Yeast Extract-Polypeptone-Glucose (YEPG) agar plates, which were then sprayed with a 1% PAH acetone solution (Kiyohara et al., 1982). After solvent vaporization, the plates were incubated at 28 ℃ and observed daily. The second spray-plated method was to spray a 1% PAH acetone solution on the MM agar plates. After solvent vaporization, isolates were spotted on the plates and incubated at 28 ℃. Isolates producing significantly clear zones by utilization of the PAH were scored as positive. The turbidity test was that pre-grown cells on the nutrient agar plate was inoculated into sterilized culture tubes containing 10 ml of MM and 400 μg of PAH. The culture tube was incubated at 28 ℃ while shaking at 150 rpm. When turbidity occurred, cultures were scored as positive and marked as PAH-degrader candidates (표 1).

Table 1. Bacterial identification by 16S rRNA gene sequences and the results of screening test for several PAHs

Isolate ID	Best match in GenBank		Screening test (YEPG/SMM/LMM)				
	Strains	Accession No.	FLE	PHE	FLA	PYR	BAP
JS1-2	Achromobacter xylosoxidans	AY946288	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS1-1	Achromobacter xylosoxidans strain NFRI-A1	AB161691	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
P1-1	Arthrobacter sp. CAB1	AB039736	-/-/-	+/+/+	-/-/-	-/-/-	n/+/-
JS18	Bacillus cereus biovar toyoi	AJ310100	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS23b	Bacillus sp. CJ11076	AF500212	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS15	Bacillus sp. DU	AJ842963	-/-/+	-/-/-	-/-/-	-/-/-	n/-/-
JS13	Bacillus thuringiensis	D16281	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS2	Bosea sp. MN 51a	AJ313022	-/-/-	-/+/-	-/-/-	-/-/-	n/-/-
JS11	Bosea thiooxidans	AJ250796	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
C3	Burkholderia sp. 56	AY177370	-/-/-	+/+/+	-/-/-	-/-/-	n/-/-
JS12	Mesorhizobium loti MAFF303099	BA000012	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS6	Mesorhizobium plurifarium	Y14161	-/-/+	-/+/-	-/-/-	-/-/-	n/-/-
JS9	Mesorhizobium plurifarium subsp. americium strain Ls29	AF516881	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS7	Mesorhizibium sp. ORS3165	AY875973	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-
JS14	Mycobacterium sp. PX3.15	AY337605	-/-/-	-/+/-	+/+/+	-/-/-	n/-/-
JS19b1	Mycobacterium sp. RJGII.135	U30661	-/-/+	+/+/+	-/-/+	-/+/+	n/-/-
C4	Sinorhizobium sp. HF6	AB195269	-/-/-	-/+/+	-/-/-	-/-/-	n/-/-
C6	Stenotrophomonas maltophilia	AB008509	-/-/-	-/-/+	-/-/-	-/-/-	n/-/-
JS8	UnculturedAlcaligenaceae bacterium	AB114615	-/-/-	-/-/-	-/-/-	-/-/-	n/-/-

Analysis of PAH metabolites

Bacterial strain was grown in MM supplemented with PAH as a sole source of carbon and energy at 28 C and 150 rpm. After incubation for 14 or 30 days, the cultures were filtered through glass wool followed by

