Proteomics on environmental response of *Pseudomonas putida* F1 in soil

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Contents

Chapter 1

General introduction

Soils cover almost all the terrestrial area on Earth and have an indispensable ecological function in the global cycles of carbon, nitrogen and sulfur, and support a many bacterial species. A single gram of soil has been estimated to contain between 1,000 and 1,000,000 taxa (Torsvik, *et al.*, 2002, Gans, *et al.*, 2005). The total density may reach 1.5×10^{10} bacteria per gram (Torsvik, *et al.*, 1990, Torsvik & Ovreas, 2002). Soil microbes carry out many important functions in environment. However, the explicit functional and ecological roles of individual taxa remain uncertain because soil is a difficult medium for the analysis of bacterial structure and function. Therefore, the most key questions in microbiology ‘‘who is out there?’’ and ‘‘what are they doing?’’ are still unanswered for many environments and for many microbial taxa.

In microbiology, the application of post-genomic analyses has been limited mainly to laboratory studies of pure cultures. The properties of an organism cultivated in the laboratory may not necessary reflect its activity and physiology in the original environment where factors such as resource competition, physico-chemical and biological environmental heterogeneity and predation. (Brock, 1987).

Recently, to understand the structure and function of microbial community, metagenomic approaches based on direct isolation of nucleic acids from environmental samples have proven to be powerful tools for comparing and for exploring the microbial community structure (Biddle, *et al.*, 2008) and metabolic profiling of complex environmental microbial communities (Tringe, *et al.*, 2005, DeLong, *et al.*, 2006), as well as for predicting novel biomolecules by use of libraries constructed from isolated nucleic acids (Handelsman, 2004, Daniel, 2005, Ferrer, *et al.*, 2009). However, metagenomic approaches do not provide information about the expression levels of the genes. Therefore, the functional role of genes or organisms in environments remains uncertain.

Microbial functionality can be characterized either by the analysis of mRNA and/or

proteins. Major limitations related to the short half-life of mRNA, low correlation between RNA levels and syntheses of the corresponding proteins have hampered the study of indigenous microbial communities. The proteome represents the product of global gene expression (transcription and translation), protein stability, protein processing and turnover. Proteome analyses therefore extend beyond genomic analyses, which only describe the theoretical capability of an organism or community by providing a direct measurement.

Metaproteomics studies the collective proteins from all the microorganisms in a community (Gans, *et al.*, 2005) and provides information about the actual functionality in relation to metabolic pathways and regulation cascades (Torsvik, *et al.*, 1990, Torsvik & Ovreas, 2002). Therefore, proteomics is an ideal supplement to functional genomics (Torsvik & Ovreas, 2002). The combination of genomics and proteomics should provide the knowledge between microbial community-structure and soil function.

Although there are studies focusing on the microbial community, there is no reported case of proteome analysis focused on one species of bacterium in soil environments. In presently, the metaproteome approach was considered difficult to understand particularly of the role and lifestyle of one species of bacterium in habitat environments.

In this study, I aimed to understand the environmental response of a soil bacterium *Pseudomonas putida* F1 in soil environment using proteome analysis. First, the protein involved in aromatic hydrocarbon degradation of *P. putida* F1 was identified in pure liquid culture (Chapter 2). Second, the proteins involved in the degradation of aromatic hydrocarbon of *P. putida* F1 detected in soil, and revealed response to aromatic hydrocarbon in this bacterium grown in soil (Chapter 3). Finally, in order to understand the environmental response of *P. putida* F1 in environmental soil, I identified the specifically expressed proteins in the soils (Chapter 4).

Chapter 2

Genome-wide analytical approaches using semi-quantitative expression proteomics for aromatic hydrocarbon metabolism in *Pseudomonas putida* F1

2.1 Introduction

Pseudomonas putida is a gram-negative gammaproteobacterium that lives in various environments and is capable of degrading aromatic compounds. The genomes of 5 *P. putida* strains, viz., F1 (GenBank, CP000712.1), KT2440 (Nelson, *et al.*, 2002), GB-1 (GenBank, CP000926.1), W619 (GenBank, CP000949.1), and S16 (Yu, *et al.*, 2011), have been completely sequenced. These strains appear to have common pathways for the metabolism of aromatic compounds, based on analysis of data from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/) and Microbial Genome Database for comparative analysis (MBGD; http://mbgd.genome.ad.jp/) (Uchiyama, 2003, Uchiyama, *et al.*, 2010). These include the catechol (*cat* genes) and protocatechuate (*pca* genes) branches of the *β*-ketoadipate pathway for benzoate degradation, and the 2-methylcitrate (2-MC) cycle (*prp* genes) for propanoate degradation. Of the 5 strains, only the F1 strain of *P. putida* is known to have the capacity to degrade toluene, ethylbenzene, benzene, and *p*-cymene (Parales, *et al.*, 2000, Wu, *et al.*, 2011).

This capacity for degradation of aromatic hydrocarbons results from a 47-kb gene cluster (named an aromatics degradation island [ADI]), which has probably been inserted into the chromosome (Fig. 2.1; KEGG, MBGD). The ADI is composed of 42 genes, and is organized into a *tod* operon, a *sep* cluster, and a two-component system for metabolism of toluene, ethylbenzene, and benzene (Zylstra, *et al.*, 1988, Zylstra & Gibson, 1989, Menn, *et al.*, 1991, Wang, *et al.*, 1995, Lau, *et al.*, 1997, Phoenix, *et al.*, 2003), and *cym* and *cmt* operons for *p*-cymene metabolism (Eaton, 1996, Eaton, 1997).

The degradation pathways for toluene, ethylbenzene, benzene, and *p*-cymene in *P. putida* F1 are shown on the basis of the KEGG pathway database (Fig. 2.2). Toluene, ethylbenzene, and benzene will be degraded via the *tod* pathway (reaction steps 1 to 7), 2-MC cycle (steps 14 to 19), and *β*-ketoadipate pathway (steps 8 to 13), and *p*-cymene in the *cym*/*cmt* pathway (steps

20 to 27, and 4 to 7). The final metabolites transferred from the aromatic hydrocarbons enter the tricarboxylic acid (TCA) cycle. Genes encoding the key enzymes for each of the steps in the degradation process have been assigned by genome analysis. Some of the enzymes involved in particular steps are present as paralogues and complexes, all of which need to be identified and their roles understood, in order to fully comprehend a given metabolic pathway.

Proteome analysis has been reported for identified proteins related to metabolic pathways in bacterial cells, e.g. those involved in the degradation of succinate, benzoate, *p*-hydroxybenzoate, phenylacetate, vanilline, phenol, and chlorophenoxy herbicides in *P. putida* KT2440 (Santos, *et al.*, 2004, Benndorf, *et al.*, 2006, Kim, *et al.*, 2006, Yun, *et al.*, 2011), degradation of fluoranthene in *Mycobacterium* sp. JS14 (Lee, *et al.*, 2007) and of γ-caprolactone in *Rhodococcus erythropolis* R138 (Barbey, *et al.*, 2012), and aromatic compounds catabolism in thermophilic *Geobacillus thermodenitrificans* NG80-2 (Li, *et al.*, 2012). Generally, in proteomic analysis, the altered spots on two-dimensional electrophoresis (2-DE) gels are analyzed and identified by mass spectrometry (MS) (Santos, *et al.*, 2004, Benndorf, *et al.*, 2006, Kim, *et al.*, 2006, Kurbatov, *et al.*, 2006, Hecker, *et al.*, 2008, Moreno, *et al.*, 2009, Roma-Rodrigues, *et al.*, 2010, Gong, *et al.*, 2012). This method, however, is not adequate for identifying all proteins involved in a given pathway, because only those spots that appear different are analyzed, and thus all the proteins expressed in the cells are not comprehensively identified.

For more comprehensive proteome analysis, the expressed proteins are identified in slices of a one-dimensional (1-D) SDS-PAGE gel, cut from top to bottom (Kuwana, *et al.*, 2002, Mastroleo, *et al.*, 2009, Yun, *et al.*, 2011, Li, *et al.*, 2012), combined with a liquid chromatography–tandem mass spectrometry (LC–MS/MS). A drawback of this method is that it is difficult to quantitatively compare expression levels between samples by the number of

peptides detected. The methods most appropriate for quantitative analyses of expressed proteins are isotope-coded affinity-tags (ICAT) (Gygi, *et al.*, 1999), isobaric-tags for relative and absolute quantitation (iTRAQ) (Ross, *et al.*, 2004)and isotope coded protein labeling (ICPL) (Schmidt, *et al.*, 2005) which involves labeling of proteins with chemical reagents, as well as the exponentially modified protein abundance index (emPAI), which is a label-free method and is the technique most convenient to use (Ishihama, *et al.*, 2005, Shinoda, *et al.*, 2010).

In this study, I performed comprehensive proteome analysis, using a method that combined 1-D SDS-PAGE and nanoLC–MS/MS, and semi-quantitative analysis, using protein content (PC) calculated from the emPAI, not using raw emPAI value, to delineate the metabolism of glucose, toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate. Based on a comparison of the proteome data set for each substrate, I identified (i) all key enzymes in the degradation pathway of toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate, (ii) the proteins involved in the various metabolic pathways, and (iii) substrate-specific expressed proteins. Moreover, my results suggest that the approaches I use here are ideal for a primary analysis of the various physiological characteristics and processes of bacterial cells, which is comparable to a DNA-array analysis approach.

2.2 Materials and methods

2.2.1 Bacterial strain and growth conditions

P. putida F1 was purchased from ATCC, and was grown on mineral salt medium (18.3 mM $NaHPO_4 \cdot 12H_2O$, 11.2 mM KH_2PO_4, 4.8 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4 \cdot 7H_2O$, and 0.3 mM $CaCl_2$) (Munoz, *et al.*, 2007) supplemented with trace elements (14.9 μM EDTA-2Na, 7.2 μM $FeSO_4 \cdot 7H_2O$, 0.35 μM $ZnSO_4 \cdot 7H_2O$, 0.15 μM $MnCl_2 \cdot 4H_2O$, 4.9 μM H_3BO_3, 0.84 μM $CoCl_2 \cdot 6H_2O$, 0.06 μM $CuCl_2 \cdot 2H_2O$, 0.08 μM $NiCl_2 \cdot 6H_2O$, and 0.14 μM $NaMoO_4 \cdot 2H_2O$), and

contained 0.2% (w/v) glucose, 0.1% (v/v) toluene, 0.3% (v/v) ethylbenzene, 0.25% (v/v) benzene, 0.2% (v/v) *p*-cymene, or 0.033% (v/v) *p*-cumate as sole sources of carbon and energy. Ten milliliters of each of these cultures were incubated at 30°C with vigorous shaking. The bacteria cells were harvested after 25–35 h of incubation time by centrifugation.

2.2.2 1-D SDS-PAGE and in-gel digestion

Harvested cells were lysed with ReadyPrep Protein Extraction Kit (Bio-Rad, CA, USA). Proteins (50 μg) were separated using 12.5% SDS-PAGE (90 mm × 85 mm) and stained with Coomassie. The gel lanes were cut into 60 strips of ~ 1 mm. The gel strips were completely de-stained with 30% acetonitrile (ACN) in 25 mM NH_4HCO_3, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide. After these gel strips had been completely dried, in-gel digestion was performed with 40 μL of sequencing-grade modified trypsin (12.5 μg/mL in 50 mM NH_4HCO_3), at 37°C overnight. The digested peptides were extracted with 25 mM NH_4HCO_3 in 60% ACN, and twice more with 5% formic acid in 70% ACN. The peptide mixtures were used in LC–MS/MS analysis.

2.2.3 NanoLC–ESI–MS/MS analysis

LC–MS/MS analysis was performed using a LTQ ion-trap MS (Thermo Fisher Scientific, Yokohama, Japan) coupled with a multidimensional HPLC Paradigm MS2 HPLC (AMR Inc., Tokyo, Japan) and a nano-spray electrospray ionization device (Michrom Bioresources Inc., CA, USA). The tryptic peptides were loaded onto an L-column2 ODS (Chemicals Evaluation & Research Inst., Tokyo, Japan) packed with C18 modified silica particles (5 μm, 12-nm pore size), and were separated by a linear gradient of 15–65% buffer B for 40 min, followed by a gradient of 65–95% buffer B for 1 min (buffer A = 2% methanol and 0.1% formic acid in H_2O; buffer B

= 90% methanol and 0.1% formic acid in H_2O) at a flow rate of 1 μL/min. Peptide spectra were recorded in a mass range of m/z 450–1800. MS/MS spectra were acquired in data-dependent scan mode. After the full spectrum scan, 1 MS/MS spectrum of the single most intense peaks was also collected. The dynamic exclusion features were set as follows: a repeat count of 1 within 30 s, an exclusion duration of 180 s, and an exclusion list size of 50. The obtained MS/MS data were searched against the *P. putida* F1 data in NCBI (NC_009512), using the Mascot program ver. 2.3.01 (Matrix Science, London, UK) on an in-house server to identify proteins. Search parameters were set as follows: tryptic digest with a maximum of 2 missed cleavage sites; fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation; peptide masses, monoisotopic, positive charge (+ 1, + 2, + 3) of peptide; and mass tolerance of 1.2 Da for the precursor ion and 0.8 Da for product ions. To assess false-positive identifications, an automatic decoy search was performed against a randomized database with a default significance threshold of $p < 0.05$; the false discovery rate at the identity threshold was below 3.4%.

2.2.4 Protein quantitative analysis

Label-free quantitative analysis of the abundance of the identified proteins was performed using the emPAI values that are provided by the Mascot program (Ishihama, *et al.*, 2005, Shinoda, *et al.*, 2010). The emPAI compares the number of observed unique parent ions per protein with the number of observable peptides per protein. PC in molar percentages within the cultured was calculated as

$$PC\ (mol\%) = emPAI / \Sigma(emPAI) \times 100$$

where Σ(emPAI) is the summation of the emPAI values for all of the identified proteins (Ishihama, *et al.*, 2005).

2.2.5 Clustering analysis

To facilitate the comparison of protein expression profiles, the normalized value, Ni, of each PC for the different carbon sources series for individual proteins was calculated with the following equation:

$$N_i = (X_i - \overline{X}) / \sigma_X$$

Ni-values ranged from − 2.04 to 2.04. The MultiExperiment Viewer, which is part of the TM4 Software Suite (Saeed, *et al.*, 2003, Saeed, *et al.*, 2006), was used for figure of merit (FOM) and K-means clustering (KMC) of the proteomic data sets. The FOM was analyzed for the optimal number of clusters of the KMC. KMC was only performed for statistically significant protein expression profiles. For both analyses, sample selection was based on a selected gene cluster. For the FOM, the parameters were set as: 100 of FOM iterations, calculate means, 50 of maximum KMC iterations, and Euclidean distance. For KMC, protein expression profiles were subdivided into 20 clusters, and the Euclidean distance was used as the current metric, and 50 maximum iterations were calculated.

2.3 Results and discussion

2.3.1 Protein identification and label-free quantitative analysis

Proteins of *P. putida* F1 cells grown in the presence of glucose, toluene, ethylbenzene, benzene, *p*-cymene, or *p*-cumate were separated by SDS-PAGE, and gels were cut into 60 strips for in-gel digestion (Fig. 2.3). Each peptide mixture was analyzed by nanoLC–MS/MS for protein identification.

In cells grown with glucose, toluene, ethylbenzene, benzene, *p*-cymene and *p*-cumate, 2022, 1733, 1802, 1801, 2368, and 2314 proteins were identified, respectively, with more than 2 unique peptide-filtering criteria, covering 33.0%–45.1% of the 5250 proteins of *P. putida* F1. PC

was calculated using the emPAI value of the identified protein to compare the level of expressed proteins. In the protein data sets derived from cells grown on each substrate, 1331 proteins were present in all data sets, and 2463 of the known *P. putida* proteins were not identified in any of these cultures. Proteins specific to glucose, toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate-substrate data sets were also identified, numbering 68, 27, 46, 32, 129, and 100, respectively.

In this analysis, the proteins detected only 1 unique peptide filtering criterion numbered 560, 649, 625, 607, 580, and 511, for the glucose, toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate data sets, respectively. These proteins will be candidates for the more comprehensive analysis of protein expression as related to metabolism.

2.3.2 The metabolic degradation pathway of aromatic hydrocarbons

2.3.2.1 Toluene metabolic pathway

In *P. putida* F1, toluene, ethylbenzene, and benzene are degraded to acetaldehyde, pyruvate, and acetyl-CoA via 7 enzymes (Fig. 2.2), which are encoded in the *tod* operon (Fig. 2.1). In cells cultured in the presence of toluene, ethylbenzene, and benzene, as expected, the 10 genes encoding these degradation enzymes were up-regulated (Fig. 2.4), while the expression of an aromatic hydrocarbon-degradation membrane protein (Pput_2883, TodX), was also observed. These results delineate the enzymes and genes that catalyze the reaction steps in the toluene/ethylbenzene/benzene metabolic pathway (Fig. 2.6).

Furthermore, the expression of a response-regulator receiver protein (Pput_2871, TodT) and a sensor histidine-kinase (Pput_2872, TodS) were specifically found to be up-regulated in the presence of toluene, ethylbenzene, and benzene (Fig. 2.4). These proteins comprise a

two-component system that regulates the expression of the *tod* operon and proteins involved in solvent efflux pumps (Pput_2866, 2867, 2868, and 2869, SepRABC) in the *sep* cluster, which is located downstream of the *tod* operon. These results indicate that the *tod* operon is positively regulated by TodST (Lau, *et al.*, 1997).

2.3.2.2 Ethylbenzene metabolic pathway

The ethylbenzene degradation pathway also involves a pathway in which propanoate is catabolized to succinate (Fig. 2.2). The pathway is known as the 2-MC cycle and is widespread among bacteria, based on sequence analysis of the completed genomes of a number of different bacteria (Horswill & Escalante-Semerena, 1997, Horswill & Escalante-Semerena, 1999, Bramer & Steinbuchel, 2001, Claes, *et al.*, 2002, Grimek & Escalante-Semerena, 2004). In *P. putida* F1, expression analysis of the 2-MC cycle has not yet been performed. Enzymes involved in the degradation of propanoate were annotated on the KEGG pathway database (Fig. 2.5).

The 2-MC cycle is driven by the proteins encoding the propanoate utilization operon (*prp*) (Horswill & Escalante-Semerena, 1997, Horswill & Escalante-Semerena, 1999, Bramer & Steinbuchel, 2001). The genome of *P. putida* F1 also contains the *prp* operon, which is composed of Pput_3432, 3433, 3435, 3436 and 3437, and Pput_3431, which is transcribed in the opposite direction to the *prp* operon. The proteins encoded by these genes could be designated as PrpD, PrpF, AcnD, PrpC, PrpB, PrpR and AcnB, respectively, from the ortholog gene analysis.

In the culture containing ethylbenzene, the expression of all the genes in the *prp* operon was clearly up-regulated (Fig. 2.5). It is difficult to assign Pput_3433 (PrpF) and 3433 (AcnD) proteins to a reaction step in the 2-MC cycle, because the 2-MC cycle itself has not yet been clearly defined. Pput_3525 and Pput_3345 were also expressed at lower levels in the

ethylbenzene culture, as compared to the other cultures. The expression of Pput_1428 and 4567, which comprise acetyl-CoA synthetase, was up-regulated less in this culture than in the culture with toluene. The acetyl-CoA synthetase engages in various pathways, such as glycolysis/gluconeogenesis, pyruvate metabolism, and methane metabolism. Therefore, these proteins may have little relation to propanoate degradation. Taken together, Pput_3525, 3345, 3435, 3432, 3431, 3436, 3433, and 3434 play a role in the 2-MC cycle, and could be designated as, FadDx, PrpE, PrpC, PrpD, AcnB, PrpB, PrpF, and AcnD, respectively (Fig. 2.5 and 2.6).

2.3.2.3 *p*-Cymene and *p*-cumate metabolic pathway

p-Cymene and *p*-cumate, which is an intermediate product of *p*-cymene metabolism, are finally degraded to isobutyrate, pyruvate, acetaldehyde, and acetyl-CoA via cis-2-hydroxypenta-2,4-dienoate (Fig. 2.2). The genes involved in the degradation of *p*-cymene and *p*-cumate are located in the *cym* operon and *cmt* operon within the ADI (Fig. 2.1). These genes were assigned to the 11 key enzymes of these pathways (Fig. 2.2). In the cultures utilizing *p*-cymene and *p*-cumate, all genes of the *cym* and the *cmt* operons were up-regulated (Fig. 2.4). The expression of the genes of the *cym* operon, which is involved in the dissimilation of *p*-cymene to *p*-cumate was also observed in the presence of *p*-cumate. This result indicates that the *cym* and the *cmt* operons are under the same regulatory control.

The Pput_2900 gene (named as *cym*E) in the *cym* operon is not assigned to the key enzymes of the *p*-cymene metabolism pathway, and has been defined as propionyl-CoA synthetase (named as *prp*E) in the NCBI database. In the presence of ethylbenzene, however, the Pput_2900 gene was poorly expressed (Fig. 2.5). Therefore, the reaction step in which the CymE protein is involved is not clear on metabolic map.

The Pput_2887 gene encoding enoyl-CoA hydratase/isomerase (EC:4.2.1.17) and the

Pput_2906 gene encoding carboxymethylenebutenolidase (EC:3.1.1.45) were also only slightly up-regulated, but their functions are unknown.

The Pput_2908 (CymR) protein of the TetR family transcriptional regulators acts as a repressor to both operons that have *p*-cymene and *p*-cumate as effectors (Eaton, 1996), and is under inducible negative-control. The PC of the CymR protein in cultures, including *p*-cymene and *p*-cumate, was less than those observed with other substrates. This result may indicate that the expression of the *cym*R gene is repressed after binding to the effector.

There are 3 pairs of paralogous proteins in the *tod* and the *cmt* operons, viz., Pput_2875 (TodG) and Pput_2890 (CmtF) protein (54.5% identity), Pput_2873 (TodH) and Pput_2888 (CmtG) (75.7% identity), and Pput_2874 (TodI) and Pput_2889 (CmtH) (69.4% identity) that catalyze the same reaction steps, viz. (5), (6), and (7), respectively (see Fig. 2.2). Although these were paralogous, the expression of Tod and Cmt proteins are strictly controlled by the substrate; consequently, the paralogues, which were annotated by genome analysis, will be easily assigned to a key enzyme and function using this comprehensive semi-quantitative method.

In the ADI, expression of Pput_2870, 2885, and 2906, which encode hypothetical proteins, and Pput_2884 of the LysR family transcriptional regulators, was not observed in the presence of any of the substrates. This may have resulted from several causes, which may relate to the detection-sensitivity for the ionized fragment by the mass spectrometer, a low level of protein expression, a possibility of pseudo-genes, and so on.

Kurbatov et al. reported that when *P. putida* KT2440 was grown in the presence of phenol, acetyl-CoA was metabolized via glyoxylate rather than via the TCA cycle, utilizing the end products of phenol degradation to provide metabolites (oxaloacetate, malate, pyruvate, and phosphoenolpyruvate) for anabolic pathways (Kurbatov, *et al.*, 2006). In this study, the proteins involved in the glyoxylate pathway were not observed to be up-regulated during growth in the

presence of aromatic compounds, although the end-products are the same as those involved in the phenol pathway.

2.3.3 Proteins specifically up-regulated in each substrate-culture

The normalized value, Ni, of the 2787 proteins in the combined 6 protein data sets was calculated and ranged from − 2.04 to 2.04. The protein with a Ni-value of 2.00 or more in each substrate-culture was defined as a specifically up-regulated protein.

For the glucose culture, 94 of specific proteins were identified. For the toluene culture, 62 specific proteins were identified, but no markedly up-regulated proteins were observed. For the ethylbenzene culture, among 164 of the specifically up-regulated proteins, PrpF, AcnD, PrpB, and Pput_3437 (GntR family transcriptional regulator) in the propanoate metabolic pathway were observed. Pput_2140 (dihydrodipicolinate synthase), Pput_3977 (hypothetical protein), and Pput_4670 (rare lipoprotein B), of which the functions relevant to ethylbenzene degradation were not clear, were also up-regulated. For the benzene culture, among 52 specifically up-regulated proteins, Pput_2058 (aldo/keto reductase), Pput_2406 (sulfatase), and Pput_4346 (β-ketoadipyl CoA thiolase) were identified, in addition to CatA, CatB, CatC, PcaI, PcaJ, and PcaF.

Among 137 and 109 specifically up-regulated proteins for *p*-cymene and *p*-cumate cultures, respectively, Pput_1022 and 3227 (cold-shock DNA-binding protein) and Pput_3466 (DNA-binding protein HU-beta), and Pput_5222 (DNA-binding protein HU-alpha) were seen. The relationship between the function of the proteins and p-cymene and p-cumate degradation is unknown.

Additionally for toluene, ethylbenzene, and benzene cultures, Pput_0580 (N-acetyltransferase), Pput_0581 and 0582 (ethanolamine ammonia-lyase small subunit and

large subunit) and Pput_0583 (ethanolamine transporter), and for toluene and ethylbenzene cultures, Pput_3976 (acetate permease, ActP) and Pput_3977 (hypothetical protein), were also up-regulated.

Most of the specifically up-regulated proteins have no direct relation to the degradation of each substrate, but may be related to secondary or tertiary metabolism. Furthermore, the role of the specifically up-regulated proteins in metabolism would need to be clarified. Nevertheless, because some of these proteins were markedly increased, it may be possible to use them as biomarkers for the activity to degrade aromatic hydrocarbons.