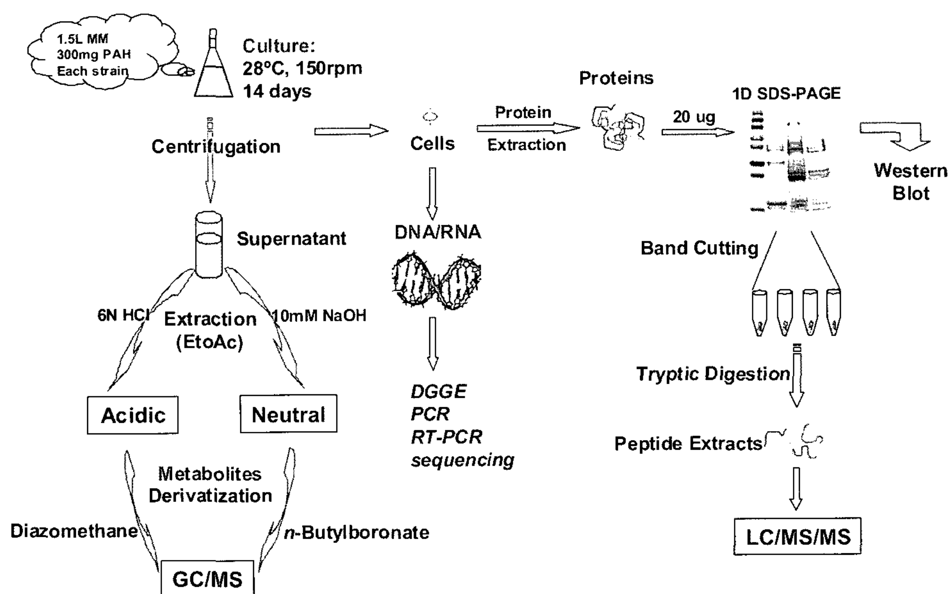
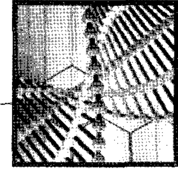


Figure 1. Scheme of catabolic, proteomic, and genomic experiments



centrifugation (6,000.g, 10 min) to remove the unreacted crystalline PAH, if any left, and bacterial cells. The pellet was discarded. The supernatant was acidified to pH 2.3 with 6 N HCl, and then extracted with ethyl acetate. The combined organic layer was extracted with 10 mM sodium hydroxide. The remaining organic phase was dried over anhydrous sodium sulfate and concentrated to 5 ml (neutral fraction). The aqueous NaOH extracts were acidified to pH 2.3, and then extracted with of ethyl acetate (acidic fraction). Gas chromatography-mass spectrometry (GC-MS) analysis of the neutral fraction was done with and without derivatization. After removal of ethyl acetate from the neutral fraction, the residue was dissolved in acetone (10 ml) followed by addition of 50 mg of n-butylboronic acid. After refluxing for 30 min, the mixture was concentrated to 1 ml and analyzed by GC-MS. Diols and cis-dihydrodiols in the residue were derivatized with diazomethane and re-analyzed with GC-MS (Keum et al., 2006). Metabolites in the acidic fraction were also derivatized with diazomethane and detected with GC-MS (그림 1).

Phenanthrene degradation by Mycobacterium sp. JS19b1

Mycobacterium sp. JS-19b1 grew with phenanthrene as a sole source of carbon and energy. The metabolites were isolated and characterized by gas chromatography-mass spectrometry. Strain JS19b1 could degrade phenanthrene through highly branched metabolic pathways, including dioxygenation on multiple position and ring opening by both ortho- and meta-cleavage (그림 2). The presence of novel metabolic pathways was confirmed by replacement culture with synthetic metabolites. Phenanthrene degradation initiated with dioxygenation on C-1,2, C-3,4, and C-9,10. Both ortho and meta-cleavage products of 1,2- and 3,4-dihydroxyphenanthrene were detected. Two novel ortho-cleavage products, 1-[(E)-2-carboxyvinyl]-2-naphthoic acid and 2-[(E)-2-carboxyvinyl]-1-napthoic acid were further metabolized to naphthalene-1,2-dicarboxylic acid, and then to 1,2-dihydroxynaphthalene, which can also be produced from hydroxynaphthoic acids. These results suggests that part of the branched pathways is converged into 1,2-dihydroxynaphthalene. Products from K-region dioxygenation were more dominant than those from other pathways. The metabolites, however, gradually degraded to phthalic acid through decarboxylation and mono-/di-oxygenation.

Proteomics of PAH-degrading bacteria

Proteomic techniques have been proven to be the most powerful method for identification of proteins in complex mixtures. It is suitable to study the alteration of protein expression and physiological response of bacterial cellsunder different environmental conditions. Also, the proteomic studies have been applied for exploring the structure or function of a specific protein in microorganisms.

There are two common approaches for analyzing complex protein mixtures. One is the separation of whole proteins by two-dimensional gel electrophoresis (2-DE) and subsequent identification of individual proteins using mass spectrometry. The other is the separation of proteolytic peptides by liquid chromatography and their identification by directly coupled electrospray ionization-tandem mass spectrometry, often called multidimensional protein identification technology (MUDPIT). Since 2-DE

can resolve hundreds of proteins in a gel, most microbial proteomics research involves the 2-DE method. However, one dimensional SDS-polyacrylamide (1D-PAGE)was used directly prior to In-gel digestion to profilethe bacterial proteins in conjunction with liquid-chromatography tandem nanoelectrospray mass spectrometry (LC-MS/MS)for the identification of proteins.

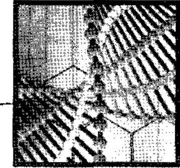
There are several reports that proteomic analysis has been performed to detect enzymes in response to bacterial exposure to specific chemicals such as monocyclic aromatic compounds and PAHs. Kim et al. (2004) reported the protein profiles of Mycobacterium vanbaaleniiPYR-1 grown in the presence of pyrene, pyrene-4,5-quinoline, phenanthrene, anthracene, and fluoranthene using 2DE. From this experiment, they found PAH-induced proteins (catalase-peroxidase, putative monooxygenase, dioxygenase small subunit, small subunit of naphthalene-inducible dioxygenase, and aldehyde dehydrogenase), carbohydrate metabolism related proteins (enolase, 6-phosphogluconate dehydrogenase, indole-3-glycerol phosphate synthase, and fumarase), DNA translation proteins, heat shock proteins, and energy production protein (ATP synthase). However,limited proteome analysis involvingPAH bacterial degradation has been reported.

Proteomic experiment

Approximately 20 μg of protein extracted using bacterial protein extraction kit were loaded to 10-20% gradient SDS-PAGE followed by Coomassie blue staining. Each gel lane was cut into 20 even slices, destained with 50% (v/v) acetonitrile in 50 mM NH_4HCO_3, and then completely dried in a speed- vacuum centrifuge. The dried gel slices were treated with 50 μL of 10 mM dithiothreitol and 55 mM iodoacetamide. After removal of the unabsorbed solution, 30 μL of sequencing-grade modified trypsin (20 μg/mL in 25 mM NH_4HCO_3) was added to each rehydrated slice. These samples were incubated at 37 oC overnight. Tryptic digestion was stopped by adding 5 μL of 2% trifluoroacetic acid (TFA). The digested peptides were extracted from each gel slice with 30 μL of 0.1% TFA and then with 30 μL of 50% acetonitrile/0.1% TFA by sonication for 15 min at the ice bath. Both extracts were combined for LC-MS/MS analysis (그림 1).

LC-MS/MS analyses were carried out on an UltimateTM nano LC system interfaced to an esquireHCTplus quadrople ion trap mass spectrometer (Bruker Dlatonics, Billerica, MA). The mobile phase gradient (A = 0.1% formic acid; B = 0.1% formic acid in acetonitrile) was changed from 95% A and 5% B for 5 min, to 40% A and 60% B in 88 min, 5% A and 95% B in 10 min, then 95% A and 5% B in 15 min, and finally was held at 95% A and 5% B for 20 min. Peptide spectra were recorded over a mass range of m/z 300-2500 while MS/MS spectra were recorded in information dependent data acquisition over a mass range of m/z 50-1600. One peptide spectrum was recorded followed by two MS/MS spectra; the accumulation time was 1 sec for peptide spectra and 2 sec for MS/MS spectra. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Doubly or triply charged ions were selected for product ion spectra. MS/MS spectra were interpreted by Mascot (Matrix Science Ltd, London, UK) via Biotools 2.2 software (Bruker Daltonics, Billerica, MA).