2.3.4 K-means clustering of regulated proteins

The expression profiles of 2787 proteins were divided into 20 groups by FOM and KMC (Fig. 2.7 and Table 2.1). From the analysis, most of the proteins of the *tod* and the *sep* operons, and the two-component system (TodST), were affiliated to groups L, N, O, and Q, the proteins in the propanoate metabolic pathway to groups I and J, and the proteins in the 3-oxoadipate pathway to group H. The proteins of the *cym* and the *cmt* operons were affiliated to groups E and F. The proteins up-regulated in the 4 degradation pathways were apparently differentiated according to their functions. Similarly, it will be possible to cluster the down-regulated and constitutively expressed proteins by KMC analysis.

In this study, I analyzed the comprehensive expression of the proteomes in cultures using different substrates by a semi-quantitative method, using PC-values converted from the emPAI of identified proteins. Our analysis approach has some advantages: firstly, it allows for easier identification and validation of the proteins involved in the degradation metabolism compared with 2-DE proteomics. The proteins encoded by key enzyme genes are detected not from the changed spots and bands on a gel, but from the genomic information derived from a

functionally annotated gene. This is supported by the fact that I was able to identify all of the key enzymes in 4 metabolic pathways (Fig. 2.6), as well as the regulator and transporter proteins.

Secondly, the expression of proteins can be analyzed by focusing on operons. Generally, many gene clusters related to metabolic pathways comprise an operon, e.g. besides the operons investigated in this study, the *pha* and *paa* operons for phenylalanine degradation (Jimenez, *et al.*, 2002, Teufel, *et al.*, 2010), the *ure* operon for urea degradation, the *suc* and *sdh* operons for the TCA cycle, and the *cop*, *cus*, and *ars* operons for metal resistance (Wu, *et al.*, 2011). This advantage is supported by the finding that CmtI and CmtAd proteins were either not expressed, or expressed at low levels, but were estimated to be expressed at much the same levels as that of other proteins in the *cmt* operon.

Finally, this approach makes it possible to obtain information regarding expression profiles of proteins identified genome-wide, and to analyze the differential display proteome between total cellular proteins. This will correspond to expression analysis via an array approach for transcriptomics. Sequential analysis during cell growth would also be informative. From comparison between the proteome data sets of logarithmic and stationary phase cultures of *P. putida* F1 in the presence of glucose, 21 and 73 phase-specific proteins, respectively, were identified. These included Pput_0388 (biotin synthase) and Pput_3906 (capsule polysaccharide biosynthesis protein) in the log phase, and Pput_3644 (ribosome modulation factor), which is a stationary phase-specific inhibitor of ribosome functions (Wada, *et al.*, 1995, Wada, *et al.*, 2000), in stationary phase cultures (data not shown).

2.4 Conclusion

In conclusion, in order to obtain a genome-wide understanding of the degradation of aromatic hydrocarbons in the *P. putida* F1 strain, I have demonstrated the utility of semi-quantitative analysis using PC values converted from the emPAI. The main pathways and many proteins involved in the degradation were identified by comparative expression proteomics. Moreover, the proteome data set obtained by this analysis can be processed as a general array-analysis data. Our results suggest that these demonstrated approaches will be ideal as a primary investigation into the various physiological characteristics of different bacterial strains.

Table 2.1 List of genes in each group divided by K-means clustering of proteome data sets.

Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T
Pput_0022	Pput_0342	Pput_1118	Pput_0028	Pput_0009	Pput_0023	Pput_0044	Pput_0071	Pput_0013	Pput_0001	Pput_0015	Pput_0106	Pput_0090	Pput_0002	Pput_0043	Pput_0003	Pput_0068	Pput_0119	Pput_0082	Pput_0004
Pput_0040	Pput_0452	Pput_1445	Pput_0501	Pput_0017	Pput_0069	Pput_0060	Pput_0104	Pput_0021	Pput_0074	Pput_0076	Pput_0209	Pput_0110	Pput_0079	Pput_0077	Pput_0027	Pput_0115	Pput_0151	Pput_0143	Pput_0097
Pput_0099	Pput_0745	Pput_2086	Pput_0691	Pput_0120	Pput_0073	Pput_0167	Pput_0324	Pput_0036	Pput_0088	Pput_0177	Pput_0215	Pput_0145	Pput_0086	Pput_0081	Pput_0030	Pput_0138	Pput_0162	Pput_0175	Pput_0137
Pput_0100	Pput_1098	Pput_2342	Pput_0796	Pput_0132	Pput_0101	Pput_0182	Pput_0357	Pput_0067	Pput_0118	Pput_0184	Pput_0301	Pput_0213	Pput_0103	Pput_0083	Pput_0084	Pput_0149	Pput_0339	Pput_0413	Pput_0222
Pput_0135	Pput_1158	Pput_3218	Pput_0851	Pput_0139	Pput_0200	Pput_0220	Pput_0359	Pput_0072	Pput_0131	Pput_0208	Pput_0347	Pput_0265	Pput_0109	Pput_0089	Pput_0129	Pput_0207	Pput_0356	Pput_0437	Pput_0229
Pput_0158	Pput_1235	Pput_3219	Pput_0866	Pput_0183	Pput_0226	Pput_0274	Pput_0411	Pput_0108	Pput_0133	Pput_0210	Pput_0348	Pput_0368	Pput_0111	Pput_0098	Pput_0144	Pput_0228	Pput_0362	Pput_0469	Pput_0260
Pput_0179	Pput_1710	Pput_3941	Pput_0867	Pput_0242	Pput_0239	Pput_0318	Pput_0425	Pput_0124	Pput_0136	Pput_0284	Pput_0412	Pput_0394	Pput_0157	Pput_0128	Pput_0146	Pput_0230	Pput_0381	Pput_0502	Pput_0268
Pput_0261	Pput_1812	Pput_4594	Pput_0885	Pput_0273	Pput_0245	Pput_0337	Pput_0427	Pput_0125	Pput_0150	Pput_0365	Pput_0429	Pput_0440	Pput_0178	Pput_0140	Pput_0173	Pput_0267	Pput_0493	Pput_0512	Pput_0449
Pput_0264	Pput_2310		Pput_0994	Pput_0291	Pput_0248	Pput_0355	Pput_0433	Pput_0163	Pput_0159	Pput_0366	Pput_0456	Pput_0442	Pput_0185	Pput_0141	Pput_0205	Pput_0280	Pput_0504	Pput_0551	Pput_0473
Pput_0316	Pput_2569		Pput_1019	Pput_0432	Pput_0251	Pput_0397	Pput_0507	Pput_0235	Pput_0186	Pput_0367	Pput_0483	Pput_0463	Pput_0211	Pput_0147	Pput_0225	Pput_0314	Pput_0553	Pput_0577	Pput_0475
Pput_0423	Pput_4692		Pput_1070	Pput_0472	Pput_0303	Pput_0444	Pput_0517	Pput_0250	Pput_0199	Pput_0390	Pput_0487	Pput_0562	Pput_0224	Pput_0206	Pput_0259	Pput_0406	Pput_0570	Pput_0636	Pput_0484
Pput_0426	Pput_4742		Pput_1214	Pput_0495	Pput_0305	Pput_0519	Pput_0599	Pput_0279	Pput_0233	Pput_0396	Pput_0494	Pput_0596	Pput_0243	Pput_0240	Pput_0276	Pput_0420	Pput_0622	Pput_0654	Pput_0503
Pput_0447	Pput_5091		Pput_1249	Pput_0497	Pput_0327	Pput_0615	Pput_0645	Pput_0323	Pput_0241	Pput_0409	Pput_0506	Pput_0672	Pput_0257	Pput_0258	Pput_0300	Pput_0431	Pput_0692	Pput_0706	Pput_0510
Pput_0489			Pput_1270	Pput_0556	Pput_0346	Pput_0727	Pput_0665	Pput_0345	Pput_0249	Pput_0421	Pput_0511	Pput_0766	Pput_0262	Pput_0263	Pput_0304	Pput_0476	Pput_0789	Pput_0753	Pput_0641
Pput_0496			Pput_1349	Pput_0646	Pput_0373	Pput_0750	Pput_0720	Pput_0364	Pput_0281	Pput_0430	Pput_0609	Pput_0783	Pput_0275	Pput_0307	Pput_0306	Pput_0478	Pput_0802	Pput_0816	Pput_0687
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Pput_0573			Pput_1529	Pput_0873	Pput_0450	Pput_1043	Pput_0918	Pput_0392	Pput_0360	Pput_0455	Pput_0715	Pput_0831	Pput_0309	Pput_0350	Pput_0386	Pput_0490	Pput_0891	Pput_0883	Pput_0755
Pput_0574			Pput_1580	Pput_0875	Pput_0461	Pput_1091	Pput_0924	Pput_0422	Pput_0369	Pput_0480	Pput_0775	Pput_1020	Pput_0310	Pput_0361	Pput_0404	Pput_0514	Pput_0925	Pput_0936	Pput_0756
Pput_0633			Pput_1826	Pput_0903	Pput_0474	Pput_1134	Pput_0928	Pput_0546	Pput_0370	Pput_0491	Pput_0826	Pput_1156	Pput_0343	Pput_0372	Pput_0424	Pput_0516	Pput_0984	Pput_1000	Pput_0793
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Pput_0674			Pput_2211	Pput_1031	Pput_0499	Pput_1225	Pput_0995	Pput_0592	Pput_0382	Pput_0515	Pput_1065	Pput_1289	Pput_0349	Pput_0435	Pput_0434	Pput_0589	Pput_0999	Pput_1018	Pput_0804
Pput_0675			Pput_3133	Pput_1037	Pput_0505	Pput_1259	Pput_1084	Pput_0593	Pput_0403	Pput_0527	Pput_1165	Pput_1315	Pput_0363	Pput_0438	Pput_0446	Pput_0668	Pput_1099	Pput_1047	Pput_0840
Pput_0681			Pput_3230	Pput_1122	Pput_0513	Pput_1299	Pput_1102	Pput_0594	Pput_0451	Pput_0549	Pput_1239	Pput_1326	Pput_0383	Pput_0439	Pput_0457	Pput_0894	Pput_1188	Pput_1048	Pput_0843
Pput_0709			Pput_3236	Pput_1175	Pput_0521	Pput_1309	Pput_1108	Pput_0647	Pput_0454	Pput_0583	Pput_1324	Pput_1439	Pput_0400	Pput_0441	Pput_0458	Pput_0952	Pput_1205	Pput_1050	Pput_0993
Pput_0728			Pput_3652	Pput_1194	Pput_0525	Pput_1532	Pput_1257	Pput_0655	Pput_0557	Pput_0597	Pput_1440	Pput_1486	Pput_0407	Pput_0443	Pput_0466	Pput_0992	Pput_1211	Pput_1057	Pput_1128
Pput_0749			Pput_3993	Pput_1203	Pput_0579	Pput_1536	Pput_1295	Pput_0726	Pput_0581	Pput_0598	Pput_1461	Pput_1621	Pput_0445	Pput_0460	Pput_0471	Pput_1015	Pput_1252	Pput_1061	Pput_1251
Pput_0813			Pput_4033	Pput_1280	Pput_0595	Pput_1589	Pput_1338	Pput_0777	Pput_0582	Pput_0606	Pput_1551	Pput_1638	Pput_0482	Pput_0465	Pput_0477	Pput_1024	Pput_1253	Pput_1071	Pput_1316
Pput_0864			Pput_4043	Pput_1284	Pput_0604	Pput_1607	Pput_1388	Pput_0890	Pput_0586	Pput_0623	Pput_1599	Pput_1760	Pput_0528	Pput_0467	Pput_0486	Pput_1109	Pput_1260	Pput_1119	Pput_1424
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Pput_0956			Pput_4520	Pput_1336	Pput_0762	Pput_1662	Pput_1610	Pput_0976	Pput_0740	Pput_0663	Pput_2659	Pput_2489	Pput_0580	Pput_0545	Pput_0531	Pput_1186	Pput_1476	Pput_1170	Pput_1530
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Pput_1076			Pput_4714	Pput_1510	Pput_0880	Pput_1684	Pput_1778	Pput_1034	Pput_0771	Pput_0772	Pput_3122	Pput_3017	Pput_0616	Pput_0584	Pput_0554	Pput_1432	Pput_1583	Pput_1608	Pput_1652
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Pput_1110			Pput_4780	Pput_1613	Pput_1001	Pput_1816	Pput_1871	Pput_1060	Pput_0801	Pput_0792	Pput_3165	Pput_3458	Pput_0619	Pput_0632	Pput_0625	Pput_1452	Pput_1661	Pput_1742	Pput_1689
Pput_1112			Pput_4804	Pput_1627	Pput_1002	Pput_1877	Pput_1884	Pput_1062	Pput_0811	Pput_0809	Pput_3237	Pput_3507	Pput_0620	Pput_0637	Pput_0626	Pput_1453	Pput_1678	Pput_1949	Pput_1690
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Pput_1173			Pput_4973	Pput_1699	Pput_1139	Pput_1959	Pput_1978	Pput_1150	Pput_0876	Pput_0943	Pput_3542	Pput_3622	Pput_0662	Pput_0757	Pput_0676	Pput_1535	Pput_1714	Pput_2596	Pput_1820
Pput_1178			Pput_5057	Pput_1723	Pput_1164	Pput_1999	Pput_2034	Pput_1206	Pput_0961	Pput_0953	Pput_3590	Pput_3637	Pput_0702	Pput_0761	Pput_0680	Pput_1585	Pput_1749	Pput_2778	Pput_1976
Pput_1195			Pput_5072	Pput_1815	Pput_1213	Pput_2008	Pput_2050	Pput_1217	Pput_0964	Pput_0957	Pput_3627	Pput_3696	Pput_0718	Pput_0763	Pput_0717	Pput_1630	Pput_1784	Pput_3442	Pput_1995
Pput_1212			Pput_5087	Pput_1822	Pput_1216	Pput_2016	Pput_2053	Pput_1221	Pput_0991	Pput_0978	Pput_3765	Pput_3735	Pput_0738	Pput_0768	Pput_0724	Pput_1663	Pput_1800	Pput_3468	Pput_2091
Pput_1271			Pput_5103	Pput_1823	Pput_1233	Pput_2017	Pput_2054	Pput_1254	Pput_0997	Pput_1097	Pput_3767	Pput_3805	Pput_0746	Pput_0774	Pput_0730	Pput_1718	Pput_1909	Pput_3574	Pput_2594
Pput_1293			Pput_5212	Pput_1831	Pput_1298	Pput_2018	Pput_2055	Pput_1266	Pput_1003	Pput_1104	Pput_3847	Pput_3837	Pput_0760	Pput_0791	Pput_0765	Pput_1745	Pput_1955	Pput_3808	Pput_2764
Pput_1322			Pput_5228	Pput_1845	Pput_1410	Pput_2021	Pput_2058	Pput_1281	Pput_1101	Pput_1126	Pput_3903	Pput_3838	Pput_0770	Pput_0799	Pput_0786	Pput_1802	Pput_1989	Pput_3945	Pput_2765
Pput_1327			Pput_5296	Pput_1858	Pput_1444	Pput_2244	Pput_2144	Pput_1288	Pput_1115	Pput_1137	Pput_3904	Pput_3868	Pput_0779	Pput_0810	Pput_0817	Pput_1825	Pput_2287	Pput_3949	Pput_3324
Pput_1348				Pput_1859	Pput_1471	Pput_2393	Pput_2149	Pput_1290	Pput_1116	Pput_1157	Pput_3908	Pput_3985	Pput_0790	Pput_0868	Pput_0835	Pput_1950	Pput_2559	Pput_4018	Pput_3465
Pput_1382				Pput_1943	Pput_1472	Pput_2418	Pput_2150	Pput_1300	Pput_1147	Pput_1169	Pput_3926	Pput_4075	Pput_0815	Pput_0872	Pput_0848	Pput_1963	Pput_2590	Pput_4047	Pput_3550
Pput_1414				Pput_1957	Pput_1496	Pput_2419	Pput_2216	Pput_1319	Pput_1152	Pput_1222	Pput_3930	Pput_4229	Pput_0869	Pput_0884	Pput_0942	Pput_2002	Pput_2752	Pput_4053	Pput_3603
Pput_1423				Pput_2070	Pput_1509	Pput_2501	Pput_2223	Pput_1333	Pput_1154	Pput_1268	Pput_3943	Pput_4230	Pput_0871	Pput_0886	Pput_0946	Pput_2061	Pput_2755	Pput_4199	Pput_3605
Pput_1430				Pput_2157	Pput_1524	Pput_2547	Pput_2275	Pput_1341	Pput_1172	Pput_1302	Pput_3998	Pput_4233	Pput_0882	Pput_0887	Pput_0958	Pput_2113	Pput_2813	Pput_4287	Pput_3769
Pput_1503				Pput_2249	Pput_1531	Pput_2627	Pput_2371	Pput_1396	Pput_1177	Pput_1306	Pput_4175	Pput_4334	Pput_0888	Pput_0902	Pput_0987	Pput_2205	Pput_3089	Pput_4362	Pput_3770
Pput_1527				Pput_2269	Pput_1577	Pput_2731	Pput_2385	Pput_1406	Pput_1196	Pput_1405	Pput_4289	Pput_4375	Pput_0889	Pput_0927	Pput_0989	Pput_2242	Pput_3109	Pput_4378	Pput_3773
Pput_1628				Pput_2279	Pput_1620	Pput_2779	Pput_2405	Pput_1425	Pput_1198	Pput_1428	Pput_4320	Pput_4415	Pput_0954	Pput_0932	Pput_1013	Pput_2311	Pput_3142	Pput_4592	Pput_3774
Pput_1639				Pput_2303	Pput_1631	Pput_2790	Pput_2406	Pput_1454	Pput_1238	Pput_1435	Pput_4407	Pput_4433	Pput_0979	Pput_0941	Pput_1014	Pput_2329	Pput_3144	Pput_4640	Pput_3901
Pput_1688				Pput_2332	Pput_1640	Pput_2797	Pput_2407	Pput_1460	Pput_1243	Pput_1437	Pput_4425	Pput_4501	Pput_0981	Pput_0948	Pput_1053	Pput_2348	Pput_3146	Pput_4768	Pput_3934
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Pput_1872				Pput_2443	Pput_1680	Pput_2971	Pput_2691	Pput_1592	Pput_1389	Pput_1485	Pput_4871	Pput_4874	Pput_1111	Pput_0980	Pput_1228	Pput_2763	Pput_3353	Pput_4984	Pput_4187
Pput_1910				Pput_2574	Pput_1700	Pput_2974	Pput_2857	Pput_1654	Pput_1390	Pput_1571	Pput_4899	Pput_4895	Pput_1121	Pput_0982	Pput_1230	Pput_2881	Pput_3357	Pput_5056	Pput_4236
Pput_1930				Pput_2602	Pput_1720	Pput_2994	Pput_2872	Pput_1655	Pput_1392	Pput_1587	Pput_4909	Pput_4944	Pput_1140	Pput_0996	Pput_1236	Pput_2883	Pput_3451	Pput_5114	Pput_4277
Pput_1983				Pput_2636	Pput_1750	Pput_3024	Pput_3039	Pput_1695	Pput_1397	Pput_1593	Pput_5140	Pput_5006	Pput_1162	Pput_1005	Pput_1246	Pput_3222	Pput_3473	Pput_5123	Pput_4291
Pput_1998				Pput_2645	Pput_1789	Pput_3065	Pput_3054	Pput_1719	Pput_1416	Pput_1653	Pput_5181		Pput_1167	Pput_1006	Pput_1250	Pput_3223	Pput_3536	Pput_5198	Pput_4434
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Pput_2104				Pput_2727	Pput_1928	Pput_3178	Pput_3334	Pput_2000	Pput_1500	Pput_1806			Pput_1308	Pput_1072	Pput_1314	Pput_3579	Pput_3892		Pput_4544
Pput_2131				Pput_2784	Pput_1938	Pput_3326	Pput_3367	Pput_2003	Pput_1544	Pput_1808			Pput_1335	Pput_1073	Pput_1325	Pput_3660	Pput_3897		Pput_4612
Pput_2170				Pput_2818	Pput_1973	Pput_3348	Pput_3453	Pput_2004	Pput_1582	Pput_1913			Pput_1340	Pput_1077	Pput_1391	Pput_3802	Pput_3923		Pput_4678
Pput_2214				Pput_2889	Pput_2037	Pput_3527	Pput_3517	Pput_2013	Pput_1590	Pput_1935			Pput_1350	Pput_1078	Pput_1412	Pput_3822	Pput_3925		Pput_4697
Pput_2230				Pput_2890	Pput_2043	Pput_3538	Pput_3576	Pput_2039	Pput_1601	Pput_1952			Pput_1403	Pput_1107	Pput_1429	Pput_3865	Pput_4258		Pput_4698
Pput_2248				Pput_2891	Pput_2072	Pput_3594	Pput_3723	Pput_2042	Pput_1703	Pput_2005			Pput_1480	Pput_1120	Pput_1443	Pput_3928	Pput_4293		Pput_4856
Pput_2264				Pput_2893	Pput_2073	Pput_3644	Pput_3777	Pput_2044	Pput_1741	Pput_2067			Pput_1501	Pput_1127	Pput_1467	Pput_3951	Pput_4338		Pput_4872
Pput_2394				Pput_2895	Pput_2094	Pput_3646	Pput_3831	Pput_2085	Pput_1748	Pput_2257			Pput_1525	Pput_1133	Pput_1475	Pput_3953	Pput_4365		Pput_4900
Pput_2471				Pput_2896	Pput_2095	Pput_3655	Pput_3834	Pput_2110	Pput_1774	Pput_2258			Pput_1548	Pput_1138	Pput_1491	Pput_3959	Pput_4416		Pput_4907
Pput_2493				Pput_2897	Pput_2151	Pput_3717	Pput_4207	Pput_2112	Pput_1775	Pput_2341			Pput_1603	Pput_1144	Pput_1498	Pput_3997	Pput_4450		Pput_4920
Pput_2526				Pput_2900	Pput_2197	Pput_3718	Pput_4268	Pput_2140	Pput_1791	Pput_2388			Pput_1612	Pput_1209	Pput_1499	Pput_4000	Pput_4547		Pput_4929
Pput_2546				Pput_2901	Pput_2219	Pput_3726	Pput_4303	Pput_2143	Pput_1792	Pput_2537			Pput_1656	Pput_1210	Pput_1504	Pput_4152	Pput_4569		Pput_5062
Pput_2607				Pput_2903	Pput_2224	Pput_3797	Pput_4321	Pput_2173	Pput_1796	Pput_2555			Pput_1674	Pput_1234	Pput_1528	Pput_4174	Pput_4599		Pput_5067
Pput_2687				Pput_2904	Pput_2274	Pput_3807	Pput_4336	Pput_2207	Pput_1834	Pput_2573			Pput_1691	Pput_1242	Pput_1547	Pput_4178	Pput_4605		Pput_5210
Pput_2705				Pput_2905	Pput_2280	Pput_3816	Pput_4340	Pput_2220	Pput_1886	Pput_2589			Pput_1693	Pput_1248	Pput_1559	Pput_4208	Pput_4685		Pput_5223
Pput_2724				Pput_2906	Pput_2319	Pput_3909	Pput_4342	Pput_2221	Pput_1907	Pput_2653			Pput_1694	Pput_1297	Pput_1570	Pput_4226	Pput_4743		Pput_5295
Pput_2816				Pput_2952	Pput_2320	Pput_3920	Pput_4343	Pput_2222	Pput_1934	Pput_2774			Pput_1697	Pput_1337	Pput_1581	Pput_4231	Pput_4859		Pput_5297
Pput_2894				Pput_3040	Pput_2352	Pput_3958	Pput_4344	Pput_2228	Pput_2015	Pput_2787			Pput_1712	Pput_1339	Pput_1600	Pput_4243	Pput_4862		
Pput_2950				Pput_3052	Pput_2401	Pput_3991	Pput_4346	Pput_2236	Pput_2084	Pput_3083			Pput_1727	Pput_1345	Pput_1604	Pput_4245	Pput_4926		
Pput_2960				Pput_3117	Pput_2420	Pput_4054	Pput_4396	Pput_2239	Pput_2141	Pput_3132			Pput_1765	Pput_1351	Pput_1609	Pput_4259	Pput_4952		
Pput_3002				Pput_3143	Pput_2622	Pput_4209	Pput_4422	Pput_2268	Pput_2182	Pput_3225			Pput_1783	Pput_1366	Pput_1615	Pput_4278	Pput_4972		
Pput_3011				Pput_3207	Pput_2623	Pput_4256	Pput_4427	Pput_2270	Pput_2227	Pput_3228			Pput_1785	Pput_1378	Pput_1617	Pput_4309	Pput_5105		
Pput_3038				Pput_3226	Pput_2631	Pput_4275	Pput_4428	Pput_2304	Pput_2323	Pput_3319			Pput_1788	Pput_1436	Pput_1622	Pput_4395	Pput_5119		
Pput_3071				Pput_3231	Pput_2649	Pput_4281	Pput_4435	Pput_2322	Pput_2334	Pput_3439			Pput_1790	Pput_1441	Pput_1632	Pput_4399	Pput_5196		
Pput_3119				Pput_3262	Pput_2651	Pput_4318	Pput_4478	Pput_2328	Pput_2347	Pput_3460			Pput_1797	Pput_1455	Pput_1651	Pput_4405	Pput_5234		
Pput_3152				Pput_3273	Pput_2665	Pput_4393	Pput_4553	Pput_2335	Pput_2444	Pput_3462			Pput_1809	Pput_1462	Pput_1668	Pput_4412	Pput_5282		
Pput_3160				Pput_3309	Pput_2722	Pput_4402	Pput_4757	Pput_2337	Pput_2560	Pput_3489			Pput_1819	Pput_1481	Pput_1669	Pput_4479			
Pput_3330				Pput_3317	Pput_2725	Pput_4440	Pput_4868	Pput_2343	Pput_2588	Pput_3553			Pput_1828	Pput_1508	Pput_1743	Pput_4481			
Pput_3393				Pput_3349	Pput_2803	Pput_4482	Pput_4883	Pput_2356	Pput_2671	Pput_3585			Pput_1835	Pput_1533	Pput_1772	Pput_4518			
Pput_3428				Pput_3445	Pput_2810	Pput_4541	Pput_4910	Pput_2359	Pput_2786	Pput_3597			Pput_1885	Pput_1591	Pput_1821	Pput_4576			
Pput_3444				Pput_3463	Pput_2814	Pput_4551	Pput_4933	Pput_2389	Pput_2828	Pput_3606			Pput_1891	Pput_1594	Pput_1830	Pput_4577			
Pput_3448				Pput_3491	Pput_2839	Pput_4560	Pput_5011	Pput_2392	Pput_3070	Pput_3624			Pput_1936	Pput_1619	Pput_1838	Pput_4578			
Pput_3486				Pput_3584	Pput_2887	Pput_4580	Pput_5013	Pput_2429	Pput_3163	Pput_3642			Pput_1991	Pput_1660	Pput_1844	Pput_4589			
Pput_3488				Pput_3602	Pput_2888	Pput_4674	Pput_5068	Pput_2539	Pput_3191	Pput_3643			Pput_2009	Pput_1664	Pput_1846	Pput_4597			
Pput_3548				Pput_3604	Pput_2892	Pput_4680	Pput_5080	Pput_2544	Pput_3195	Pput_3706			Pput_2010	Pput_1696	Pput_1852	Pput_4615			
Pput_3611				Pput_3634	Pput_2898	Pput_4683	Pput_5121	Pput_2562	Pput_3235	Pput_3768			Pput_2025	Pput_1698	Pput_1932	Pput_4669			
Pput_3629				Pput_3688	Pput_2899	Pput_4719	Pput_5174	Pput_2563	Pput_3246	Pput_3796			Pput_2032	Pput_1704	Pput_1948	Pput_4759			
Pput_3689				Pput_3731	Pput_2902	Pput_4779	Pput_5250	Pput_2568	Pput_3292	Pput_3806			Pput_2065	Pput_1715	Pput_1958	Pput_4760			
Pput_3743				Pput_3775	Pput_2968	Pput_4828		Pput_2586	Pput_3345	Pput_3844			Pput_2079	Pput_1721	Pput_1996	Pput_4769			
Pput_3745				Pput_3792	Pput_3041	Pput_4919		Pput_2587	Pput_3432	Pput_3857			Pput_2080	Pput_1725	Pput_1997	Pput_4807			
Pput_3827				Pput_3823	Pput_3042	Pput_4922		Pput_2591	Pput_3449	Pput_3867			Pput_2081	Pput_1739	Pput_2082	Pput_4815			
Pput_3840				Pput_3842	Pput_3048	Pput_4923		Pput_2660	Pput_3528	Pput_3895			Pput_2189	Pput_1753	Pput_2093	Pput_4840			
Pput_3855				Pput_3894	Pput_3058	Pput_4925		Pput_2679	Pput_3541	Pput_3905			Pput_2193	Pput_1766	Pput_2097	Pput_4850			
Pput_3861				Pput_3984	Pput_3116	Pput_4957		Pput_2686	Pput_3554	Pput_3950			Pput_2229	Pput_1776	Pput_2155	Pput_4853			
Pput_3992				Pput_3987	Pput_3118	Pput_5073		Pput_2698	Pput_3598	Pput_4050			Pput_2234	Pput_1777	Pput_2164	Pput_4913			
Pput_4089				Pput_4180	Pput_3121	Pput_5215		Pput_2703	Pput_3610	Pput_4062			Pput_2243	Pput_1795	Pput_2179	Pput_4918			
Pput_4198				Pput_4201	Pput_3215	Pput_5276		Pput_2711	Pput_3617	Pput_4158			Pput_2247	Pput_1817	Pput_2186	Pput_4949			
Pput_4242				Pput_4204	Pput_3227			Pput_2713	Pput_3618	Pput_4173			Pput_2321	Pput_1818	Pput_2212	Pput_4962			
Pput_4247				Pput_4219	Pput_3229			Pput_2723	Pput_3641	Pput_4189			Pput_2349	Pput_1833	Pput_2278	Pput_4994			
Pput_4254				Pput_4361	Pput_3250			Pput_2735	Pput_3734	Pput_4195			Pput_2358	Pput_1836	Pput_2301	Pput_5082			
Pput_4255				Pput_4411	Pput_3274			Pput_2770	Pput_3764	Pput_4197			Pput_2378	Pput_1956	Pput_2318	Pput_5099			
Pput_4274				Pput_4476	Pput_3308			Pput_2773	Pput_3789	Pput_4203			Pput_2425	Pput_2041	Pput_2345	Pput_5214			
Pput_4406				Pput_4488	Pput_3314			Pput_2783	Pput_3798	Pput_4215			Pput_2473	Pput_2087	Pput_2346	Pput_5218			
Pput_4438				Pput_4489	Pput_3340			Pput_2800	Pput_3811	Pput_4227			Pput_2530	Pput_2111	Pput_2375	Pput_5291			
Pput_4449				Pput_4530	Pput_3351			Pput_2801	Pput_3851	Pput_4240			Pput_2542	Pput_2181	Pput_2376				
Pput_4464				Pput_4531	Pput_3414			Pput_2809	Pput_3884	Pput_4246			Pput_2564	Pput_2307	Pput_2377				
Pput_4483				Pput_4535	Pput_3466			Pput_2822	Pput_3929	Pput_4250			Pput_2566	Pput_2524	Pput_2379				

Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T
Pput_4549				Pput_4563	Pput_3534			Pput_2856	Pput_3938	Pput_4288			Pput_2567	Pput_2531	Pput_2380				
Pput_4555				Pput_4574	Pput_3547			Pput_2862	Pput_3969	Pput_4290			Pput_2575	Pput_2557	Pput_2382				
Pput_4572				Pput_4586	Pput_3555			Pput_3009	Pput_3976	Pput_4292			Pput_2579	Pput_2576	Pput_2400				
Pput_4600				Pput_4635	Pput_3586			Pput_3014	Pput_3989	Pput_4298			Pput_2710	Pput_2634	Pput_2410				
Pput_4601				Pput_4729	Pput_3680			Pput_3073	Pput_4030	Pput_4389			Pput_2751	Pput_2760	Pput_2450				
Pput_4603				Pput_4734	Pput_3703			Pput_3079	Pput_4068	Pput_4404			Pput_2758	Pput_2762	Pput_2460				
Pput_4657				Pput_4745	Pput_3716			Pput_3090	Pput_4155	Pput_4424			Pput_2775	Pput_2767	Pput_2470				
Pput_4668				Pput_4789	Pput_3758			Pput_3101	Pput_4171	Pput_4477			Pput_2799	Pput_2836	Pput_2507				
Pput_4677				Pput_4811	Pput_3799			Pput_3128	Pput_4182	Pput_4504			Pput_2808	Pput_2838	Pput_2514				
Pput_4706				Pput_4870	Pput_3803			Pput_3176	Pput_4220	Pput_4516			Pput_2824	Pput_2866	Pput_2525				
Pput_4767				Pput_4880	Pput_3813			Pput_3181	Pput_4225	Pput_4556			Pput_2863	Pput_2873	Pput_2571				
Pput_4770				Pput_4903	Pput_3815			Pput_3184	Pput_4251	Pput_4562			Pput_2867	Pput_2875	Pput_2593				
Pput_4818				Pput_5012	Pput_3832			Pput_3194	Pput_4262	Pput_4567			Pput_2869	Pput_2876	Pput_2595				
Pput_4898				Pput_5063	Pput_3882			Pput_3200	Pput_4264	Pput_4575			Pput_2880	Pput_2878	Pput_2652				
Pput_4940				Pput_5108	Pput_3936			Pput_3202	Pput_4285	Pput_4591			Pput_2882	Pput_2879	Pput_2733				
Pput_4961				Pput_5110	Pput_3963			Pput_3245	Pput_4308	Pput_4611			Pput_2908	Pput_3102	Pput_2745				
Pput_5020				Pput_5134	Pput_4002			Pput_3252	Pput_4322	Pput_4637			Pput_3013	Pput_3134	Pput_2746				
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Pput_5124				Pput_5189	Pput_4202			Pput_3263	Pput_4421	Pput_4764			Pput_3161	Pput_3170	Pput_2864				
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Pput_5165					Pput_4409			Pput_3300	Pput_4536	Pput_4817			Pput_3187	Pput_3244	Pput_3193				
Pput_5171					Pput_4431			Pput_3302	Pput_4537	Pput_4875			Pput_3189	Pput_3431	Pput_3224				
Pput_5208					Pput_4432			Pput_3305	Pput_4538	Pput_4894			Pput_3217	Pput_3443	Pput_3232				
Pput_5222					Pput_4494			Pput_3318	Pput_4585	Pput_4902			Pput_3259	Pput_3446	Pput_3260				
Pput_5227					Pput_4495			Pput_3346	Pput_4620	Pput_4904			Pput_3327	Pput_3469	Pput_3277				
Pput_5253					Pput_4533			Pput_3352	Pput_4655	Pput_4906			Pput_3331	Pput_3478	Pput_3299				
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					Pput_4662			Pput_3434	Pput_4773	Pput_4983			Pput_3398	Pput_3578	Pput_3399				
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					Pput_4876			Pput_3477	Pput_4825	Pput_5101			Pput_3502	Pput_3596	Pput_3470				
					Pput_4884			Pput_3484	Pput_4834	Pput_5111			Pput_3508	Pput_3599	Pput_3481				
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					Pput_4924			Pput_3546	Pput_4864	Pput_5175			Pput_3524	Pput_3619	Pput_3539				
					Pput_4927			Pput_3561	Pput_4877	Pput_5200			Pput_3658	Pput_3628	Pput_3549				
					Pput_4960			Pput_3589	Pput_4885	Pput_5209			Pput_3659	Pput_3645	Pput_3608				
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					Pput_5213			Pput_3742	Pput_5193	Pput_5298			Pput_3751	Pput_3776	Pput_3804				
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								Pput_3814	Pput_5261				Pput_3786	Pput_3800	Pput_3978				
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								Pput_3887	Pput_5304				Pput_3810	Pput_3812	Pput_4016				
								Pput_3948	Pput_5305				Pput_3839	Pput_3843	Pput_4055				
								Pput_3977					Pput_3845	Pput_3849	Pput_4060				
								Pput_3981					Pput_3860	Pput_3872	Pput_4069				
								Pput_4004					Pput_3880	Pput_3896	Pput_4071				
								Pput_4013					Pput_3885	Pput_3922	Pput_4072				
								Pput_4031					Pput_3888	Pput_3933	Pput_4076				
								Pput_4040					Pput_3890	Pput_3946	Pput_4091				
								Pput_4067					Pput_3944	Pput_3947	Pput_4153				
								Pput_4084					Pput_3962	Pput_3955	Pput_4163				
								Pput_4154					Pput_3982	Pput_3988	Pput_4165				
								Pput_4159					Pput_4006	Pput_3994	Pput_4177				
								Pput_4200					Pput_4026	Pput_3999	Pput_4183				
								Pput_4232					Pput_4063	Pput_4023	Pput_4185				
								Pput_4299					Pput_4064	Pput_4073	Pput_4186				
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								Pput_4302					Pput_4082	Pput_4161	Pput_4216				
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								Pput_4328					Pput_4131	Pput_4176	Pput_4221				
								Pput_4331					Pput_4156	Pput_4184	Pput_4252				
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								Pput_4373					Pput_4160	Pput_4194	Pput_4297				
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								Pput_4496					Pput_4217	Pput_4257	Pput_4350				
								Pput_4517					Pput_4222	Pput_4294	Pput_4353				
								Pput_4522					Pput_4223	Pput_4295	Pput_4357				
								Pput_4534					Pput_4234	Pput_4301	Pput_4363				
								Pput_4564					Pput_4260	Pput_4348	Pput_4372				
								Pput_4602					Pput_4267	Pput_4351	Pput_4394				
								Pput_4644					Pput_4337	Pput_4352	Pput_4401				
								Pput_4670					Pput_4354	Pput_4377	Pput_4436				
								Pput_4675					Pput_4356	Pput_4379	Pput_4506				
								Pput_4676					Pput_4374	Pput_4381	Pput_4510				
								Pput_4689					Pput_4376	Pput_4383	Pput_4542				
								Pput_4708					Pput_4382	Pput_4385	Pput_4550				
								Pput_4710					Pput_4387	Pput_4386	Pput_4557				
								Pput_4724					Pput_4398	Pput_4391	Pput_4593				
								Pput_4746					Pput_4410	Pput_4392	Pput_4618				
								Pput_4796					Pput_4423	Pput_4408	Pput_4621				
								Pput_4800					Pput_4451	Pput_4413	Pput_4623				
								Pput_4821					Pput_4507	Pput_4414	Pput_4639				
								Pput_4829					Pput_4508	Pput_4503	Pput_4643				
								Pput_4845					Pput_4546	Pput_4519	Pput_4651				
								Pput_4848					Pput_4584	Pput_4532	Pput_4671				
								Pput_4878					Pput_4596	Pput_4543	Pput_4693				
								Pput_4886					Pput_4634	Pput_4548	Pput_4705				
								Pput_4888					Pput_4652	Pput_4561	Pput_4712				
								Pput_4893					Pput_4656	Pput_4565	Pput_4716				
								Pput_4911					Pput_4658	Pput_4566	Pput_4717				
								Pput_4943					Pput_4663	Pput_4573	Pput_4735				
								Pput_4959					Pput_4665	Pput_4581	Pput_4748				
								Pput_4982					Pput_4673	Pput_4582	Pput_4754				
								Pput_5051					Pput_4682	Pput_4583	Pput_4755				
								Pput_5131					Pput_4686	Pput_4590	Pput_4776				
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								Pput_5167					Pput_4771	Pput_4660	Pput_4826				
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								Pput_5188					Pput_4782	Pput_4694	Pput_4842				
								Pput_5254					Pput_4787	Pput_4726	Pput_4854				
								Pput_5265					Pput_4790	Pput_4740	Pput_4866				
								Pput_5272					Pput_4793	Pput_4747	Pput_4881				
								Pput_5275					Pput_4795	Pput_4772	Pput_4891				
								Pput_5306					Pput_4803	Pput_4778	Pput_4928				
													Pput_4806	Pput_4783	Pput_4930				
													Pput_4814	Pput_4784	Pput_4947				
													Pput_4822	Pput_4785	Pput_4948				
													Pput_4861	Pput_4788	Pput_4974				
													Pput_4879	Pput_4791	Pput_5000				
													Pput_4896	Pput_4809	Pput_5024				
													Pput_4915	Pput_4810	Pput_5037				
													Pput_4937	Pput_4816	Pput_5059				
													Pput_4941	Pput_4819	Pput_5060				
													Pput_4967	Pput_4823	Pput_5078				
													Pput_4977	Pput_4837	Pput_5088				

Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K	Group L	Group M	Group N	Group O	Group P	Group Q	Group R	Group S	Group T
													Pput_4980	Pput_4838	Pput_5100				
													Pput_4985	Pput_4873	Pput_5129				
													Pput_4986	Pput_4908	Pput_5130				
													Pput_4987	Pput_4914	Pput_5158				
													Pput_4997	Pput_4931	Pput_5177				
													Pput_4999	Pput_4936	Pput_5232				
													Pput_5026	Pput_4950	Pput_5292				
													Pput_5043	Pput_4951	Pput_5311				
													Pput_5052	Pput_4968					
													Pput_5053	Pput_4971					
													Pput_5089	Pput_4992					
													Pput_5090	Pput_5007					
													Pput_5092	Pput_5016					
													Pput_5106	Pput_5054					
													Pput_5107	Pput_5071					
													Pput_5126	Pput_5086					
													Pput_5137	Pput_5093					
													Pput_5138	Pput_5104					
													Pput_5162	Pput_5136					
													Pput_5168	Pput_5180					
													Pput_5169	Pput_5192					
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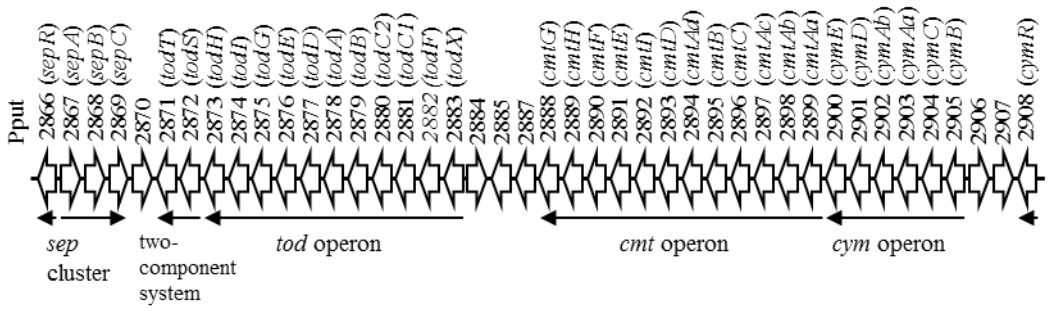

Fig.2.1 Genetic map of *sep*, *tod*, *cmt*, and *cym* operons in aromatics degradation island (AGI) on the chromosome in *P. putida* F1. The arrowheads represent the directions of transcription.

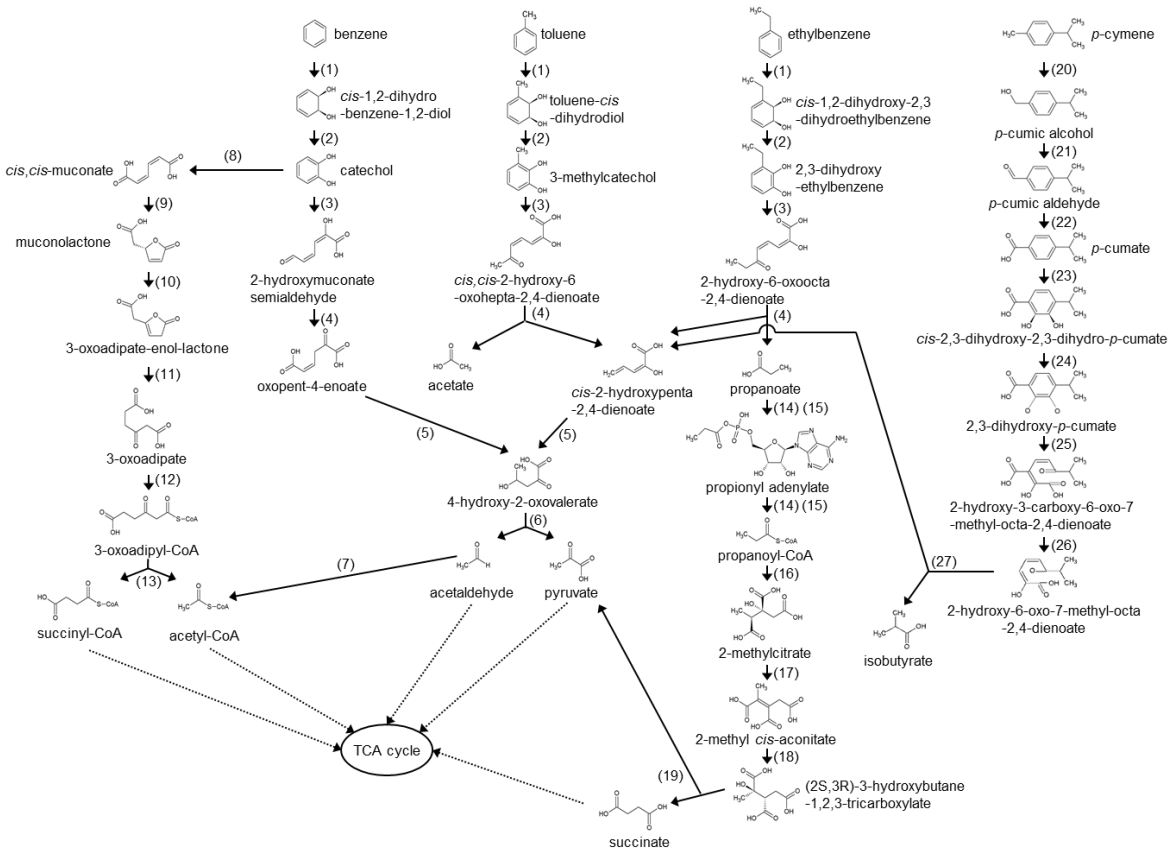

Fig. 2.2 The metabolic pathway of toluene, ethylbenzene, benzene, and p-cymene in *P. putida* F1. The number in parentheses indicates a reaction step and a key enzyme: (1) toluene dioxygenase (EC:1.14.12.11), (2) cis-benzene glycol dehydrogenase (EC:1.3.1.19), (3) catechol 2,3-dioxygenase (EC:1.13.11.2), (4) 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (EC:3.7.1.9), (5) 2-keto-4-pentenoate hydratase (EC:4.2.1.80), (6) 4-hydroxy 2-oxovalerate aldolase (EC:4.1.3.39), (7) acetaldehyde dehydrogenase (EC:1.2.1.10), (8) catechol 1,2-dioxygenase (EC:1.13.11.1), (9) muconate cycloisomerase (EC:5.5.1.1), (10) muconolactone isomerase (EC:5.3.3.4), (11) 3-oxoadipate enol-lactonase (EC:3.1.1.24), (12) 3-oxoacid CoA-transferase (EC:2.8.3.6), (13) β-ketoadipyl CoA thiolase (EC:2.3.1.174, EC:2.3.1.16), (14) acetyl-CoA synthetase (EC:6.2.1.1), (15) propionyl-CoA synthetase (EC:6.2.1.17), (16) methylcitrate synthase (EC:2.3.3.5), (17) 2-methylcitrate dehydratase (EC:4.2.1.79), (18) 2-methylisocitrate

dehydratase (EC:4.2.1.99), (19) 2-methylisocitrate lyase (EC:4.1.3.30), (20) *p*-cymene monooxygenase (EC:1.14.13.-), (21) *p*-cumic alcohol dehydrogenase (EC:1.1.1.90), (22) *p*-cumic aldehyde dehydrogenase (EC:1.2.1.29), (23) *p*-cumate 2,3-dioxygenase (EC:1.13.11.-), (24) 2,3-dihydroxy-2,3-dihydro-*p*-cumate dehydrogenase (EC:1.3.1.58), (25) 2,3-dihydroxy-*p*-cumate-3,4-dioxygenase (EC:1.13.11.-), (26) 2-hydroxy-3-carboxy-muconic semialdehyde decarboxylase (EC:4.1.1.-), and (27) 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase hydrolase (EC:3.7.1.-).

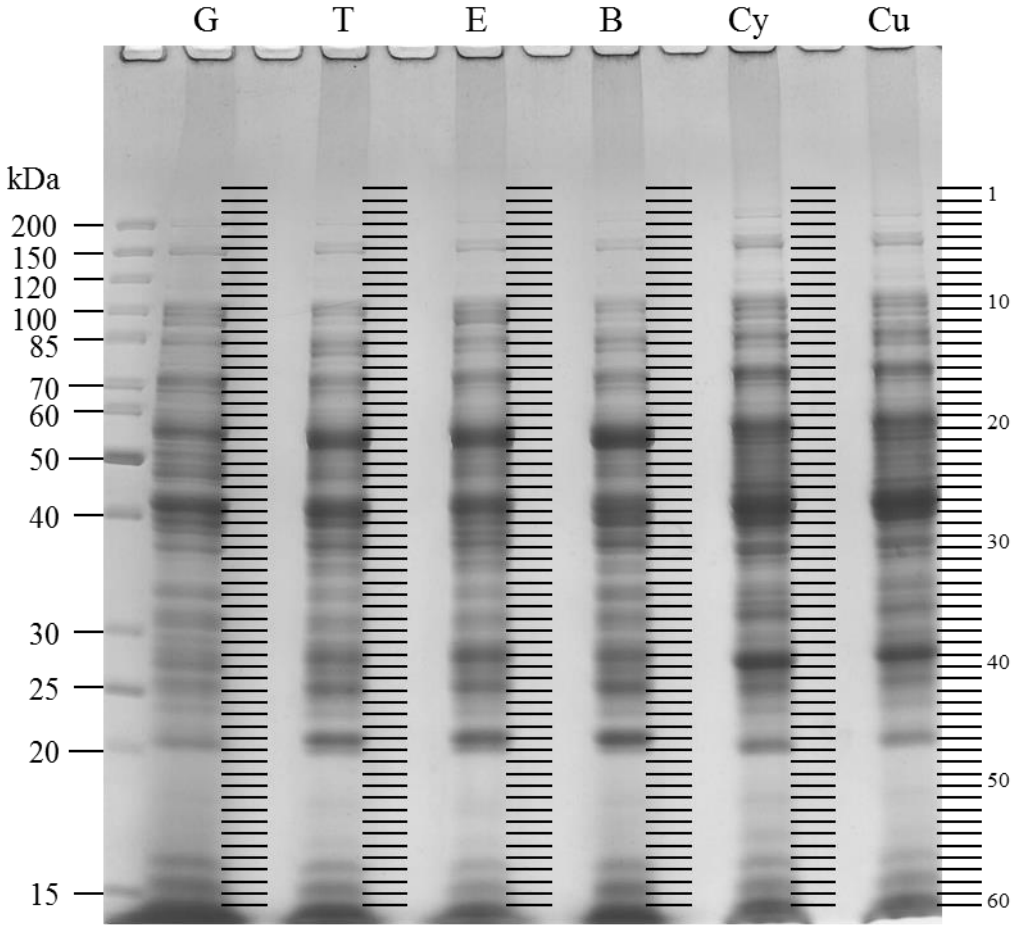

Fig. 2.3 SDS-PAGE of proteins of *P. putida* F1 cells grown with glucose (G), toluene (T), ethylbenzene (E), benzene (B), *p*-cymene (Cy) and *p*-cumate (Cu). Gels were cut into 52-68 slices for in-gel digestion.

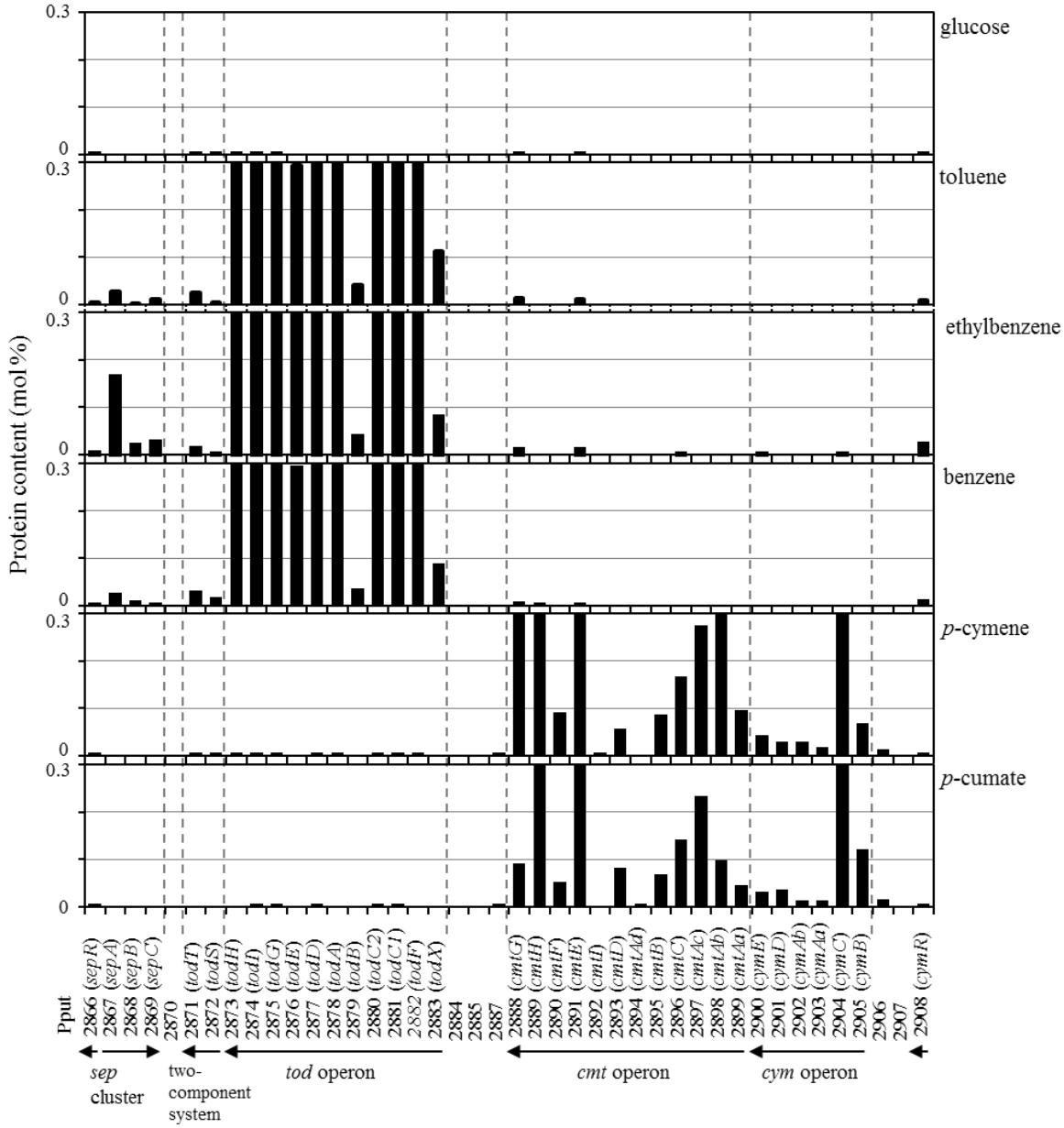

Fig. 2.4 Protein content values of cellular proteins in the culture with glucose, toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate. The aromatics degradation island (ADI). Arrows indicate the direction of transcription. The numbers in parentheses indicate a reaction step in Fig. 2.2.

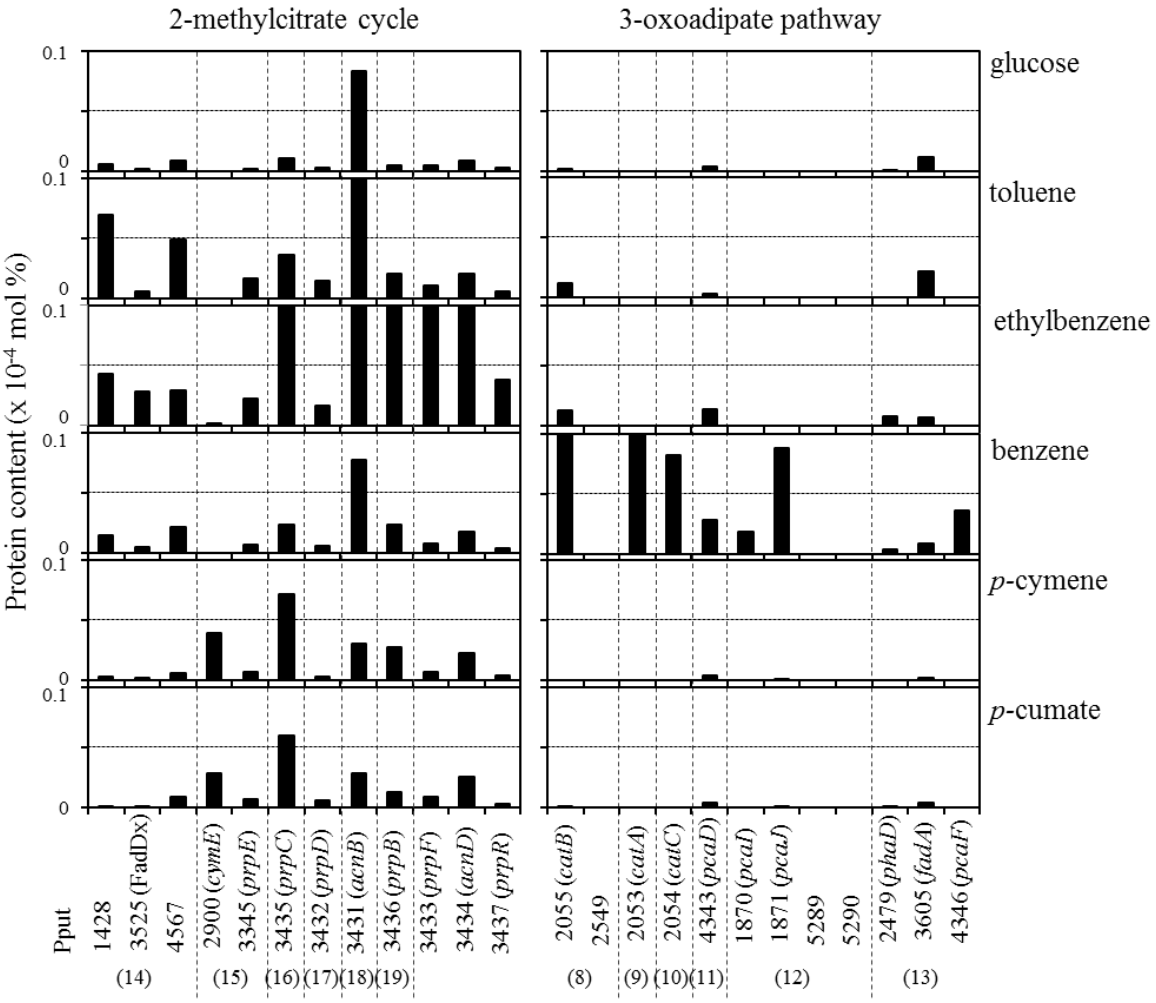

Fig. 2.5 Protein content values of cellular proteins in the culture with glucose, toluene, ethylbenzene, benzene, *p*-cymene, and *p*-cumate. The 2-methylcitrate cycle and 3-oxoadipate pathway. Arrows indicate the direction of transcription. The numbers in parentheses indicate a reaction step in Fig. 2.2.

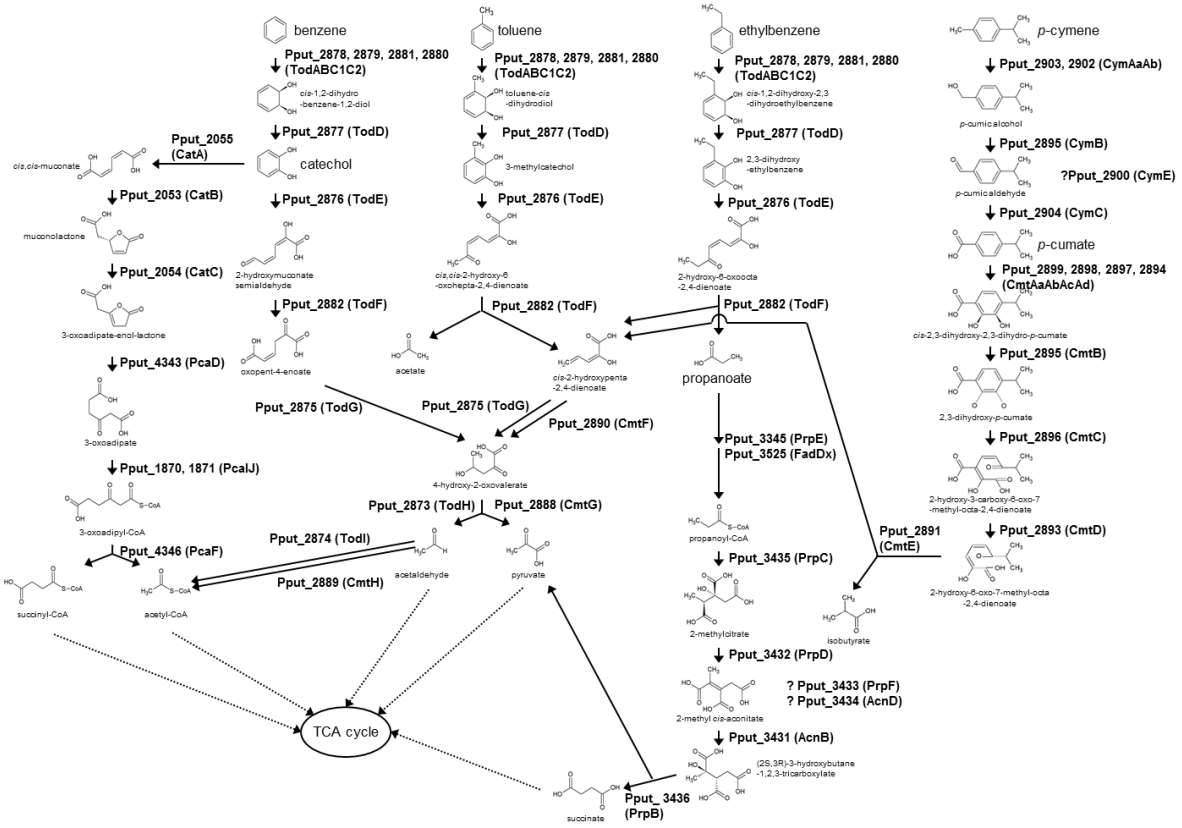

Fig. 2.6 The metabolic pathway of toluene, ethylbenzene, benzene and *p*-cymene in *P. putida* F1. The metabolites and enzymes responsible for pathways are indicated. Identified proteins in this study are highlighted with bold letters.

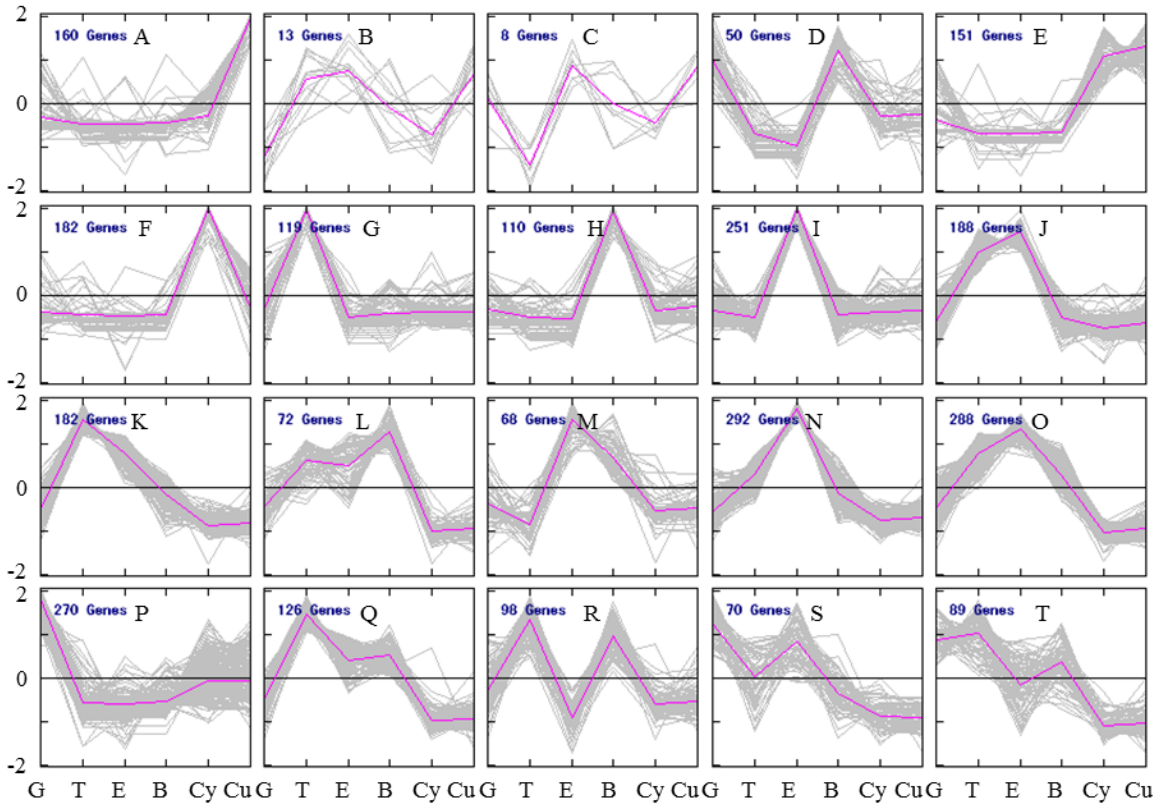

Fig. 2.7 Result of *K*-means clustering of proteome data sets of glucose (G), toluene (T), ethylbenzene (E), benzene (B), *p*-cymene (Cy) and *p*-cumate (Cu).

Chapter 3

Gene expression profiling of *Pseudomonas putida* F1 after exposure to aromatic hydrocarbon in soil by using proteome analysis

3.1 Introduction

Metabolic pathways of bacterial isolates capable of degrading crude oil, agricultural chemicals, and aromatic hydrocarbons have been intensively investigated. A recent whole genome bioinformatics analysis identified genes involved in catabolic pathways (Ogata, *et al.*, 1999, Caspi, *et al.*, 2012). The soil bacterium *Pseudomonas putida* F1 strain can use toluene, ethylbenzene, and benzene as sole carbon and energy sources for growth (Gibson, *et al.*, 1968). The genome of this strain has been completely sequenced (GenBank, CP000712.1). In *P. putida* F1, toluene, ethylbenzene, and benzene were degraded to intermediate products such as tricarboxylic acid (TCA) cycle via the toluene degradation (*tod* genes) (Zylstra, *et al.*, 1988, Zylstra & Gibson, 1989), propanoate (*prp* genes), and *β*-ketoadipate (*pca* and *cat* genes) pathways. I previously identified key enzymes involved in the degradation of toluene, ethylbenzene, and benzene in *P. putida* F1 in liquid media (Fig. 3.1) (Kasahara, *et al.*, 2012). Additionally, I detected two protein systems (transport and sensor) important in aromatic hydrocarbon degradation, including the solvent efflux pump system (SepRABC; Pput_2866, 2867, 2868 and 2869) (Phoenix, *et al.*, 2003) and the two-component system (TodST; Pput2872 and Pput_2871), which regulate the toluene degradation pathway (Lau, *et al.*, 1997). However, it is unclear whether laboratory analyses of the bacterial activity and catabolism accurately reflect the soil environment (Xu, 2006). Thus, it is important to detect proteins involved in aromatic hydrocarbon degradation by *P. putida* F1 in soil formed the complex ecosystem..

Proteomics is a powerful approach that can be used for the large-scale characterization of the proteins in a cell (Graves & Haystead, 2002, Graham, *et al.*, 2011). Mass spectrometry-based proteomics is widely used in bacterial sciences (Jimenez, *et al.*, 2002, Thompson, *et al.*, 2010). Proteomics-based approaches can be used to analyze the function of indigenous microbial communities in soil environments (Bastida, *et al.*, 2010, Williams, *et al.*,

2010, Wang, *et al.*, 2011, Wu, *et al.*, 2011, Knief, *et al.*, 2012), while no approach is available to analyze the function of a single bacterial strain in soil environments.

In this study, I identified proteins involved in the degradation of toluene, ethylbenzene, and benzene by *P. putida* F1 in soil by using two-dimensional gel electrophoresis (2-DE) or standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS). Proteome analyses revealed that *P. putida* F1 responded to aromatic hydrocarbons in the soil in a manner similar to that observed in liquid media.

3.2 Materials and methods

3.2.1 Bacterial culture conditions in soil

P. putida F1 strain was grown at 30°C for 16 h in 100 mL of mineral salt medium (MSM) containing 0.2% (w/v) glucose with vigorous shaking shaking (190 rpm) (Munoz, *et al.*, 2007). The cells were collected by centrifugation at 6,000 ×g for 10 min and washed twice with 100 mL of 0.8% (w/v) NaCl. Finally, cells were suspended in 10 mL of 0.8% NaCl, and diluted to 1.0 x 10^9 colony-forming unit (CFU) mL^{-1}. Aliquot (5 ml) of the cell suspension was inoculated into 50 g of unsterilized garden soil (N: 340 mg L^{-1}, P_2O_5: 1350 mg L^{-1}, K_2O: 220 mg L^{-1}, MgO: 150 mg L^{-1}, pH 6.2, no. of viable bacterial cells: 1.1 × 10^7 cells g^{-1} soil) (Hokusan, Hokkaido, Japan) containing 0.2% (w/v) glucose in a petri dish. Soils inoculated were incubated at 30°C for 3 days. Next, toluene (T), ethylbenzene (E), or benzene (B) were added to the soil at final concentrations of 0.5% (v/w), 1.5% (v/w), and 1.25% (v/w), respectively, and mixed thoroughly. The soils were subsequently incubated at 30°C for 18 days. Soil samples were taken at 0, 1, 3, 6, 12, and 18 days. The 0-day sample was collected prior to adding aromatic hydrocarbons. The moisture content (50%) of the soil samples was gravimetrically controlled using distilled water

during incubation. The soil sample was taken from three random locations in the petri dish, and then these samples were mixed.

3.2.2 Measurement of toluene, ethylbenzene, and benzene degradation in soil

The concentrations of toluene, ethylbenzene, and benzene in the incubated soil was measured using gas chromatography (GC) (Oldenhuis, *et al.*, 1989). Soil samples incubated with and without *P. putida* F1 cells were used for these measurements. To extract toluene, ethylbenzene, and benzene from the soils, 2 mL of 0.8% NaCl and 2 mL of pentane were added to 3 g of incubated soils. Next, the mixture was shaken for 24 h at room temperature and centrifuged at 3,000 ×g for 3 min. The pentane layer was diluted using acetone. The mixture was analyzed using GC (GC-2014; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a DB-5MS column (length 30 m, internal diameter 0.25 mm: J & W Scientific, CA, USA). The initial oven temperature was maintained at 50°C for 10 min, and then increased to 250°C at a rate of 5°C min^{-1} and then held for 60 min. The injector and detector temperatures were maintained at 250°C. All samples were measured in triplicate.

3.2.3 Viable count of *P. putida* F1 in inoculated soil

Viable cell numbers of *P. putida* F1 and indigenous bacteria were determined using the dilution plate method. The soil inoculated and non-incubated samples were serially diluted in sterilized water at 10^5–10^6 and 10^4–10^5 fold, respectively. The soil suspension was inoculated onto Luria-Bertani (LB) ager (5 g yeast extract L^{-1}, 10 g tryptone L^{-1}, 5 g NaCl L^{-1}, pH 7, and 1.5 wt% agar) plate. Five replicates were prepared in all cases. Bacterial colony-forming units (CFU) in the samples were counted at 30°C after incubation for 24 h for *P. putida* F1 and for 7 days for indigenous bacteria.

3.2.4 Separation of bacterial cells from soil and protein extraction

Bacterial cells were separated from the soil samples by using Nycodenz density gradient centrifugation (Rickwood, *et al.*, 1982, Lindahl & Bakken, 1995). A total of 12 g (wet weight) of incubated soil samples was dispersed in 24 mL of 0.8% NaCl and sonicated for 5 min by using a sonicator (VS-F100, As One, Tokyo, Japan). Next, 6.5 mL of the soil suspension was added to 6.5 mL of Nycodenz (density approximately 1.3 g mL^{-1}) (Axis-Shield PoC AS, Oslo, Norway). After the samples were centrifuged at 10,000 ×g for 40 min at 4°C, the bacterial cell layer was collected using a pipette. Collected cells that had been washed with 0.8% NaCl were lysed using the ReadyPrep Protein Extraction Kit (Total Protein) (Bio-Rad Laboratories, Hercules, CA, USA). Protein concentration was measured using a Protein Assay Kit (Bio-Rad Laboratories).

3.2.5 1-D sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Extracted bacterial proteins (50 μg) were separated using 12.5% (v/v) acrylamide gel (90 mm × 85 mm) and stained using Coomassie brilliant blue (CBB).

3.2.6 Two-dimensional gel electrophoresis

Extracted bacterial proteins were separated using 2-DE. An immobiline dry strip (pH 4–7, 18-cm long; GE Healthcare, Uppsala, Sweden) was rehydrated overnight with 5 mL of rehydration buffer (6 M urea, 2 M thiourea, 2% Triton X-100, 13 mM dithiothreitol (DTT), 1% Pharmalyte pH 3–10, 25 mM acetic acid, and 0.0025% Orange G) at 20°C. Next, 100 μg of extracted proteins were applied to the rehydrated dry strip. Isoelectric focusing (IEF) was carried out at 20°C by using a Coolphorestar IPG-IEF Type-PX (Anatech, Tokyo, Japan), with the voltage increased in a stepwise manner by using the following parameters: 500 V for 2 h,

700 V for 1 h, 1000 V for 1 h, 1500 V for 1 h, 2000 V for 1 h, 2500 V for 1 h, 3000 V for 1 h, and 3500 V for 10 h. The dry strip was then equilibrated in SDS treatment buffer (6 M urea, 32 mM DTT, 25 mM Tris-HCl [pH 6.8], 2% SDS, 0.0025% bromophenol blue (BPB), and 25% glycerol) for 30 min at room temperature with gentle shaking. The dry strip was then equilibrated in alkylating buffer (6 M urea, 240 mM iodoacetamide, 25 mM Tris-HCl [pH 6.8], 2% SDS, 0.0025% BPB, and 25% glycerol) for 15 min. SDS-PAGE was performed using a 12.5% (v/v) acrylamide gel and run at 20 mA per gel by using a electrophoresis system Coolphorestar SDS-PAGE Dual-200 (Anatech, Tokyo, Japan). The 10–200-kDa PageRulerTM Unstained Protein Ladder (Fermentas, St. Leon-Rot, Germany) were used as molecular weight markers. The gel was stained using silver nitrate and the image was captured by scanning with an Epson scanner GT-S620 (Epson, Tokyo, Japan) at a resolution of 600 dpi. The spot count was manually determined using ImageJ software version 1.44o (National Institute of Health, Bethesda, MD, USA) for each gel.

3.2.7 Proteome analysis

Proteome analysis methods were performed as described previously (Kasahara, *et al.*, 2012). Standard SDS-PAGE gel lanes were cut into 80 strips (~1 mm). CBB-stained gel strips were destained using 100 μL of 30% acetonitrile (ACN) containing 25 mM NH_4HCO_3. Differentially expressed protein spots were excised from the 2-DE gels. Silver-stained spots were destained using 100 μL of 25 mM NaS_2O_3 and 15 mM $K_3Fe(CN)_6$. Destained gels were reduced using 10 mM DTT, followed by alkylation with 55 mM iodoacetamide. After the gels were completely dried, they were digested using 40 μL of sequencing-grade modified trypsin (12.5 μg/mL in 50 mM NH_4HCO_3) at 37°C for 16 h. Digested peptides were extracted using 25 mM NH_4HCO_3 in 60% ACN and washed twice by using 5% formic acid in 70% ACN. Peptide

mixtures were used for nano liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS) analysis.