Peptide mass fingerprint (PMF) searches based on peptide masses measured were performed using the Swiss-Prot or MSDB databases with the Mascot server. PMF used the assumption that peptides are monoisotopic,



oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages.Mass tolerance of 1.0 Da was the window of error allowed for matching the peptide mass values. Probability based MOWSE scores were estimated by comparison of search results against estimated random match populations and were reported as: 10 x $\log_{10}(p)$, where pis the absolute probability. Scores in Mascot larger than the MOWSE score at p = 0.05 were considered significant, meaning that the probability of the match being a random event is lower than 0.05 when scores in Mascot were larger than the MOWSE score at p = 0.05. The algorithm used for determining the probability of a false-positive match with a given mass spectrum is described elsewhere.

Protein profiles of Mycobacterium sp. JS19b1

Mycobacterium sp. JS19b1 can utilize a very diverse set of chemicals as a sole carbon and energy source, including PAHs (anthracene, phenanthrene, pyrene, fluorene, and fluoranthene), aromatic heterocycles (dibenzothiophene and carbazole), biphenyl, and pesticide, 2,4-dichlorophenoxyacetic acid (2,4-D). According to the proteomic and metabolic analyses, catabolism of PAHs and other environmental contaminants in JS19b1 can be summarized a) diverse initial degradtive enzymes (그림 2), b) integration of different metabolic pathways through specific enzymes, c) strictly regulated specific enzymes, and d) possible gene exchange between JS19b1 and other bacteria within the community.

Microorganism has developed several different kinds of defense mechanism against the environmental stress. In relation to chemical stress, the most representative characteristic changes of bacterial physiology occurred in cellular membrane and/or cell wall structure. The defense mechanisms of bacterial cell against chemical stress can be related to a) degradation of toxicants, b) efflux of toxicants from the cell via specific/non-sepcific transporters (Nomura et al., 1992), and c) hydrophobicity increase of the cell membrane (mycolic acid layer for the case of Mycobacterium) through long-chain fatty acid deposition(Sokolovská et al., 2003) (그림 3).

Organismsconfront various types of environmental stress. Those include a) chemical (toxic chemicals or nutrient limitation) and b) physical stresses (heat, cold, friction etc.). Although the characteristics of each stress are different, the cells often show oxidative stress responses (Finkel et al., 2000). Chemical species, related to intracellular oxidative stress, encompass various types of reactive oxygens and nitrogens (Farr et al., 1991). The major proteomic sources of oxidative stress are over-reduction of respiratory chain (Chen et al., 2003).

Table 2. Proteins relevant to PAH and other environmental contaminant metabolism in Mycobacterium sp. JS19b1

Protein Classification by Substrate Metabolisms	Number of different degradation enzymes			
	NB	MM-Glu	MM-PHE	MM-PHT
PAH metabolism	0	0	9	1
Bipheny metabolism	2	2	6	2
Dibenzothiophene metabolism	0	1	2	2
2,4-D metabolism	1	1	0	0
Other aromatics metabolism	5	0	7	0
Sum number of enzymes	8	4	24	5