NanoLC-ESI-MS/MS analysis was conducted using an LTQ ion-trap MS (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Paradigm MS2 nano-flow HPLC system (AMR Inc., Tokyo, Japan) and nano-spray electrospray ionization device (Michrom Bioresources Inc., Auburn, CA, USA). The tryptic peptide mixture was loaded onto an L-column2 ODS (Chemicals Evaluation & Research Inst., Tokyo, Japan) packed with C18-modified silica particles (5 μm; pore size, 12 nm). The peptide mixture was separated using a linear gradient of 15–65% buffer B for 40 min, followed by a gradient of 65–95% buffer B for 1 min (buffer A = 2% ACN and 0.1% formic acid in H_2O; buffer B = 90% ACN and 0.1% formic acid in H2O) at a flow rate of 1 μL min^{-1}. Peptide spectra were recorded over a mass range of m/z 450–1800. MS/MS spectra were acquired in data-dependent scan mode. After the full spectrum scan, one MS/MS spectrum of the most intense single peaks was also collected. Dynamic exclusion features were set as follows: a repeat count of one within every 30 s, an exclusion duration of 180 s, and an exclusion list size of 50. MS/MS data were analyzed using the Mascot program ver. 2.3.01 (Matrix Science, London, UK) against the *P. putida* F1 data (NC_009512) in NCBI. Search parameters were set as follows: tryptic digest with a maximum of two missed cleavage sites; fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation; peptide masses, monoisotopic, positive charge (+1, +2, +3) of peptide; and mass tolerance of 1.2 Da for the precursor ion and 0.8 Da for product ions. To determine the false-positive rate, an automatic decoy search was performed against a randomized database with a default significance threshold of $p < 0.05$; the false discovery rate at the identity threshold was < 5.6%. Proteins with at least two unique peptides were used as filtering criteria.

3.2.8 Protein quantitative analysis

Label-free quantitative analyses of identified protein abundance were performed using the exponentially modified protein abundance index (emPAI) values obtained using the Mascot program (Ishihama, *et al.*, 2005, Shinoda, *et al.*, 2010). emPAI compares the number of observed unique parent ions per protein with the number of observable peptides per protein. Protein content (PC) in the molar fraction percentage was determined using the following equation:

$$PC\ (mol\ \%) = emPAI / \Sigma(emPAI) \times 100,$$

where Σ(emPAI) is the summation of emPAI values for all identified proteins (Ishihama, *et al.*, 2005).

3.3 Results and Discussion

3.3.1 Degradation of aromatic hydrocarbons by *P. putida* F1 in soil

Biodegradation of aromatic hydrocarbons by *P. putida* F1 in the incubated soil was measured using GC. The residual concentration of toluene, ethylbenzene, and benzene in the soil inoculated with *P. putida* F1 were less than that in the non-inoculated soil (Fig. 3.2). The decrease in concentration of aromatic hydrocarbons in the non-inoculated soil samples was not due to the degradation of indigenous bacteria, but by volatilization. For the non-inoculated and sterilized soil samples, the decrease in aromatic hydrocarbons was similar to that in the non-inoculated soil sample (data not shown). These results suggest that *P. putida* F1 degrades toluene, ethylbenzene, and benzene in soil. In the inoculated soil sample, the concentration of benzene, toluene, and ethylbenzene decreased to the limit of detection within 3, 12, and 18 days, respectively (Fig. 3.2). For proteome analyses, sampling times of the incubated soil samples were determined to be 6, 12, and 18 days.

3.3.2 Viable cell number of *P. putida* F1 in soil

During incubation, the viable cell number of *P. putida* F1 in all inoculated soil samples was 2.6×10^7 to 9.8×10^8 cells g^{-1} soil (Table 3.1). Using the Nycodenz gradient method, it is empirically difficult to collect bacterial cell present at $<1 \times 10^7$ cells g^{-1} soil, while bacterial cells are easily collected form inoculated soil samples. Viable cell number in the non-inoculated soil samples was nearly $< 1 \times 10^7$ cells g^{-1} soil. Therefore, the proteins obtained in this study were derived from inoculated *P. putida* F1cells.

3.3.3 Two-DE/LC-MS/MS analysis of soil-incubated *P. putida* F1 proteins

Two-DE gel images of extracted proteins at day 0 (T0, E0, and B0; TEB0) and day 12 (T12, E12, and B12) in the soil containing toluene, ethylbenzene, and benzene are shown in Fig. 3.3. A total of 835, 455, 589, and 500 protein spots were detected on the TEB0, T12, E12, and B12 gels, respectively. Protein spots resulting from aromatic hydrocarbon degradation were predicted on the basis of their theoretical isoelectric points (pI) and molecular weights (MW). A total of 283 protein spots of the predicted spots and differentially expressed protein spots were excised from the TEB0, T12, E12, and B12 gels, and 247 proteins were identified using more than two unique peptide filtering criteria (Table 3.2).

Close-up images of 2-DE gels are shown in Fig. 3.4 which shows detected aromatic hydrocarbon degradation proteins. Of the 11 proteins included in the *tod* cluster (*todXFC1C2BADEGIH*), nine proteins (TodA, TodC1, TodC2, TodD, TodE, TodF, TodG, TodH, and TodI) were strongly induced on the T12, E12, and B12 gel compared with TEB0 (Fig. 3.4A). For TodA, same position spots in TEB0 gel were identified the elongation factor Tu (Pput_0473, spots number: 24, 182, 183). Of the eight proteins that comprise the propanoate pathway involved in ethylbenzene degradation (Fig. 3.1), two protein spots (PrpB and PrpF) were

detected only on the E12 gel (Fig. 3.4B). Of the seven proteins comprising the *β*-ketoadipate pathway involved in benzene degradation (Fig. 3.1), two protein spots (CatA and CatB) were detected only on the B12 gel (Fig. 3.4C). Although benzene was consumed after three days of incubation (Fig. 3.2), the key enzymes involved in benzene degradation persisted after 12 days of incubation. These proteins may be induced by small amounts of benzene below the detection limit of GC analysis. Enzymes important for the degradation of toluene, ethylbenzene, and benzene in *P. putida* F1 grown in soil were detected using 2-DE/LC-MS/MS analysis. Our results indicate that *P. putida* F1 degrades aromatic hydrocarbons in soil.

The 2-DE approach is advantageous because it enables comparison of protein expression profiles and quantification of expression levels (Wilkins, *et al.*, 1998, Curreem, *et al.*, 2012). However, this approach has some limitations, including that (Wilkins, *et al.*, 1998, Curreem, *et al.*, 2012): (i) low amounts of proteins are undetectable, (ii) membrane and hydrophobic proteins are undetectable, (iii) proteins with very high or low pIs and MWs are undetectable, and (iv) proteins in overlapping spots are difficult to observe. According to the results of 2-DE, two proteins (TodB and TodX) in the *tod* cluster, six proteins in the propanoate pathway, and five proteins in the *β*-ketoadipate pathway could not be detected. However, TodB protein was induced at low levels in liquid cultures in the presence of toluene, ethylbenzene, and benzene (Kasahara, *et al.*, 2012) , while TodX is a hydrophobic membrane protein (Wang, *et al.*, 1995). Other proteins exhibited pIs of 4–7 and MWs of 15–150 kDa on 2-DE gels. These undetectable spots could not be located because they were overlapping. It would be difficult to detect all proteins involved in aromatic hydrocarbon degradation using 2-DE/LC-MS/MS analysis. The standard SDS-PAGE/LC-MS/MS analysis approach, which involves genome-wide proteome analysis (Kasahara, *et al.*, 2012) , was applied for these soil samples.

3.3.4 Standard SDS-PAGE/LC-MS/MS analysis approach of *P. putida* F1 proteins

Soils incubated with ethylbenzene were analyzed for day 0 (E0 sample), day 6 (E6 sample), and day 18 (E18 sample). Extracted proteins were separated using SDS-PAGE (Fig. 3.5). A total of 1373, 1016, and 1389 proteins were identified in E0, E6, and E18 samples, respectively. PC values were calculated for the identified proteins based on emPAI. In the E6 and E18 samples, all key enzymes in the *tod* cluster and propanoate pathway were up-regulated compared with those in the E0 sample (Fig. 3.6A and 3.6B). Proteins not detected using 2-DE/LC-MS/MS analysis were detected using SDS-PAGE/LC-MS/MS analysis. In addition to the key enzymes involved in the ethylbenzene degradation pathway, the solvent efflux pump system, SepRABC proteins, and the two-component system, TodST proteins, were identified in the E6 and E18 samples (Kasahara, *et al.*, 2012). These results indicate that *P. putida* F1 responds to ethylbenzene in soil in a similar manner as in liquid media experiments.

In the SDS-PAGE/LC-MS/MS analysis, proteins involved in ethylbenzene degradation were derived from *P. putida* F1 cells for two reasons. First, for non-inoculated soil with ethylbenzene, the bacterial layer was not fractionated by Nycodenz gradient centrifugation, and therefore, the bacterial cellular proteins could not be extracted. Second, in E6 sample, proteins were analyzed using the Mascot program against the NCBI bacteria database (2012.01.29), and the key enzyme proteins for ethylbenzene degradation were closely affiliated with *P. putida* F1.

Extracting proteins from soil is difficult because of the presence of humic substances and indigenous materials in the soil (Benndorf, *et al.*, 2007, Keiblinger, *et al.*, 2012) as well as adsorption of proteins to soil particles (Ding & Henrichs, 2002, Taylor & Williams, 2010). To remove factors inhibiting protein extraction, I fractionated bacterial cells from soil and extracted cellular proteins. I identified approximately 1260 proteins in *P. putida* F1. The number of detected proteins was approximately 70% of the number detected in pure culture (Kasahara, *et*

al., 2012). Of these, the proteins involved in the degradation of aromatic hydrocarbons as well as other proteins involved in other bioprocesses were subjected to genome-wide analysis by using bacterial strains in soil. The SDS-PAGE/LC-MS/MS analysis approach allowed the examination of bacterial response to soil environment.

3.4 Conclusion

Proteins involved in aromatic hydrocarbon degradation of *P. putida* F1 in soil were detected using 2-DE/LC-MS/MS and 1-D SDS-PAGE/LC-MS/MS analyses. I showed that *P. putida* F1 degraded aromatic hydrocarbons in the soil according to GC and proteome analyses and clarified that the response to aromatic hydrocarbons of *P. putida* F1in the soil was the same as that observed in liquid media. Thus, according to laboratory analysis, the activity and catabolism in *P. putida* F1 reflect the soil environment. Metabolism and bioprocessing by a bacterium in soil can be examined using 1-D SDS-PAGE/LC-MS/MS analysis, which is useful for understanding the autecology and lifestyle of a bacterium in its natural habitat.

Table 3.1 Viable cell number of *P. putida* F1 in soil during incubation for 18 days.

Soil sample	Incubation time (day)			
	0	6	12	18
Glucose	1.7×10^7	5.7×10^7	1.2×10^7	7.3×10^6
Toluene		7.3×10^8	7.7×10^8	2.6×10^8
Ethylbenzene		3.3×10^7	3.8×10^7	9.8×10^8
Benzene		4.6×10^8	8.8×10^7	2.6×10^7

Table 3.2 Proteins identified on the 2-DE gels in TEB0, T12, E12, and B12 samples.

No. of spots	Pput name	Accession #	Gene	Protein name	Theoretical		Mascot_score				Sequence coverage (%)				No. of peptides			
					pI	MW (kDa)	BTE0	T12	E12	B12	BTE0	T12	E12	B12	BTE0	T12	E12	B12
1	Pput_0792	gi|148546038	-	hypothetical protein Pput_0792	4.97	64757.64	nd	-	654	192	nd	-	19.9	5.6	nd	-	12	3
2	Pput_3470	gi|148548678	*Tig*	trigger factor	4.84	48429.14	697	-	-	-	35	-	-	-	27	-	-	-
3	Pput_1157	gi|148546400	-	OmpA/MotB domain-containing protein	4.95	21402.55	213	-	-	-	23	-	-	-	7	-	-	-
4	Pput_0382	gi|148545637	-	malate synthase G	5.64	78199.81	90	-	189	-	3.3	-	7.4	-	2	-	5	-
5	Pput_2061	gi|148547283	*katG*	catalase/peroxidase HPI	5.72	81955.06	81	-	196	-	3.6	-	7.3	-	2	-	5	-
6	Pput_0431	gi|148545683	-	putative serine protein kinase, PrkA	5.74	73736.69	216	-	47	-	13.4	-	4.1	-	9	-	2	-
7	Pput_1667	gi|148546905	-	dihydrolipoamide dehydrogenase	5.93	49925.1	795	-	671	-	41.6	-	34.3	-	17	-	16	-
8	Pput_4531	gi|148549733	-	methylmalonate-semialdehyde dehydrogenase	5.85	54342.1	230	-	127	-	12.8	-	8.5	-	6	-	4	-
9	Pput_1667	gi|148546905	-	dihydrolipoamide dehydrogenase	5.93	49925.1	1340	-	822	-	44.4	-	37.7	-	22	-	18	-
10	Pput_4291	gi|148549497	-	protease Do	5.82	47836.16	168	-	-	-	8.6	-	-	-	4	-	-	-
10	Pput_2904	gi|148548116	-	aldehyde dehydrogenase	5.89	53135.27	-	-	65	-	-	-	6.3	-	-	-	2	-
11	Pput_1038	gi|148546281	-	arginine deiminase	5.66	46389.67	115	-	880	-	5.3	-	40	-	2	-	21	-
12	Pput_1175	gi|148546418	-	extracellular ligand-binding receptor	5.77	37088.09	322	-	373	-	23.7	-	22.9	-	8	-	8	-
13	Pput_1053	gi|148546296	-	extracellular solute-binding protein	5.83	43041.93	530	-	-	-	30.8	-	-	-	13	-	-	-
13	Pput_2902	gi|148548114	-	oxidoreductase FAD-binding subunit	5.79	38266.35	-	-	264	-	-	-	21.8	-	-	-	8	-
14	Pput_1668	gi|148546906	*sucC*	succinyl-CoA synthetase subunit beta	5.83	41213.55	869	-	887	599	46.9	-	49.7	35.8	17	-	19	13
15	Pput_5062	gi|148550262	-	D-3-phosphoglycerate dehydrogenase	5.93	44311.37	191	-	298	100	10.5	-	19.8	4.9	4	-	7	2
16	Pput_1037	gi|148546280	-	ornithine carbamoyltransferase	5.92	37873.05	411	-	493	359	34.5	-	37.2	27.4	13	-	14	10
17	Pput_4740	gi|148549942	-	short-chain dehydrogenase/reductase SDR	5.97	27382.95	92	-	-	-	7.5	-	-	-	2	-	-	-
17	Pput_2895	gi|148548107	-	short-chain dehydrogenase/reductase SDR	5.98	27293.13	-	-	234	-	-	-	29	-	-	-	7	-
18	Pput_2882	gi|148548095	*todF*	alpha/beta hydrolase fold	5.99	30768.73	-	248	496	365	-	28.3	37	36.6	-	8	11	11
19	Pput_2874	gi|148548087	*todI*	acetaldehyde dehydrogenase	5.84	33663.45	-	644	1288	856	-	37.3	54.7	30.7	-	14	20	11
20	Pput_2873	gi|148548086	*todH*	4-hydroxy-2-ketovalerate aldolase	5.84	38449.34	-	451	555	666	-	29.8	31.5	27.8	-	12	14	12
21	Pput_1668	gi|148546906	*sucC*	succinyl-CoA synthetase subunit beta	5.83	41213.55	559	515	459	202	38.9	30.4	33.8	18	13	10	12	6
22	Pput_4243	gi|148549449	-	NADH:flavin oxidoreductase/NADH oxidase	5.63	40473.9	254	-	243	-	17.4	-	17.4	-	5	-	6	-
23	Pput_4428	gi|148549630	*aapJ*	extracellular solute-binding protein	5.54	34016.45	334	542	613	359	31	40.6	51.8	34.5	12	17	18	11
24	Pput_0473	gi|148545725	-	elongation factor Tu	5.22	43468.15	415	-	-	-	31.7	-	-	-	10	-	-	-
24	Pput_2878	gi|148548091	*TodA*	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	5.15	42784.29	-	-	447	-	-	-	22.4	-	-	-	12	-
25	Pput_0512	gi|148545764	-	DNA-directed RNA polymerase subunit alpha	4.91	36607.32	307	-	578	-	30.3	-	45	-	15	-	18	-
26	Pput_0894	gi|148546138	-	ornithine decarboxylase	4.97	43482.31	nd	-	143	-	nd	-	10.6	-	nd	-	4	-
27	Pput_3771	gi|148548977	-	3-isopropylmalate dehydrogenase	5.03	38800.05	nd	-	57	-	nd	-	7.2	-	nd	-	2	-
28	Pput_0577	gi|148545827	-	inorganic pyrophosphatase	4.77	19176.8	255	-	-	-	30.9	-	-	-	7	-	-	-
29	Pput_0097	gi|148545354	*trpA*	tryptophan synthase subunit alpha	5.17	28349.84	75	-	-	-	10.8	-	-	-	3	-	-	-
30	Pput_4079	gi|148549285	-	oxidoreductase FAD/NAD(P)-binding subunit	5.22	29690.9	141	-	-	-	23.9	-	-	-	8	-	-	-
31	Pput_3436	gi|148548644	*prpB*	2-methylisocitrate lyase	5.33	31792.27	-	-	169	-	-	-	16.6	-	-	-	5	-
32	Pput_1270	148546513	-	phosphoribosylaminoimidazole-succinocarboxamide synthase	5.37	26898.97	354	-	-	-	35.6	-	-	-	17	-	-	-
33	Pput_3521	gi|148548729	-	enoyl-CoA hydratase/isomerase	5.43	27650.33	nd	-	85	-	nd	-	7	-	nd	-	2	-
34	Pput_3469	gi|148548677	*clpP*	ATP-dependent Clp protease proteolytic subunit	5.53	23499.86	100	-	-	-	5.6	-	-	-	4	-	-	-
34	Pput_2880	gi|148548093	*todC2*	toluene dioxygenase	5.48	21999.04	-	nd	275	982	-	nd	20.3	56.7	-	nd	5	12
35	Pput_1062	gi|148546305	-	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	5.55	24069.44	141	-	nd	-	21.2	-	nd	-	5	-	nd	-
36	nd	nd	nd	nd	nd	nd	nd	-	nd	-	nd	-	nd	-	nd	-	nd	-
37	Pput_5198	gi|148550398	-	acetylglutamate kinase	5.57	31932.92	272	-	-	-	13.6	-	-	-	7	-	-	-
38	Pput_4185	gi|148549391	*tsf*	elongation factor Ts	5.14	30398.88	1272	-	-	-	49.8	-	-	-	51	-	-	-
39	Pput_4591	gi|148549793	-	dihydrodipicolinate reductase	5.68	28395.85	123	-	68	-	8.2	-	7.9	-	2	-	2	-
40	Pput_4216	gi|148549422	*adk*	adenylate kinase	5.59	23208.07	94	-	109	-	17.1	-	12.5	-	3	-	2	-
41	Pput_2876	gi|148548089	*todE*	glyoxalase/bleomycin resistance protein/dioxygenase	5.79	32057.92	-	283	282	355	-	16.5	21	19.6	-	4	7	6
42	Pput_4543	gi|148549745	*ilvH*	acetolactate synthase 3 regulatory subunit	5.93	17773.62	57	-	nd	-	11.7	-	nd	-	2	-	nd	-
43	Pput_3777	gi|148548983	*ibpA*	heat shock protein Hsp20	5.94	16127.36	262	-	151	-	45.6	-	32.7	-	7	-	5	-
44	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
45	Pput_1669	gi|148546907	-	succinyl-CoA synthetase subunit alpha	5.89	30091.57	545	34	nd	47	29.6	3.1	nd	9.5	10	nd	11	2
46	Pput_0755	gi|148546001	-	50S ribosomal protein L25/general stress protein Ctc	5.72	21285.19	321	103	270	75	37.1	11.2	33	9.6	10	2	7	2
47	Pput_1669	gi|148546907	-	succinyl-CoA synthetase subunit alpha	5.89	30091.57	56	-	225	-	6.5	-	20.4	-	2	-	8	-
48	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
49	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
50	Pput_4578	gi|148549780	*nusA*	transcription elongation factor NusA	4.58	54640.39	459	-	-	-	22.1	-	-	-	19	-	-	-
51	Pput_3942	gi|148549148	*rpsA*	30S ribosomal protein S1	4.82	61517.94	701	-	-	-	34.8	-	-	-	32	-	-	-
52	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
53	Pput_5295	gi|148550495		F0F1 ATP synthase subunit beta	4.88	49328.34	69	-	-	-	4.6	-	-	-	2	-	-	-
54	Pput_4005	gi|148549211	-	peptidyl prolyl isomerase, FKBP-type	4.88	23958.53	327	-	-	-	34	-	-	-	10	-	-	-
55	Pput_0539	gi|148545789	-	OmpW family protein	4.91	21941.15	129	-	181	-	15.4	-	23.3	-	3	-	4	-
56	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
57	Pput_0936	gi|148546179	-	tartrate/fumarate subfamily Fe-S type hydro-lyase alpha subunit	5.19	54673.94	91	-	-	-	4.1	-	-	-	2	-	-	-
58	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	335	584	341	-	13.1	22	17.6	-	6	17	8
59	Pput_4497	gi|148549699	-	trans-2-enoyl-CoA reductase	5.16	44237.56	121	-	-	-	10.4	-	-	-	4	-	-	-
60	Pput_2764	gi|148547978	-	saccharopine dehydrogenase	5.17	45335.06	57	-	-	-	4.6	-	-	-	2	-	-	-
61	Pput_1666	gi|148546904	-	dihydrolipoamide succinyltransferase	5.2	42410.11	94	-	-	-	7.9	-	-	-	2	-	-	-
62	Pput_4383	gi|148549588	-	cell division protein FtsA	5.2	44864.36	242	-	-	-	11.7	-	-	-	5	-	-	-
63	Pput_0002	gi|148545261	-	DNA polymerase III subunit beta	5.16	40693.32	264	-	-	-	21	-	-	-	8	-	-	-
64	Pput_1442	gi|148546682	-	aspartate kinase	5.25	44577.33	262	-	-	-	24.3	-	-	-	11	-	-	-
65	Pput_1748	gi|148546986	-	isocitrate lyase	5.39	48631.06	168	-	-	-	14.7	-	-	-	7	-	-	-
66	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
67	Pput_1003	gi|148546246	-	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	5.35	44944.5	71	-	291	-	7.1	-	14.3	-	3	-	10	-
68	Pput_1057	gi|148546300	-	carbohydrate-selective porin OprB	5.43	46777	497	-	-	-	27.1	-	-	-	27	-	-	-
69	Pput_3770	gi|148548976	-	aspartate-semialdehyde dehydrogenase	5.46	40645.82	147	-	-	-	11.4	-	-	-	4	-	-	-
70	Pput_4542	gi|148549744	-	ketol-acid reductoisomerase	5.48	36347.35	197	-	-	-	10.7	-	-	-	3	-	-	-
71	Pput_4833	gi|148550035	-	fructose-1,6-bisphosphate aldolase	5.48	38428.15	309	-	399	-	23.2	-	20.3	-	10	-	13	-
72	Pput_1053	gi|148546296	-	extracellular solute-binding protein	5.83	43041.93	102	-	-	-	8.6	-	-	-	3	-	-	-
73	Pput_3574	gi|161936355	-	transaldolase B	5.37	34720.93	175	-	-	-	14.9	-	-	-	4	-	-	-
73	Pput_2055	gi|148547277	*catA*	catechol 1,2-dioxygenase	5.14	34243.1	-	-	-	85	-	-	-	8.4	-	-	-	3
74	Pput_4594	gi|148549796	-	GrpE protein	4.93	20532.44	92	-	-	-	11.4	-	-	-	2	-	-	-
75	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
76	Pput_1125	gi|148545354	-	alkylhydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	5.06	21715.92	146	-	-	-	18	-	-	-	3	-	-	-
77	Pput_1745	gi|148546905	-	NADH dehydrogenase subunit B	5.18	25506.7	120	-	-	-	15.6	-	-	-	4	-	-	-
78	Pput_1667	gi|148546370	-	dihydrolipoamide dehydrogenase	5.93	49925.1	166	-	-	-	9.6	-	-	-	4	-	-	-
79	Pput_0702	gi|148545948	*glyA*	serine hydroxymethyltransferase	5.85	44868.96	125	-	-	-	9.6	-	-	-	5	-	-	-
80	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
81	Pput_0229	gi|148545485	-	4-aminobutyrate aminotransferase	5.85	44795.52	250	-	-	-	18.1	-	-	-	6	-	-	-
82	Pput_1668	gi|148546906	*sucC*	succinyl-CoA synthetase subunit beta	5.83	41213.55	124	-	-	-	6.7	-	-	-	2	-	-	-
83	Pput_4914	gi|148550116		fructose-1,6-bisphosphatase	5.71	37194.87	245	-	-	-	24.1	-	-	-	12	-	-	-
84	Pput_1949	gi|148547174	-	UTP-glucose-1-phosphate uridylyltransferase	5.46	30937.79	83	-	-	-	7.9	-	-	-	2	-	-	-
85	Pput_4684	gi|148549886	-	iojap-like protein	5.25	17633.72	73	nd	-	-	10.1	nd	-	-	3	nd	-	-
86	Pput_4596	gi|148549798	-	ferric uptake regulator family protein	5.43	15189.73	104	80	-	-	16.4	14.9	-	-	2	2	-	-
87	Pput_0879	gi|148546123	*ndk*	nucleoside-diphosphate kinase	5.45	15034.67	166	-	-	-	25.5	-	-	-	4	-	-	-
88	Pput_1235	gi|148546478	*oprD*	outer membrane porin	4.75	43598.4	521	887	466	848	31.8	38	36.1	37.5	14	19	17	20
89	Pput_3651	gi|148548857	-	OmpF family protein	4.63	34548.55	439	537	111	573	34	41.9	6.1	38.7	11	16	3	13
90	Pput_1140	gi|148546383	*Dcd*	deoxycytidine triphosphate deaminase	5.5	21219.56	123	-	-	-	11.7	-	-	-	2	-	-	-
91	Pput_0954	gi|148546197	-	superoxide dismutase	5.55	21924.73	54	-	-	-	7.1	-	-	-	2	-	-	-
92	Pput_4958	gi|148550160	-	malate dehydrogenase	5.09	45428.55	436	-	-	-	30.8	-	-	-	11	-	-	-
93	Pput_4428	gi|148549630	*aapJ*	extracellular solute-binding protein	5.54	34016.45	91	99	-	161	8.8	10.5	-	15.2	3	3	-	5
94	Pput_4958	gi|148550160	-	malate dehydrogenase	5.09	45428.55	nd	-	-	-	nd	-	-	-	nd	-	-	-
95	nd	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-
96	Pput_0886	gi|148546130	-	Pyrrolo-quinoline quinone	4.74	38015.59	107	-	-	-	10	-	-	-	5	-	-	-
97	Pput_4745	gi|148549947	-	extracellular ligand-binding receptor	5.74	37297.97	474	-	-	-	22	-	-	-	15	-	-	-
98	Pput_3651	gi|148548857	-	OmpF family protein	4.63	34548.55	-	-	-	295	-	-	-	21.5	-	-	-	8
99	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
100	Pput_1235	gi|148546478	*oprD*	outer membrane porin	4.75	43598.4	-	-	-	167	-	-	-	18.8	-	-	-	9