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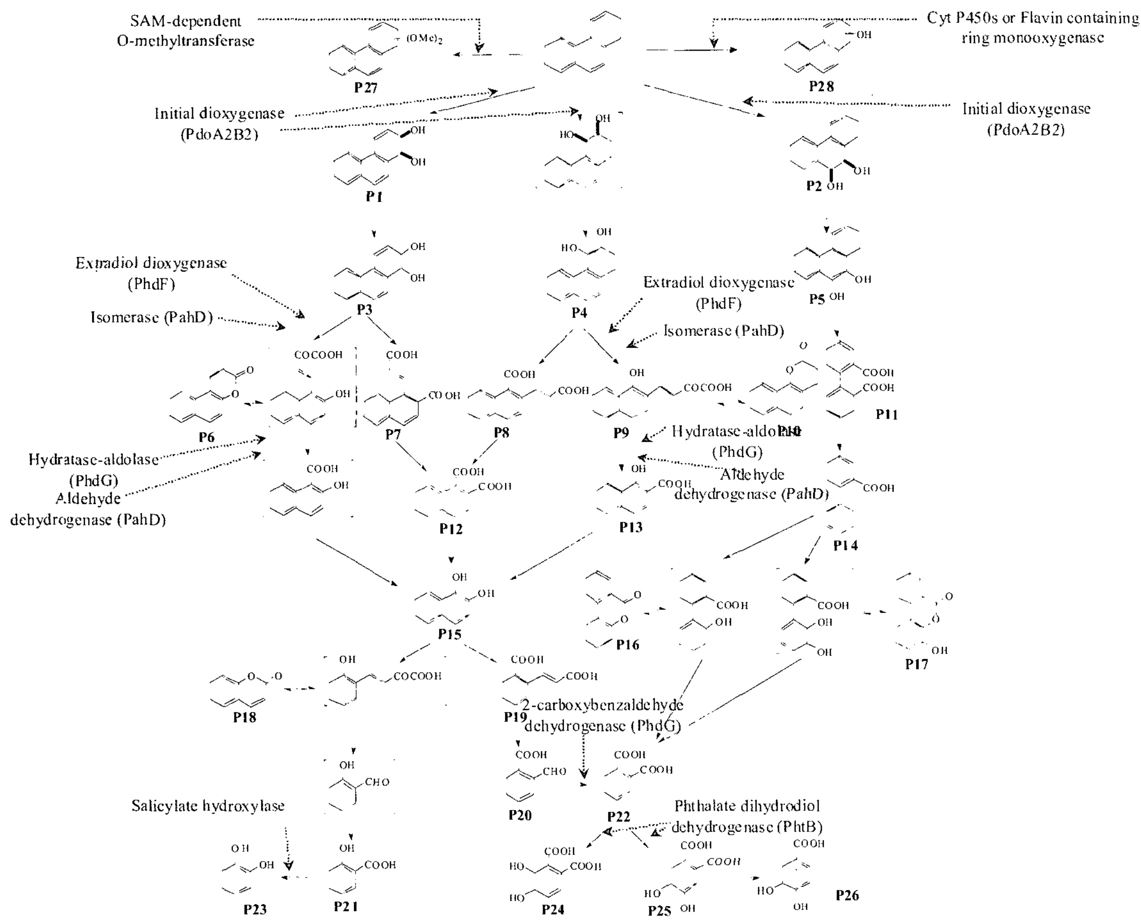
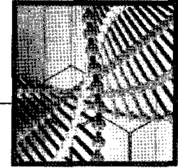


Figure 2. Metabolic pathways of phenanthrene and enzymes detected from proteomic analysis

Although the researches to identify the relationships between cellular physiology and generation of oxidative stress have long been performed, a clear answer has yet been provided. Proteomic data related to oxidative stress are grouped into four classes of proteins: a) source proteins of oxidative stress, b) sensors and regulators of specific stress, c) proteins related to the removal of reactive oxygens and d) proteins related to the removal or repair of damaged biomolecules (e.g., lipids, denatured proteins, DNA, and RNA).

The biological role of cytochrome P450 covers biochemical reactions from primary metabolism (e.g., cellular steroid metabolism) to degradation of xenobiotic chemicals (e.g., pesticides, PAHs etc.) (Kullman et al., 1996). There are extremey diverse set of isozymes in fungi and higher organisms (Nelson et al., 2004) and most of degradative reactions of xenobiotic chemicals are catalyzed by cytochrome P450s. However, the number of isozymes and their biological roles in bacteria are not clear in most cases. Eighteen different cytochrome P450s were identified from the proteomic analysis. The expression level and identities of isozymes differed largely among treatments. In consideration of strong and wide expression, CYP138is probably related general metabolism. In comparison, the CYP126, 137, and 144 may not be related to primary metabolism. Because