101	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
102	Pput_4363	gi|148549568	*groEL*	chaperonin GroEL	4.97	56693.68	-	-	-	233	-	-	-	9.9	-	-	-	4
103	Pput_4363	gi|148549568	*groEL*	chaperonin GroEL	4.97	56693.68	-	-	-	246	-	-	-	12.3	-	-	-	7
104	Pput_4363	gi|148549568	*groEL*	chaperonin GroEL	4.97	56693.68	-	-	-	89	-	-	-	5.1	-	-	-	2
105	Pput_5295	gi|148550495	-	F0F1 ATP synthase subunit beta	4.88	49328.34	-	-	-	94	-	-	-	4.6	-	-	-	2
106	Pput_5295	gi|148550495	-	F0F1 ATP synthase subunit beta	4.88	49328.34	-	-	-	388	-	-	-	21.2	-	-	-	8
107	Pput_1123	gi|148546366	-	bacterioferritin	4.74	17960.12	-	-	-	157	-	-	-	21.7	-	-	-	3
108	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
109	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
110	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
111	Pput_5088	gi|148550288	-	extracellular solute-binding protein	5.85	37653.22	-	-	-	73	-	-	-	6.8	-	-	-	2
112	Pput_2873	gi|148548086	*todH*	4-hydroxy-2-ketovalerate aldolase	5.84	38449.34	-	203	219	175	-	17.6	23	14.5	-	6	7	5
113	Pput_2053	gi|148547275	*catB*	muconate and chloromuconate cycloisomerase	5.89	40179.22	-	-	-	140	-	-	-	5.4	-	-	-	2
114	Pput_1175	gi|148546418	-	extracellular ligand-binding receptor	5.77	37088.09	-	-	-	393	-	-	-	23.2	-	-	-	9
115	Pput_2053	gi|148547275	*catB*	muconate and chloromuconate cycloisomerase	5.89	40179.22	-	-	-	256	-	-	-	12.6	-	-	-	5
116	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
117	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
118	nd	nd	nd	nd	nd	nd	-	-	-	nd	-	-	-	nd	-	-	-	nd
119	Pput_2874	gi|148548087	*todI*	acetaldehyde dehydrogenase	5.84	33663.45	-	372	344	282	-	26.6	20.6	11.1	-	8	6	3
120	Pput_2877	gi|148548090	*todD*	2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase	5.29	28763.94	-	410	132	223	-	36.4	19.3	32.7	-	12	5	7
121	Pput_4734	gi|148549936	-	Ferritin, Dps family protein	4.82	20048.03	-	121	-	-	-	23.9	-	-	-	4	-	-
122	Pput_2785	gi|148547999	-	integral membrane sensor signal transduction histidine kinase	nd	nd	-	nd	-	-	-	nd	-	-	-	nd	-	-
123	Pput_4745	gi|148549947	-	extracellular ligand-binding receptor	5.74	37297.97	-	341	-	-	-	24.6	-	-	-	9	-	-
124	nd	nd	nd	nd	nd	nd	-	nd	-	-	-	nd	-	-	-	nd	-	-
125	Pput_2765	gi|148547979	-	methyltransferase type 12	5.52	37387.42	-	40	-	-	-	5.5	-	-	-	2	-	-
126	Pput_1175	gi|148546418	-	extracellular ligand-binding receptor	5.77	37088.09	-	156	-	-	-	14.6	-	-	-	5	-	-
127	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	166	-	-	-	6.4	-	-	-	3	-	-
128	Pput_3651	gi|148548857	-	OmpF family protein	4.63	34548.55	-	-	129	-	-	-	10.8	-	-	-	3	-
129	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	-	353	-	-	-	18.4	-	-	-	9	-
130	Pput_2898	gi|148548110	-	ring hydroxylating dioxygenase, alpha subunit	5.09	48936.89	-	-	489	-	-	-	29.7	-	-	-	12	-
131	Pput_2898	gi|148548110	-	ring hydroxylating dioxygenase, alpha subunit	5.09	48936.89	-	-	511	-	-	-	22.1	-	-	-	10	-
132	Pput_0473	gi|148545725	-	elongation factor Tu	5.22	43468.15	-	-	2110	-	-	-	42.8	-	-	-	77	-
133	Pput_0473	gi|148545725	-	elongation factor Tu	5.22	43468.15	-	-	248	-	-	-	22.7	-	-	-	8	-
134	nd	nd	nd	nd	nd	nd	-	-	nd	-	-	-	nd	-	-	-	nd	-
135	Pput_4363	gi|148549568	*groEL*	chaperonin GroEL	4.97	56693.68	-	-	118	-	-	-	8.1	-	-	-	3	-
136	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	-	869	-	-	-	34	-	-	-	27	-
137	Pput_2875	gi|148548088	*todG*	4-oxalocrotonate decarboxylase	4.9	28221.78	-	-	478	-	-	-	36.6	-	-	-	8	-
138	Pput_2890	gi|148548102	-	4-oxalocrotonate decarboxylase	4.88	28098.63	-	-	103	-	-	-	7.6	-	-	-	3	-
139	Pput_2878	gi|148548091	*todA*	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	5.15	42784.29	-	-	981	-	-	-	42.2	-	-	-	16	-
140	Pput_2823	gi|148548037	-	azoreductase	4.85	21309.93	-	-	289	-	-	-	36.9	-	-	-	7	-
141	nd	nd	nd	nd	nd	nd	-	-	nd	-	-	-	nd	-	-	-	nd	-
142	Pput_3579	gi|148548785	-	VacJ family lipoprotein	4.96	22823.67	-	-	229	-	-	-	24.7	-	-	-	5	-
143	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	-	707	-	-	-	18.9	-	-	-	19	-
144	Pput_2905	gi|148548117	-	short-chain dehydrogenase/reductase SDR	5.37	26129.32	-	-	450	-	-	-	22.2	-	-	-	7	-
145	Pput_2877	gi|148548090	*todD*	2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase	5.29	28763.94	-	-	114	-	-	-	13.5	-	-	-	3	-
146	Pput_2880	gi|148548093	*todC2*	toluene dioxygenase	5.48	21999.04	-	-	260	-	-	-	25.1	-	-	-	6	-
147	Pput_2876	gi|148548089	*todE*	glyoxalase/bleomycin resistance protein/dioxygenase	5.79	32057.92	-	-	88	-	-	-	10.7	-	-	-	3	-
148	nd	nd	nd	nd	nd	nd	-	-	nd	-	-	-	nd	-	-	-	nd	-
149	Pput_2893	gi|148548105	-	class II aldolase/adducin family protein	5.7	26158.33	-	-	160	-	-	-	22.2	-	-	-	5	-
150	Pput_2880	gi|148548093	*todC2*	toluene dioxygenase	5.48	21999.04	-	-	562	-	-	-	40.1	-	-	-	9	-
151	Pput_2880	gi|148548093	*todC2*	toluene dioxygenase	5.48	21999.04	-	-	705	-	-	-	40.1	-	-	-	10	-
152	Pput_2891	gi|148548103	-	alpha/beta hydrolase fold	6.05	32485.86	-	-	403	-	-	-	33	-	-	-	11	-
153	Pput_2891	gi|148548103	-	alpha/beta hydrolase fold	6.05	32485.86	-	-	946	-	-	-	34.4	-	-	-	15	-
154	Pput_2896	gi|148548108	-	glyoxalase/bleomycin resistance protein/dioxygenase	6.09	35343.7	-	-	318	-	-	-	31.1	-	-	-	9	-
155	Pput_2896	gi|148548108	-	glyoxalase/bleomycin resistance protein/dioxygenase	6.09	35343.7	-	-	594	-	-	-	39.7	-	-	-	13	-
156	Pput_2888	gi|148548100	*mhpE2*	4-hydroxy-2-ketovalerate aldolase	5.91	38035.07	-	-	164	-	-	-	12.9	-	-	-	4	-
157	Pput_2888	gi|148548100	*mhpE2*	4-hydroxy-2-ketovalerate aldolase	5.91	38035.07	-	-	375	-	-	-	25.1	-	-	-	9	-
158	Pput_0524	gi|148545776	-	formate dehydrogenase, beta subunit	5.9	34530.82	-	-	68	-	-	-	9.2	-	-	-	3	-
159	Pput_2902	gi|148548114	-	oxidoreductase FAD-binding subunit	5.79	38266.35	-	-	110	-	-	-	8	-	-	-	3	-
160	Pput_1932	gi|148547157	-	alcohol dehydrogenase	5.5	35401.1	-	-	278	-	-	-	22.3	-	-	-	6	-
161	Pput_1667	gi|148546905	-	dihydrolipoamide dehydrogenase	5.93	49925.1	-	-	98	-	-	-	5	-	-	-	2	-
162	Pput_2881	gi|148548094	*todC1*	ring hydroxylating dioxygenase, alpha subunit	5.27	50911.76	-	-	107	-	-	-	5.1	-	-	-	2	-
163	Pput_2878	gi|148548091	*todA*	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	5.15	42784.29	-	-	673	-	-	-	21.5	-	-	-	18	-
164	Pput_1825	gi|148547060	-	ATP-dependent Clp protease, ATP-binding subunit clpA	5.59	83286.59	-	-	91	-	-	-	2.8	-	-	-	2	-
165	Pput_2900	gi|148548112	-	propionyl-CoA synthetase	5.73	71373.58	-	-	459	-	-	-	22.2	-	-	-	17	-
166	Pput_2900	gi|148548112	-	propionyl-CoA synthetase	5.73	71373.58	-	-	693	-	-	-	23	-	-	-	19	-
167	Pput_2904	gi|148548116	-	aldehyde dehydrogenase	5.89	53135.27	-	-	539	-	-	-	30	-	-	-	16	-
168	Pput_2904	gi|148548116	-	aldehyde dehydrogenase	5.89	53135.27	-	-	989	-	-	-	37.7	-	-	-	23	-
169	Pput_1663	gi|148546901	*sdhA*	succinate dehydrogenase flavoprotein subunit	5.83	63408.84	-	-	347	-	-	-	16.6	-	-	-	9	-
170	Pput_4363	gi|148549568	*groEL*	chaperonin GroEL	4.97	56693.68	-	-	347	-	-	-	19.8	-	-	-	10	-
171	nd	nd	nd	nd	nd	nd	-	-	nd	-	-	-	nd	-	-	-	nd	-
172	nd	nd	nd	nd	nd	nd	-	-	nd	-	-	-	nd	-	-	-	nd	-
173	Pput_2875	gi|148548088	*todG*	4-oxalocrotonate decarboxylase	4.9	28221.78	-	592	880	-	-	45.9	51.9	-	-	13	16	-
174	Pput_2874	gi|148548087	*todI*	acetaldehyde dehydrogenase	5.84	33663.45	-	-	110	-	-	-	7	-	-	-	2	-
175	Pput_2877	gi|148548090	*todD*	2,3-dihydroxy-2,3-dihydrophenylpropionate dehydrogenase	5.29	28763.94	-	852	639	806	-	54.9	41.5	41.5	-	16	14	15
176	Pput_0598	gi|148545848		acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	4.95	16175.35	214	-	-	-	17.6	-	-	-	6	-	-	-
177	Pput_3856	gi|148549062		elongation factor P	4.73	21289.72	440	-	-	-	32.3	-	-	-	12	-	-	-
178	Pput_4185	gi|148549391	*tsf*	elongation factor Ts	5.14	30398.88	396	-	-	-	32.4	-	-	-	17	-	-	-
179	Pput_4761	gi|148549963		extracellular solute-binding protein	5.18	33817.29	162	-	-	-	17.8	-	-	-	8	-	-	-
180	Pput_3470	gi|148548678	*tig*	trigger factor	4.84	48429.14	206	-	-	-	12.1	-	-	-	5	-	-	-
181	Pput_4593	gi|148549795	*dnaK*	molecular chaperone DnaK	4.91	65451.97	1435	-	1113	-	38	-	36.3	-	55	-	40	-
182	Pput_0473	gi|148545725		elongation factor Tu	5.22	43468.15	1387	-	-	-	42.8	-	-	-	53	-	-	-
183	Pput_0473	gi|148545725		elongation factor Tu	5.22	43468.15	3062	-	-	-	55.2	-	-	-	104	-	-	-
184	Pput_5297	gi|148550497		F0F1 ATP synthase subunit alpha	5.46	55286.66	1470	-	1097	-	38.7	-	38.7	-	48	-	37	-
185	Pput_1318	gi|148546560		cysteine synthase A	5.71	34318.74	319	-	-	-	25.3	-	-	-	11	-	-	-
186	Pput_0756	gi|148546002		ribose-phosphate pyrophosphokinase	5.56	33824.52	307	-	-	-	12.6	-	-	-	5	-	-	-
187	Pput_1316	gi|148546558		ATPase	5.69	32125.96	155	-	-	-	22.1	-	-	-	6	-	-	-
188	Pput_1267	gi|148546510		dihydrodipicolinate synthase	5.73	31634.43	188	-	-	-	8.1	-	-	-	3	-	-	-
189	Pput_2382	gi|148547603		gluconate 2-dehydrogenase	5.92	34548.14	252	-	-	-	17.5	-	-	-	13	-	-	-
190	Pput_2164	gi|148547385		phosphate ABC transporter, periplasmic phosphate-binding protein	6	34009.33	520	-	-	-	26.8	-	-	-	12	-	-	-
191	Pput_4755	gi|148549957	*rplI*	50S ribosomal protein L9	5.42	15457.23	379	-	-	-	41.2	-	-	-	18	-	-	-
192	Pput_4364	gi|148549569	*groES*	co-chaperonin GroES	5.4	10217.45	330	-	-	-	54.6	-	-	-	9	-	-	-
193	Pput_1666	gi|148546904		dihydrolipoamide succinyltransferase	5.2	42410.11	-	-	468	-	-	-	26.5	-	-	-	16	-
194	Pput_4363	gi|148549568	*groES*	chaperonin GroEL	4.97	56693.68	-	-	1224	-	-	-	36.1	-	-	-	44	-
195	Pput_4363	gi|148549568	*groES*	chaperonin GroEL	4.97	56693.68	-	-	1989	-	-	-	46	-	-	-	64	-
196	Pput_1822	gi|148547057		isocitrate dehydrogenase, NADP-dependent	5.54	45675.49	-	-	413	-	-	-	28.2	-	-	-	18	-
197	Pput_1693	gi|148546931		3-oxoacyl-(acyl carrier protein) synthase I	5.42	43235.66	-	-	354	-	-	-	18	-	-	-	9	-
198	Pput_4590	gi|148549792		carbamoyl phosphate synthase small subunit	5.48	41561.28	-	-	143	-	-	-	10.6	-	-	-	4	-
199	Pput_4542	gi|148549744		ketol-acid reductoisomerase	5.48	36347.35	-	-	597	-	-	-	36.1	-	-	-	22	-
200	Pput_1663	gi|148546901	*sdhA*	succinate dehydrogenase flavoprotein subunit	5.83	63408.84	-	-	826	-	-	-	26.9	-	-	-	27	-
201	Pput_3433	gi|148548641	*prpF*	putative AcnD-accessory protein PrpF	5.32	41401.27	-	-	440	-	-	-	32.6	-	-	-	11	-

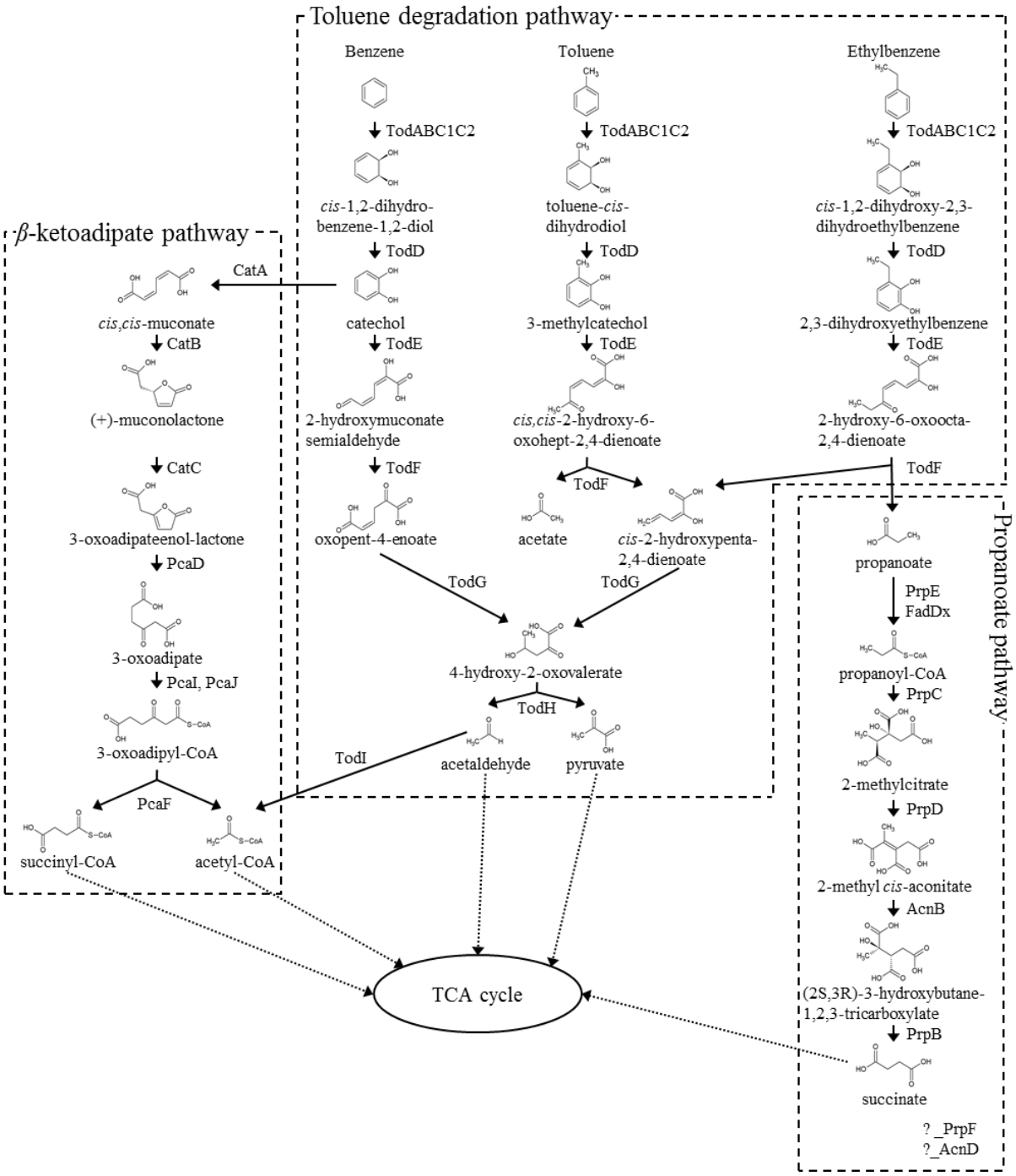

Fig. 3.1 The metabolic pathway of toluene, ethylbenzene, and benzene in P. putida F1. The proteins involved in the tod pathway is the following: TodABC1C2 (toluene dioxygenase; Pput_2878, Pput_2879, Pput_2881, and Pput_2880), TodD (cis-benzene glycol dehydrogenase; Pput_2877), TodE (catechol 2,3-dioxygenase; Pput_2876), TodF

(2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; Pput_2882), TodG (2-keto-4-pentenoate hydratase; Pput_2875), TodH (4-hydroxy 2-oxovalerate aldolase; Pput_2873), TodI (acetaldehyde dehydrogenase; Pput_2874). The proteins involved in the propanoate pathway is the following: PrpE (propionyl-CoA synthetase; Pput_3345), FasDx (AMP-dependent synthetase and ligase; Pput_3525), PrpC (methylcitrate synthase; Pput_3435), PrpD (methylcitrate synthase; Pput_3432), AcnB (bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase; Pput_3431), PrpB (2-methylisocitrate lyase; Pput_3436), PrpF (2-methylcitrate dehydratase; Pput_3433), and AcnD (2-methylisocitrate dehydratase; Pput_3434). The proteins involved in the β-ketoadipate pathway is the following: CatA (catechol 1,2-dioxygenase; Pput_2055), CatB (muconate cycloisomerase; Pput_2053), CatC (muconolactone isomerase; Pput_2054), PcaD (β-ketoadipate enol-lactone hydrolase; Pput_4343), PcaI and PcaJ (β-ketoadipate:succinyl CoA transferase; Pput_1870 and Pput_1871), PcaF (β-ketoadipyl CoA thiolase; Pput_4346), PhaD and FadA (acetyl-CoA acyltransferase; Pput_2479 and Pput_3605).

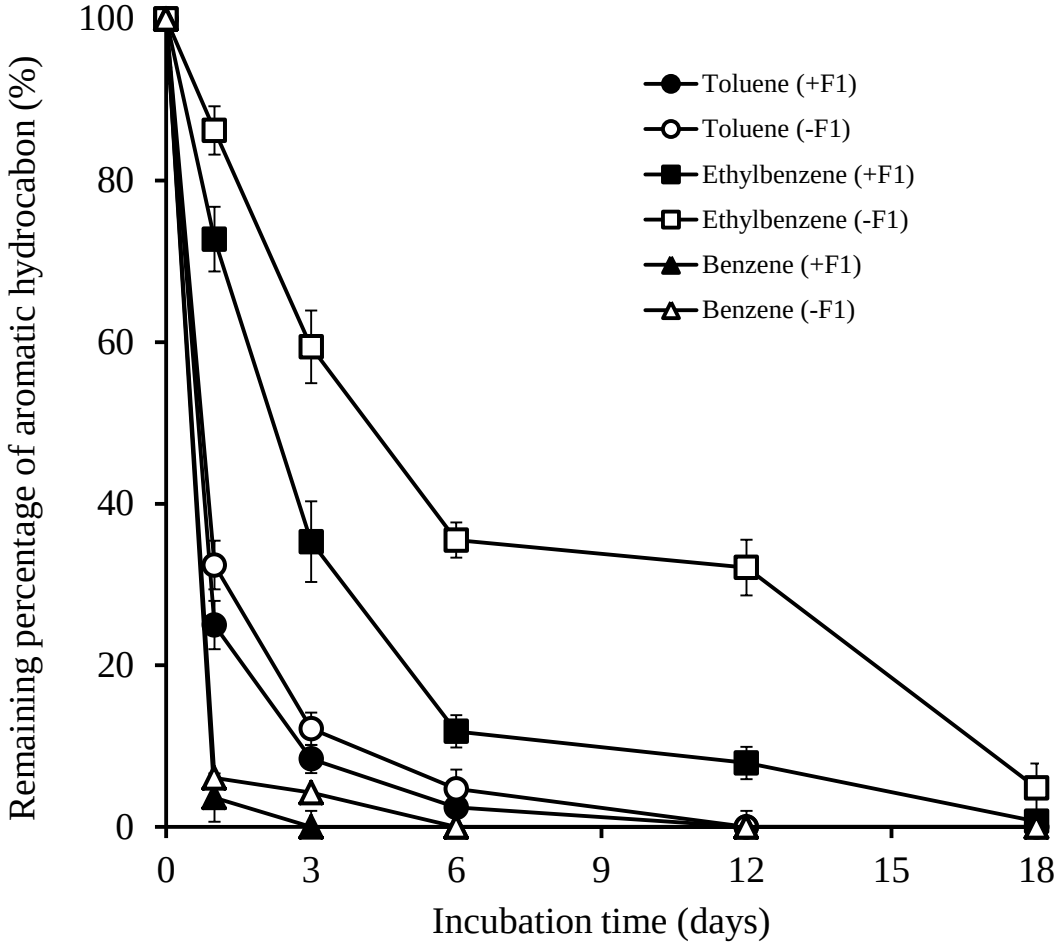

Fig. 3.2 Residual concentration of toluene, ethylbenzene, and benzene in the inoculated (closed symbol) and non-inoculated (open symbol) soil of *P. putida* F1. Error bars indicate the standard deviation of triplicate measurements.

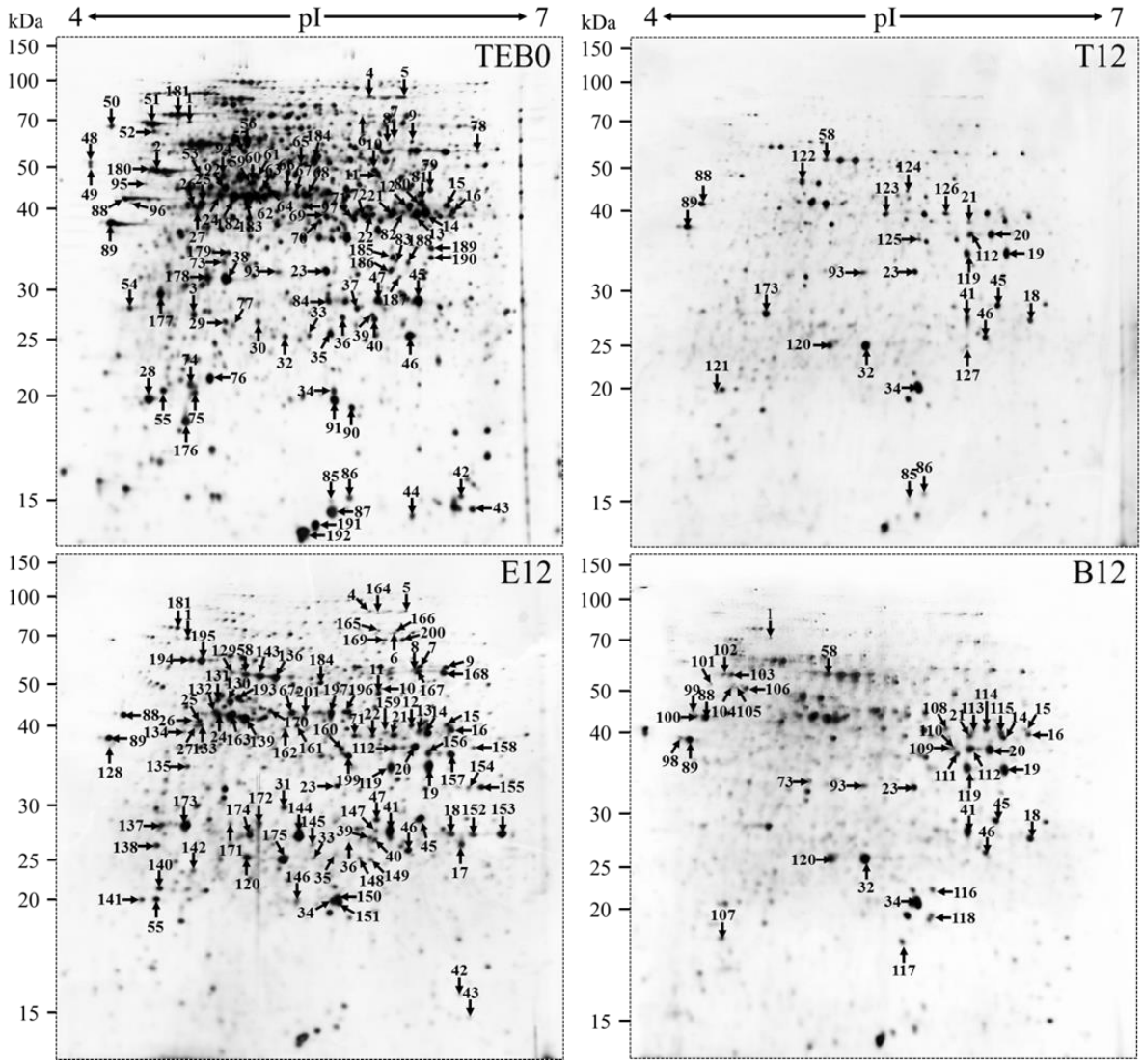

Fig. 3.3 2-DE gel images of cellular proteins obtained from *P. putida* F1 grown in soil of before (day 0; TEB0) and after addition (day 12; T12, E12 and B12) of the toluene, ethylbenzene, and benzene. Arrows indicate protein spots analyzed by LC-MS/MS. Proteins (100 μg) were loaded on an 18-cm strip with a linear gradient of pH 4–7 for IEF, following electrophoresis of 12.5% SDS-PAGE and silver staining.

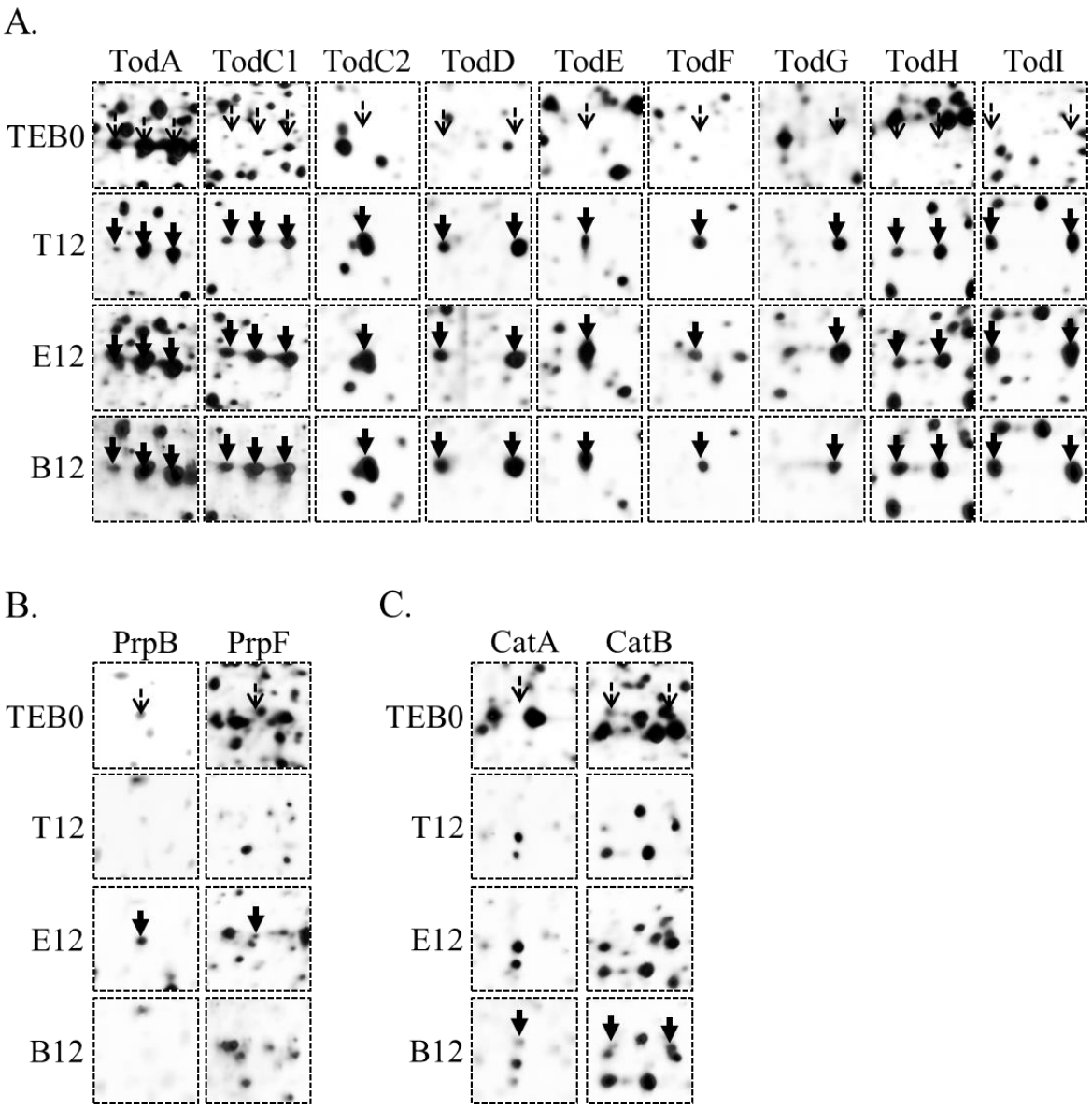

Fig. 3.4 The close-up images of the identified aromatic hydrocarbons degradation proteins in TEB0, T12, E12, and B12 gels. (A) Protein spot involved in the *tod* cluster. (B) Protein spots involved in the propanoate pathway. (C) Protein spots involved in the *β*-ketoadipate pathway.

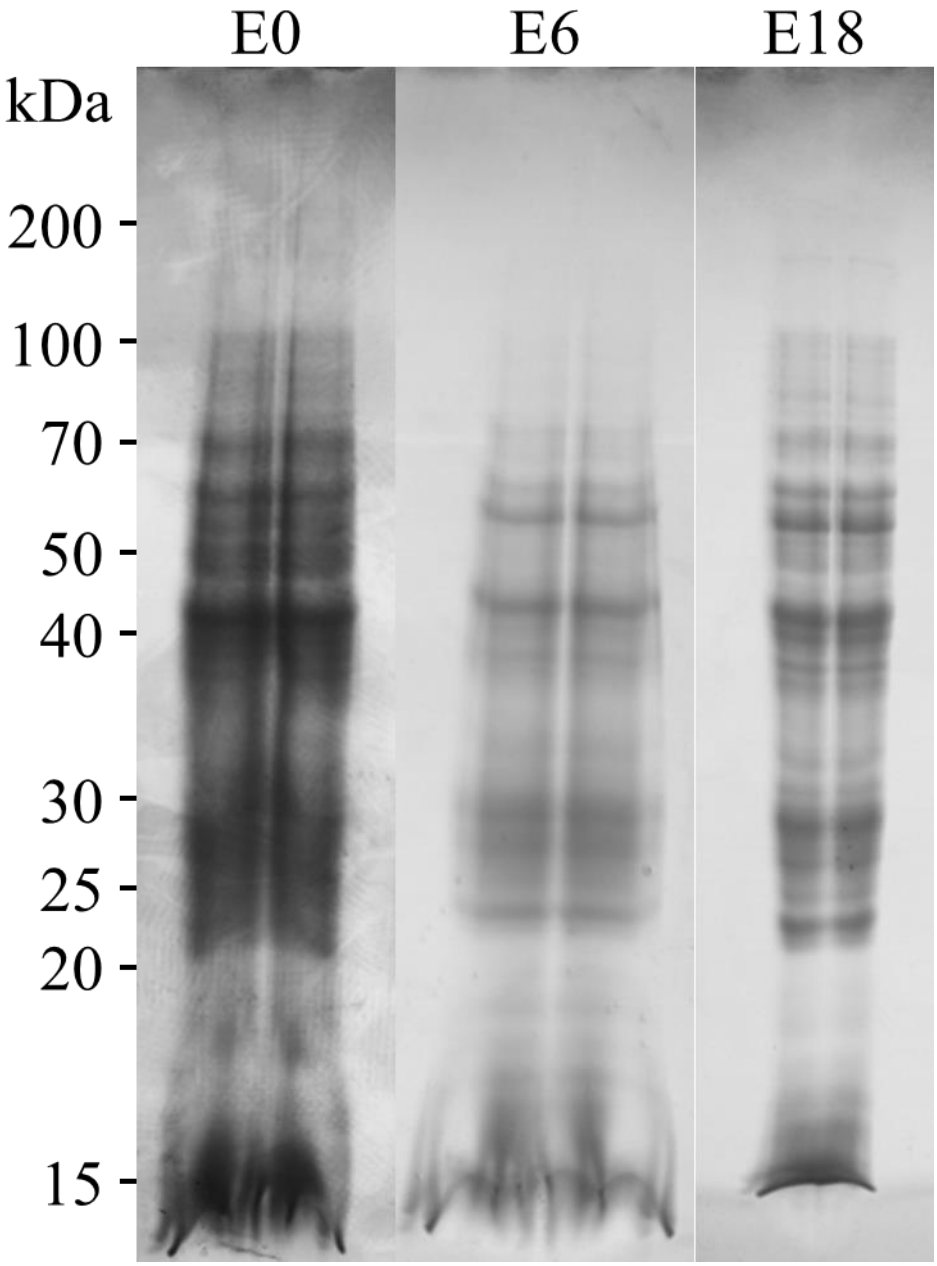

Fig. 3.5 SDS-PAGE image of extracted proteins from soil amended with ethylbenzene (E0, E6, and E18 samples). Twenty-five μg proteins per a lane were analyzed on 12.5% SDS-PAGE. The gels were stained with CBB.

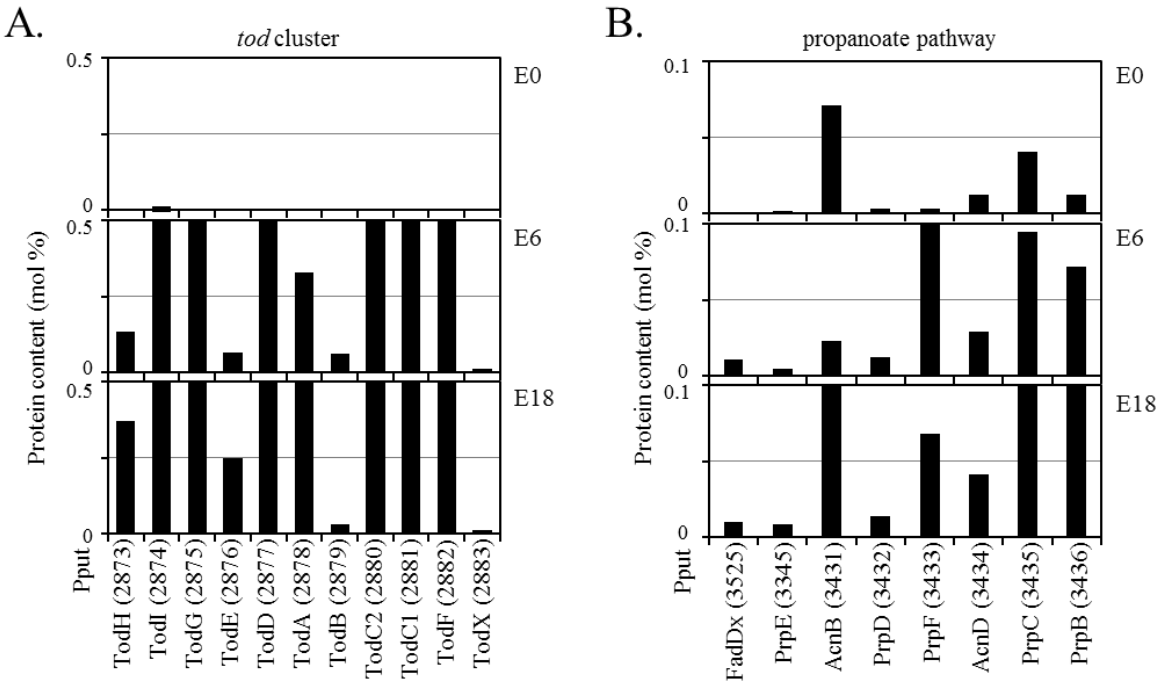

Fig. 3.6 Protein content of proteins involved in the aromatic hydrocarbon degradation in E0, E6, and E18 samples. (A) Proteins involved in the *tod* cluster. (B) Proteins involved in the propanoate pathway.

Chapter 4

Soil specific expression proteins in *Pseudomonas putida* F1

4.1. Introduction

While bacteria involved in select processes such as the nitrogen cycle and methane production and consumption have been studied extensively *in situ*, the ecophysiological characteristics of the majority of this bacteria are largely unknown (Fierer, *et al.*, 2007). Moreover, the laboratory studies have provided only limited information on survival and adaptive strategies of bacteria in various soil environments because laboratory conditions substantially differ from those of natural environments. Soil environments are heterogenous with respect to a variety of physical and chemical factors (e.g. soil particle structures, temperature, water content, pH and nutrient level) in various interactions with other indigenous organisms. Furthermore, soil environments are apparently poor in nutrient contents, and they generally provide severe survival conditions for bacteria, due in the presence of a variety of competitive organisms.

In the complete genomic sequencing bacterial strain, a many genes of unknown function are still present. In the soil bacterium *P. putida* F1, about 22% out of 5250 genes are genes of unknown function. I considered that the gene which is expressed only in the bacterial habitat environment is present in the genes of unknown function of *P. putida* F1.

In fact, *in situ* functional analysis of bacteria that live in the natural environment is difficult. However, I previously developed a novel high-throughput, non-targeted mass spectrometry (MS) approach to determine the identities of thousands of microbial proteins in the complex soil environments (Chapter 3).

In this study, to understand the bacterial response for environmental factor in soils, I identified the specific expression protein in environment soils by comparative analysis of the proteins extracted from *P. putida* F1 grown in liquid medium and soil culture system.

4.2. Material and methods

4.2.1 Growth conditions in liquid culture

P. putida F1 stain was grown aerobically at 30ºC with vigorous shaking (190 rpm) in mineral salt medium (MSM) supplemented with 0.2% glucose and Luria-Bertani (LB) medium. The bacteria cells were harvested in log phase (OD600 = 0.3) and stationary phase (25 h) by centrifugation.

4.2.2 Growth conditions in soil culture

The *P. putida* F1 strain was grown at 30°C for 16 h in 100 mL of mineral salt medium (MSM) with vigorous shaking (190 rpm) (Munoz, *et al.*, 2007). Bacterial cells were collected by centrifugation at 6,000 ×g for 10 min and washed twice with 100 mL of 0.8% (w/v) NaCl. Finally, cells were suspended in 10 mL of 0.8% NaCl. The cells were inoculated into 50 g of three types unsterilized soil (soybean, maize and forest soil) containing 0.2% (w/v) glucose in a petri dish, and adjusted to a final concentration of 1.0×10^8 cells g^{-1} soil. Inoculated soils were incubated at 30°C for 3 days. The moisture content (50%) of the soils was gravimetrically controlled using distilled water during incubation.

4.2.3 Growth conditions in static culture

P. putida F1 stain was cultured statically at 30 ºC in MSM including 0.2% glucose. The bacteria cells were harvested in log phase (OD600 = 0.3) by centrifugation.

4.2.4 Preparation of soil extract liquid medium

Soil extract liquid medium were prepared as described previously (Vilain, *et al.*, 2006).

Briefly, 60 g (dry weight) of soils from soybean field, maize field and forest field were suspended in 300 mL of sterile MOPS buffer (10 mM, pH 7.0) with shaking at 200 rpm for 1 h. To remove soil particles, the soil suspension was centrifuged at 10,000 ×g for 20 min at 4°C. The supernatant was filtered sequentially through filters with a pore size of 3.0, 0.45 and 0.2 μM in order to remove soil particles and bacteria cells.

4.2.5 Growth conditions in soil extract liquid medium

P. putida F1 stain was grown aerobically at 30 ºC with vigorous shaking (190 rpm) in soil extract liquid medium (soybean, maize and forest) supplemented with 0.2% glucose. The bacteria cells were harvested in log phase by centrifugation.

4.2.6 Separation of bacterial cells from soil

Bacterial cells were separated from soil using Nycodenz density gradient centrifugation (Rickwood, et al., 1982, Lindahl & Bakken, 1995). A total of 12 g (wet weight) of incubated soils were dispersed in 24 mL of 0.8% NaCl and sonicated for 5 min using a sonicator (VS-F100, As One, Tokyo, Japan). Next, 6.5 mL of the soil suspension was carefully added to 6.5 mL of Nycodenz (density approximately 1.3 g mL^{-1}) (Axis-Shield PoC AS, Oslo, Norway). After the samples were centrifuged at 10,000 ×g for 40 min at 4°C, the bacterial cell layer was carefully collected using a pipette. The collected cell was washed with 0.8% NaCl.

4.2.7 Protein extraction

Cold 1.6 mL of 10% TCA/acetone was added the collected cell, and rotated for 5 min at room temperature. After centrifugation (5 min, 8,000 ×g, 4°C), TCA/acetone was gently removed and discarded, and added cold 1.6 mL of 80% acetone. After centrifugation (5 min,

8,000 ×g, 4°C), TCA/acetone was gently removed and discarded. To residual acetone, the cell was evaporated for 5 min. The cell pellet was resuspended in 0.8 mL phenol (Tris-buffered, pH8.0) and 0.8 mL SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% *β*-mercaptoethanol), rotated for 30 min at room temperature. After centrifugation (10 min, 14,000 ×g, 4°C), the upper phenol phase was transferred into a fresh 15-mL Falcon tube. This extraction step was repeated twice. The transferred phenol was added 1/3 volumes of cold ethanol and 2 volumes of cold isopropanol, and stored overnight at -20°C. After centrifugation (15 min, 15,000 ×g, 4°C), the supernatant was carefully discarded. The pellet was washed once with cold 2 mL of 0.1 M ammonium acetate/methanol and twice with cold 2 mL of 80% acetone. The washed pellet was air-dried and then dissolved in solubilization buffer (5.0 M urea, 2.0 M thiourea, 2% [w/v] CHAPS, 65 mM dithiothreitol).

4.2.8 SDS-PAGE

The extracted proteins (50 μg) were separated using 12.5% (v/v) acrylamide gel (90 mm × 85 mm) and stained using Coomassie brilliant blue (CBB). The 1-DE gel lanes were cut into 60 strips of ~1 mm for protein identification.

4.2.9 In-gel digestion

Proteome analysis methods were performed as described previously (Kasahara, *et al.*, 2012). The 1-D SDS-PAGE gel lanes were cut into 80 strips of ~1 mm. CBB-stained gel strips were destained using 100 μL of 30% acetonitrile including 25 mM NH_4HCO_3. The differentially expressed protein spots were excised from the 2-DE gels. Silver-stained spots were destained using 100 μL of 50 vol% 50 mM NaS_2O_3 and 50 vol% 30 mM $K_3Fe(CN)_6$. Destained gels were reduced using 10 mM DTT, followed by alkylation with 55 mM iodoacetamide. After the gels

were completely dried, they were digested using 40 μL of sequencing-grade modified trypsin (12.5 μg/mL in 50 mM NH_4HCO_3) at 37°C for 16 h. Digested peptides were extracted using 25 mM NH_4HCO_3 in 60% ACN and washed twice using 5% formic acid in 70% ACN. The peptide mixtures were used for a nano liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS) analysis.

4.2.10 NanoLC-ESI-MS/MS analysis

A nanoLC-ESI-MS/MS analysis were performed using an LTQ ion-trap MS (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Paradigm MS2 nano-flow HPLC system (AMR Inc., Tokyo, Japan) and nano-spray electrospray ionization device (Michrom Bioresources Inc., Auburn, CA, USA). The tryptic peptide mixture was loaded onto an L-column2 ODS (Chemicals Evaluation & Research Inst., Tokyo, Japan) packed with C18-modified silica particles (5 μm, 12-nm pore size). The peptide mixture was separated using a linear gradient of 15–65% buffer B for 40 min, followed by a gradient of 65–95% buffer B for 1 min (buffer A = 2% ACN and 0.1% formic acid in H_2O; buffer B = 90% ACN and 0.1% formic acid in H_2O) at a flow rate of 1 μL min^{-1}. Peptide spectra were recorded over a mass range of m/z 450–1800. MS/MS spectra were acquired in data-dependent scan mode. After the full spectrum scan, one MS/MS spectrum of the single most intense peaks was also collected. Dynamic exclusion features were set as follows: a repeat count of one within 30 s, an exclusion duration of 180 s, and an exclusion list size of 50.

4.2.11 Protein identification

The generated MS/MS data file was analyzed using the Mascot program ver. 2.3.01 (Matrix Science, London, UK) against the *P. putida* F1 data (NC_009512) in NCBI. Search

parameters were set as follows: tryptic digest with a maximum of two missed cleavage sites; fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation; peptide masses, monoisotopic, positive charge (+1, +2, +3) of peptide; and mass tolerance of 1.2 Da for the precursor ion and 0.8 Da for product ions. To determine the false-positive rate, an automatic decoy search was performed against a randomized database with a default significance threshold of $p < 0.05$; the false discovery rate at the identity threshold was below 22.1%. Proteins with at least two unique peptides were used as filtering criteria.

4.3 Results and discussion

4.3.1 Protein identification from *P. putida* grown in soil

Proteins of *P. putida* F1 cells grown in the liquid medium and three type soils (soybean, maize and forest) were separated by SDS-PAGE (Fig. 4.1), and the gels were cut into 60 strips for in-gel digestion. Each peptide mixture was analyzed with nanoLC-MS/MS for protein indentation. In total, 2643, 1364, 1273 and 816 proteins of *P. putida* F1 were identified in liquid medium, soybean soil, maize soil and forest soil, respectively (Table 4.1).

4.3.2 Detection of soil specific expression protein

To identify specifically expressed proteins in soil environment, I performed pairwise comparisons of the liquid medium with the soil. In comparison of the proteins extracted from the liquid medium with soybean soil, 55 proteins expressed only in the soybean soil were identified, non-expressed in the liquid medium. In comparison of the proteins extracted from the liquid medium with maize soil, 56 proteins expressed only in the maize soil were identified, non-expressed in the liquid medium. In comparison of the proteins extracted from the liquid

medium with forest soil, 36 proteins expressed only in the forest soil were identified, non-expressed in the liquid medium. A comparison of the proteins expressed only in these soil environments revealed a set of 9 shared proteins (Fig. 4.2). The candidate shared protein contained a heavy metal translocating P-type ATPase (Pput_0055), a hypothetical protein (Pput_0820), a nitric oxide dioxygenase (Pput_0832), an alkaline phosphatase (Pput_0920), a hypothetical protein (Pput_3040), a short chain dehydrogenase (Pput_3041), a hypothetical protein (Pput_3042), an AMP-dependent synthetase, a ligase (Pput_3043) and a hypothetical protein (Pput_3044).