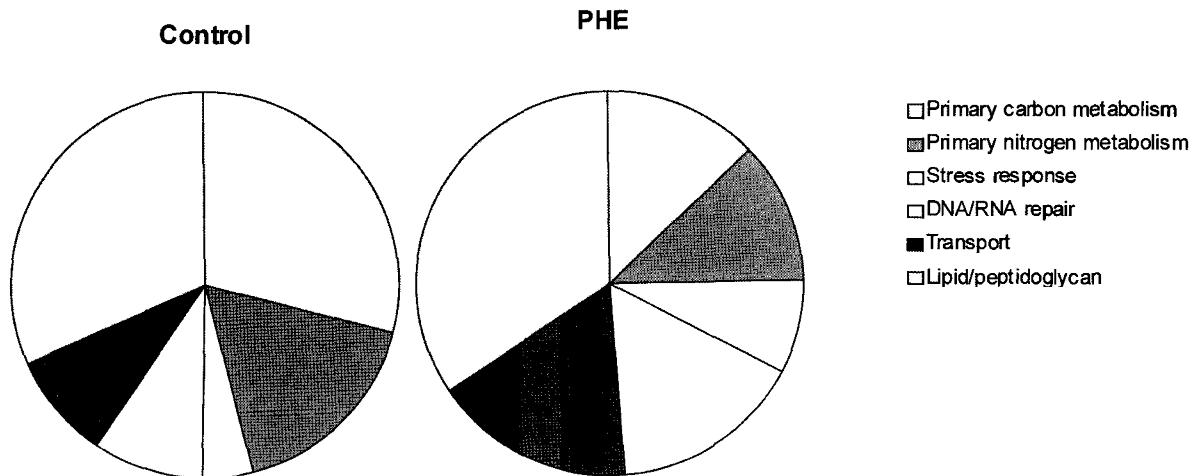


Figure 3. Changes of protein profiles in Mycobacterium sp. JS19b1

extremely diverse metabolites, which have no or limited resemblance to the primary metabolites, were produced during the metabolism of phenanthrene, the above cytochrome P450s may be involved in their metabolism.

Conclusions

Bioremediation is a major process to degrade hazardous pollutants using the metabolic versatility of microorganisms. Bacterial species that can efficiently breakdown pollutants is viewed as the key "chopping machine". Understanding metabolic pathways, catabolic mechanisms and responsible enzymes is an effective means to define important factors for efficient bioremediation of pollutants. We isolated 19 bacterial strains from a PAH contaminated soil, Hilo, Hawaii. Five strains, Burkholderia sp. C3 (AY943387), Sinorhizobium sp. C4 (AY943388), Arthrobacter sp. P1-1 (AY943390), Mycobacterium sp. JS14 (AY943385), and Mycobacterium sp. JS19b1 (AY943386), effectively degrade a wide range of aromatic compoundsincluding phenanthrene, fluorene, fluoranthene, pyrene, biphenyl, dibenzothiophene, carbazole, and dibenzofuran. Metabolic pathways and kinetics of those aromatics in the five strains were elucidated. Analysis of the metabolites revealed that aromatic compounds are decomposed through diverse pathways including mono- and di-oxygenation at multiple positions and intra- and extra-diol cleavages.

Protein profiles in Mycobacterium sp. JS19b1 have been investigated in response to aromatic compounds as well as proper controls. Approximately 2000 proteins in Mycobacterium sp. JS19b1 are identified by extensive protein profiling. The proteomic and metabolic analyses suggest that catabolism of PAHs and other aromatic environmental contaminants in strain JS19b1 is involved in (a) a suite of initial degradtive enzymes, (b) co-existence of different metabolic pathways through specific enzymes, (c) strict regulation of specific enzymes, and (d) possible gene transfer from/to other bacteria within the community.

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