The used soils were the unsterilized soil including many indigenous microorganisms. It was examined whether the soil specific proteins (SSP) derived in *P. putida* F1, using the incubated soils with and without *P. putida* F1 (Fig. 4.3). The result indicated that SSP were derived in *P. putida* F1, except a heavy metal translocating P-type ATPase (Pput_0055). Therefore, a heavy metal translocating P-type ATPase (Pput_0055) was excluded from the candidate of SSP.

Although an alkaline phosphatase (Pput_0920; DppB) expressed specifically in soils is one of the proteins composed of the dipeptide transporter (Monnet, 2003), other constituent proteins (Pput_0919; DppC, Pput_0918; DppD, Pput_0917; DppF) were undetected in soil (Fig. 4.4). Therefore, the alkaline phosphatase (Pput_0920) was excluded from the candidate of SSP.

The gene cluster (Pput_3040, Pput_3041, Pput_3042, Pput_3043 and Pput_3044) were detected in SSP. The protein cluster was suggested an operon structure from gene information that is provided by KEGG (Ogata, *et al.*, 1999). Further, a two-component regulatory system (Pput_3038; integral membrane sensor signal transduction histidine kinase and Pput_3039; two component transcriptional regulator) was located downstream of the protein cluster. However, the two-component regulatory system was undetected as SSP. Because the sensor protein

(Pput_3038) localized in inner membrane, it was considered to be difficult to detect in proteome analysis. On the other hand, the regulator protein (Pput_3039) was detected in the soybean soil (emPAI; 0.60) and the maize soil (emPAI; 0.48). In forest soil, Pput_3039 is possible that were not detected due to the inhibition of protein extraction by humic acids. The function of SSP operon was unknown.

In this study, I determined the 2 genes and 1 operon as the specific expression genes in soil (Table 4.2).

4.3.3 Analysis of expression factor in SSP

The nitric oxide dioxygenase (Pput_0832) has been proposed to function in NO detoxification (Gardner, *et al.*, 1998, Gardner, *et al.*, 1998).

To clarify the expression factor of the other SSP, I examined the effect of oxygen concentration, soil particles, soil nutrient and autochthonous microorganism, which inferred from the differences in liquid culture system and soil culture system.

The effect of oxygen concentration was investigated using a static culture in the liquid medium. The SSP was undetected in *P. putida* F1 grown in static culture (Table 4.3). This result shows that the oxygen concentration does not affect the expression of SSP.

The effect of soil particles, soil nutrient and autochthonous microorganisms were investigated using a soil extract liquid medium (soybean, maize and forest). The SSP was up-regulated in *P. putida* F1 grown in each soil extract liquid medium (Table 4.3). This result indicates that the soil nutrient is expression factor of SSP.

4.3.4 Phylogenetic analysis of SSP operon

To analyze whether the SSP operon are conserved in any bacteria, the phylogenetic

analysis of the SSP operon was performed. The analysis results were revealed that bacteria conserved all genes of SSP operon belonged to Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Fig. 4.5). In particular, SSP operon tended to be conserved in the Gammaproteobacteria.

4.4 Conclusion

In comparative proteome analysis, the 2 genes and 1 operon as the specific expression genes in soil determined. Furthermore, expression factor of SSP was estimated to be the soil components. From result of phylogenetic analysis, the SSP operon was conserved in proteobacteria. In this analysis, it was possible to detect the protein expression of *P. putida* F1 which was reflected soil environmental factors. Using this proteome analysis method, it is possible to predict the environmental information. From this research method, I was able to analyze in detail the expression protein of bacteria that survive in soil, and it was possible to understand the physiological and genetic environmental response *in situ* different from the physiological conditions *in vitro*. By applying to other microbial species this study method, a better understanding of lifestyle and autecology in each native habitat of the target bacteria is expected.

Table 4.1 Total number of identified proteins in liquid medium, soybean soil, maize soil and forest soil

Sample	Total number of identified proteins
Liquid medium	2643
Soybean soil	1364
Maize soil	1273
Forest soil	816

Table 4.2 List of soil specific proteins

		emPAI			
Pput	Function	liquid	Soybean	Maize	Forest
0820	Hypothetical protein	0	3.2	0.5	0.5
0832	Nitric oxide dioxygenase	0	1.3	1.8	0.3
3040	Hypothetical protein	0	0.9	4.0	1.6
3041	Short chain dehydrogenase	0	6.9	13.9	1.5
3042	Hypothetical protein	0	5.4	8.8	2.4
3043	AMP-dependent synthetase and ligase	0	0.5	1.8	0.2
3044	Hypothetical protein	0	0.5	1.1	0.3

* liquid: LB_L, LB_S, MSM_L, MSM_S

Table 4.3 Expression factor in soil specific proteins

	emPAI						
Sample	0820	0832	3040	3041	3042	3043	3044
MSM							
Shake culture	0	0	0	0	0	0	0
Static culture	0	0	0	0	0	0	0
Soil extract Liquid							
Soybean	0.82	0	0.73	2.62	2.27	0.48	0.69
Maize	0	0	1.12	8.14	5.64	0.81	0.44
Forest	1.22	0	10.99	30.77	12.85	2.97	1.67

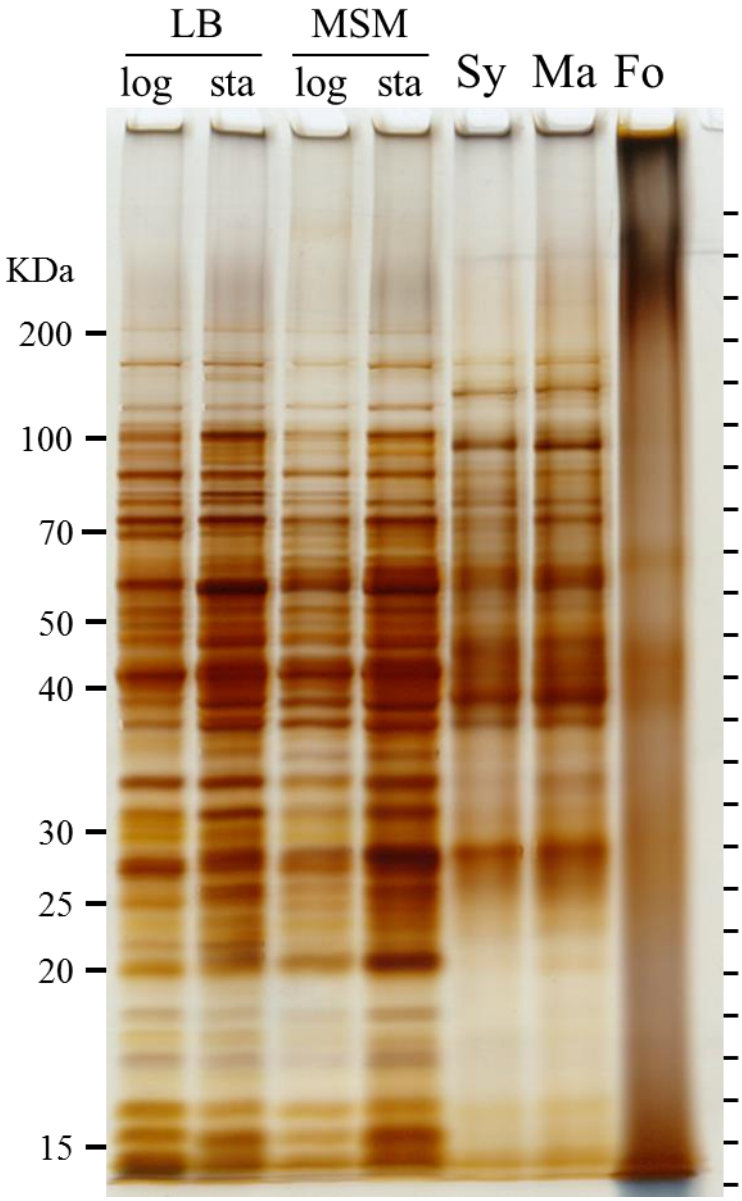

Fig. 4.1 SDS-PAGE of proteins of *P. putida* F1 cells grown in LB- log phase (LB-log), LB-stationary phase (LB-sta), MSM- log phase (MSM-log), MSM-stationary phase (MSM-sta), soybean soil (Sy), maize soil (Ma) and forest soil (Fo).

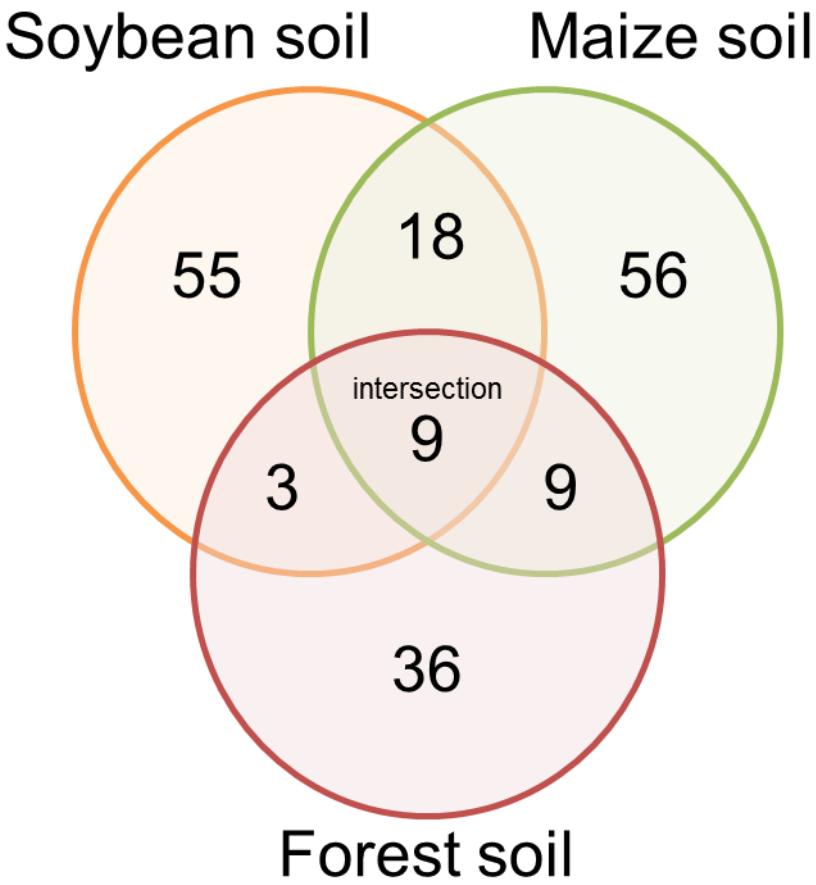

Fig. 4.2 Venn diagram showing soil specific proteins in soybean, maize and forest soils

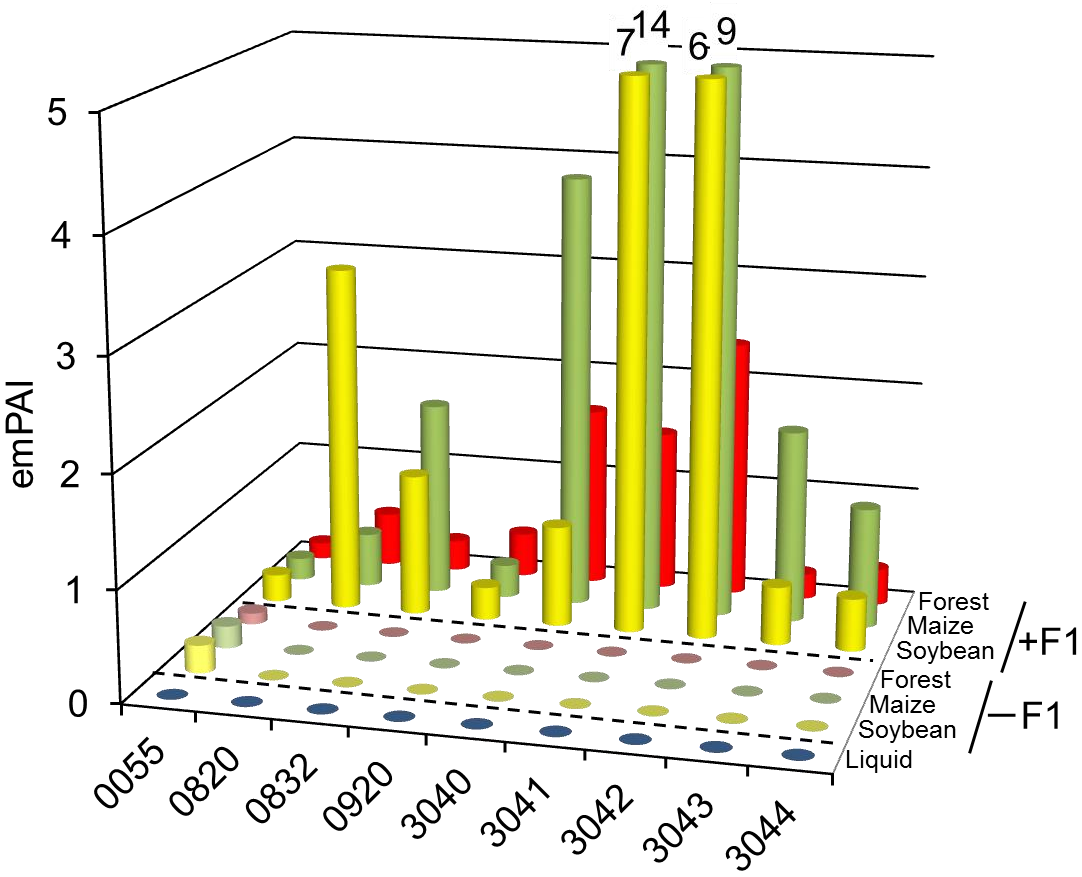

Fig. 4.3 Expression of soil specific proteins in soil with and without *P. putida* F1.

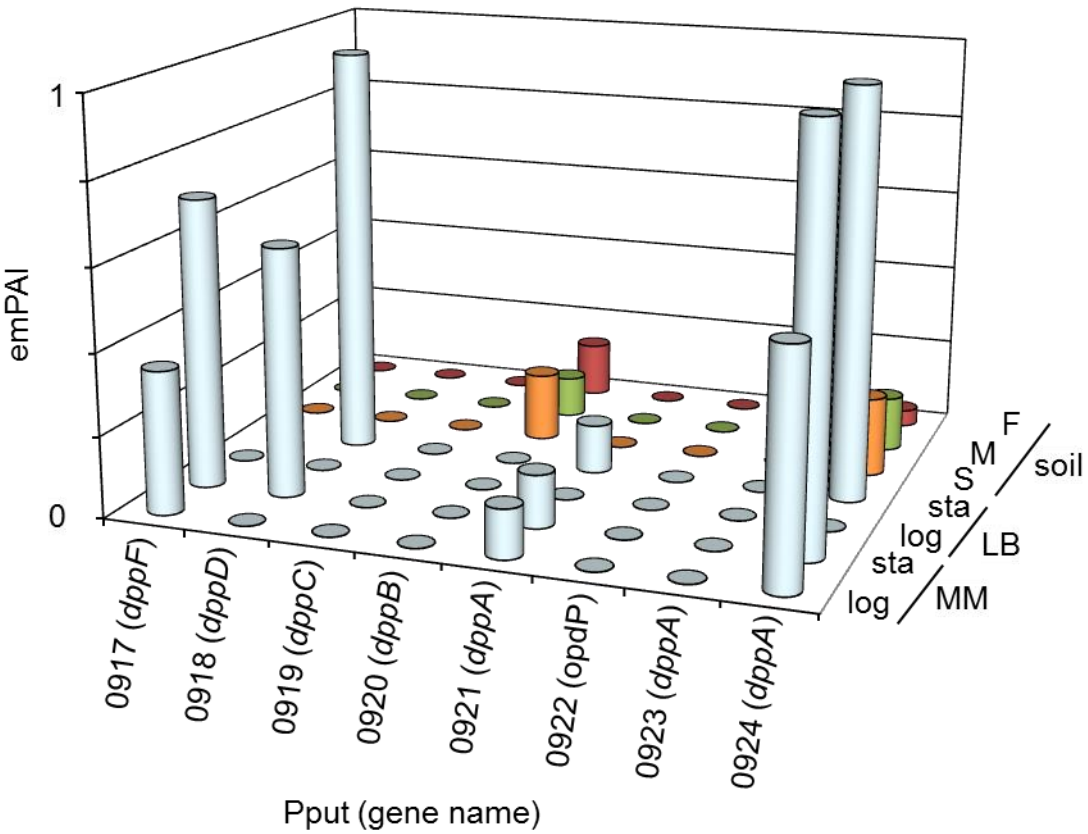

Fig 4.4 Expression profiling of the constituent genes of alkaline phosphatase of *P. putida* F1 cells grown in soil and liquid culture

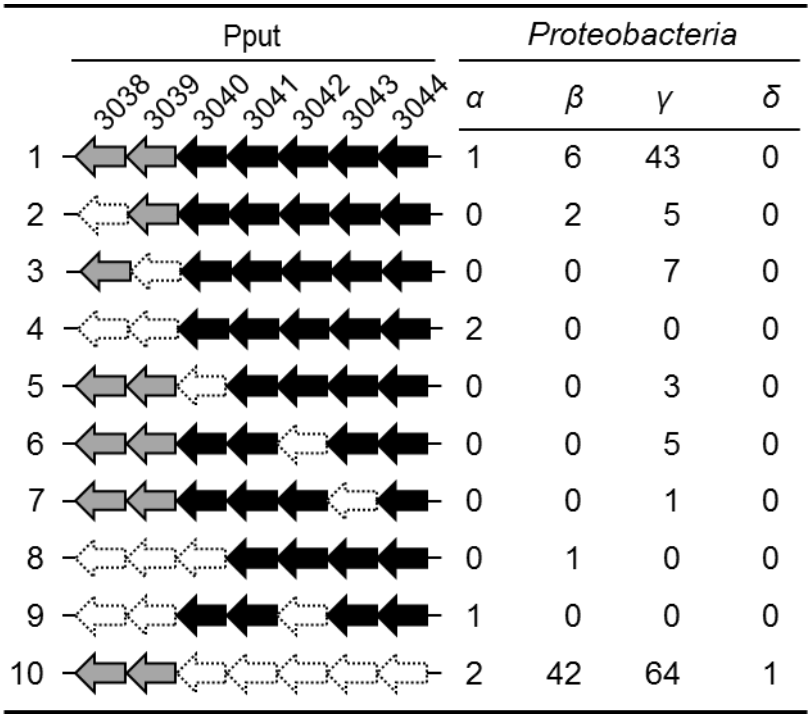

Fig. 4.5 Phylogenetic analysis of SSP operon.

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Abstract in Japanese

Proteomics on environmental response of *Pseudomonas putida* F1 in soil
（土壌における *Pseudomonas putida* F1 の環境応答に関するプロテオーム解析）

微生物は地球上のあらゆる環境に生存している。近年では、メタプロテオミクスにより、環境における微生物群集の遺伝子発現レベルにおける機能解析が行われてきているが、現状のメタプロテオミクスにおいて、複雑な複合系の中で特定個体の個生態を理解することは困難である。また、土壌環境で微生物がどのような遺伝子を発現し生存しているのかを研究した例は極めて少ない。本論文では、土壌環境における特定の微生物に着目し、土壌内における環境応答をプロテオーム解析で解析した。第 2 章では、液体培地で培養した土壌細菌 *Pseudomonas putida* F1 株の芳香族炭化水素分解に関与するタンパク質群をプロテオーム解析により明らかにした。第 3 章では、土壌内における *P. putida* F1 株の芳香族炭化水素の添加に対する応答を解析した。土壌内における *P. putida* F1 株の芳香族分解関連タンパク質発現を全て検出できたことを踏まえ、第 4 章では複雑な土壌環境に対する *P. putida* F1 株の環境応答として土壌環境でのみ特異的に発現する遺伝子の解析に着目した。

P. putida F1 株は芳香族炭化水素（トルエン、エチルベンゼン、ベンゼン）を TCA サイクルの中間代謝物に分解できることが知られている。トルエン、エチルベンゼン、ベンゼンの分解に関与するタンパク質を決定するために、これら芳香族炭化水素を唯一の炭素源として添加した液体培地で培養した *P. putida* F1 株からの細胞内タンパク質は、1-D SDS-PAGE と LC-MS/MS を用いて同定した。本論文では、ラベルフリーな emPAI に基づいた半定量的なプロテオーム解析アプローチを用いた。各種培養系からタンパク質セットを比較解析した結果、*P. putida* F1 株のトルエン、エチルベンゼン、ベンゼンの分解に関与する Toluene degradation (Tod) pathway、エチルベンゼンの分解に関与する 2-methylcitrate (2-MC) cycle およびベンゼンの分解に関与する *β*-ketoadipate pathway に関与する全ての主要タンパク質を同定した。さらに、*tod* オペロンの発現を制御する二成分制御系 (TodST) や *tod* オペロンの下流に位置する solvent effux pumps (SepRABC) についても同定した。これらの結果から、*P. putida* F1 株の液体培養におけるプロテオーム解析で半定量法による解析法を確立し、芳香族炭化水素分解に関与する遺伝子を明らかにした。

実際の土壌環境における *P. putida* F1 株の芳香族炭化水素に対する応答を解析し、実験室内で得られた知見が実際の環境において反映されているのかを明らかにすることを試みた。土壌環境中における *P. putida* F1 株の芳香族炭化水素分解関与タンパク質の発現変化を解析するため、グルコースを添加した園芸用土壌に *P. putida* F1 株を接種し 3 日間の培養を行った後、その培養土壌に芳香族炭化水素（トルエン、エチルベンゼン、ベンゼン）をそれぞれ添加し、培養を継続した。土壌培養した *P. putida* F1 株からのタンパク質抽出は、密度勾配遠心分離法を用いて土壌から細胞を分画した後、その細胞分画を用いて行った。抽出タンパク質は、二次元電気泳動法 (2-DE) および一次元電気泳動法 (1-D SDS-PAGE) による分離後、質量分析計 (nanoLC-MS/MS) および Mascot 検索を用いてタンパク質の同定を行った。2-DE/LC-MS/MS 解析は、トルエン、ベンゼン、エチルベンゼンを添加した土壌で培養した *P. putida* F1 株から、それら芳香族炭化水素の分解に主要な 22 タンパク質のうち、12 のタンパク質を検出できた。エチルベンゼンを添加した土壌の 1-D SDS-PAGE/LC-MS/MS 解析では、約 1260 の *P. putida* F1 株タンパク質を同定した。エチルベンゼン分解に関与する

全て主要タンパク質、トランスポーターおよびセンサータンパク質は、液体培養と同様に高発現した。これらの結果から、*P. putida* F1 株の土壌環境中における芳香族炭化水素の分解様式は、実験室内で得られた結果と同様であることを示した。従って、*P. putida* F1 株の芳香族化合物の分解は、実験室内で得られた知見を実際の環境で反映していることを明らかにした。本解析アプローチは、土壌内での *P. putida* F1 株の環境変化に対する応答をタンパク質レベルで解析できたことから、土壌に生息する微生物の理解に貢献できる可能性を示した。

未だ細菌ゲノム解析株において多くの機能未知遺伝子が存在する。これらの機能未知遺伝子の中には、細菌が生来生息する環境でのみ発現・機能する遺伝子が存在すると考えられた。本研究では、*P. putida* F1 株の土壌培養系と液体培養系に対して、比較プロテオーム解析を行い、土壌環境特異的な発現タンパク質の検出および解析を行った。*P. putida* F1 株は、0.5%グルコースを添加した土壌（大豆畑、コーン畑、森林）に接種し、3 日間、30℃で培養した。土壌培養した *P. putida* F1 株からのタンパク質抽出は、密度勾配遠心分離法と SDS-フェノール法を用いて行った。抽出タンパク質は 1-D SDS-PAGE 後、質量分析計および Mascot 検索を用いて同定した。プロテオーム解析により、大豆土壌、野菜土壌および森林土壌において、それぞれ 1364、1273、816 タンパク質を同定した。比較プロテオーム解析において、土壌特異的に発現する 2 つの遺伝子および 1 オペロンを特定した。それら遺伝子は、一酸化窒素ジオキシゲナーゼおよび機能未知遺伝子を含んだ。一方のオペロンは、二成分制御遺伝子を含む 7 つの遺伝子で構成された機能未知のオペロンであった。また、土壌特異的オペロンの発現因子について、多種微生物間相互作用の生物学的側面や土壌の物理的・化学的側面から検討を行った結果、土壌成分がその発現に関与していることが推測された。さらに、この土壌特異的オペロンについて系統解析を行った結果、主にプロテオバクテリア門に保存されている遺伝子群であることが示された。

本論文において、土壌内で *P. putida* F1 株の芳香族炭化水素に対する応答をタンパク質レベルで検出することができ、液体培養と比較において同様の主要分解タンパク質を発現することを示した。また、土壌という環境要因による *P. putida* F1 株の土壌特異的発現タンパク質を特定することができた。本研究手法より、土壌に生息する細菌の発現タンパク質を詳細に解析することができ、試験管内での生理状態とは異なった現場での生理学的・遺伝学的な環境応答を理解することが可能となった。本研究手法を他の微生物種に応用することで、対象とする微生物のそれぞれの生息環境における個生態学やライフスタイルの理解を深めることが期待される。