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

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Abstract

Pseudomonas putida F1 can metabolize toluene, ethylbenzene, and benzene for growth. Previously, we identified proteins involved in the utilization of these compounds by *P. putida* F1 through culture in liquid media. However, it was unclear whether laboratory analysis of bacterial activity and catabolism accurately reflected the soil environment. We identified proteins involved in the degradation of toluene, ethylbenzene, and benzene growth in soil using two-dimensional gel electrophoresis (2-DE) or standard SDS-PAGE combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS). According to 2-DE/LC-MS/MS analysis, 12 of 22 key enzymes involved in the degradation of toluene, ethylbenzene, and benzene were detected. In standard SDS-PAGE/LC-MS/MS analysis of soil with ethylbenzene, approximately 1,260 cellular proteins were identified in *P. putida* F1. All key enzymes and transporter and sensor proteins involved in ethylbenzene degradation were up-regulated similar to that noted in liquid cultures. In *P. putida* F1, aromatic hydrocarbon response in soil is the same as that observed in liquid media.

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 (Caspi et al. 2012; Ogata et al. 1999). The soil bacterium *Pseudomonas putida* F1 strain can use toluene, ethylbenzene, and benzene as the sole sources of carbon and energy 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 through the tricarboxylic acid (TCA) cycle via the toluene degradation (involving *tod* genes) (Zylstra and Gibson 1989; Zylstra et al. 1988), propanoate (*prp* genes), and *β*-ketoadipate (*pca* and *cat* genes) pathways. We previously identified key enzymes involved in the degradation of toluene, ethylbenzene, and benzene in *P. putida* F1 in liquid media (Fig. 1) (Kasahara et al. 2012). We 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; Pput_2872 and 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). Therefore, it is important to identify the 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 (Graham et al. 2011; Graves and Haystead 2002). Mass spectrometry-based proteomics is widely used in bacterial sciences (Thompson et al. 2010; Jimenez et al. 2002). Proteomics-based approaches can be used to

61 analyze the function of indigenous microbial communities in soil environments (Wang et al. 2011; Wu et al.
62 2011; Williams et al. 2010; Bastida et al. 2010; Knief et al. 2012), while no approach is available to analyze the
63 function of a single bacterial strain in soil environments.

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

69

70 **Materials and methods**

71 Bacterial culture conditions in soil

72 *P. putida* F1 was grown at 30°C for 16 h in 100 mL of mineral salt medium (MSM) containing 0.2% (w/v)
73 glucose with vigorous shaking (190 rpm) (Munoz et al. 2007). The cells were collected by centrifugation at
74 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
75 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
76 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:
77 150 mg L^{-1}, pH 6.2, no. of viable bacterial cells: 1.1 × 10^7 cells g^{-1} soil) (Hokusan, Hokkaido, Japan) containing
78 0.2% (w/v) glucose in a petri dish. Soils inoculated were incubated at 30°C for 3 days. Next, toluene (T),
79 ethylbenzene (E), or benzene (B) were added to the soil at final concentrations of 0.5% (v/w), 1.5% (v/w), and
80 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.

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.

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.

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 and 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).

Standard SDS-PAGE

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).

2-DE

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 PageRuler^{TM} 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.

Proteome analysis

Proteome analysis methods were performed as described previously (Kasahara et al. 2012). Standard

141 SDS-PAGE gel lanes were cut into 80 strips (~1 mm). CBB-stained gel strips were destained using 100 μL of
142 30% acetonitrile (ACN) containing 25 mM NH_4HCO_3. Differentially expressed protein spots were excised from
143 the 2-DE gels. Silver-stained spots were destained using 100 μL of 25 mM NaS_2O_3 and 15 mM $K_3Fe(CN)_6$.
144 Destained gels were reduced using 10 mM DTT, followed by alkylation with 55 mM iodoacetamide. After the
145 gels were completely dried, they were digested using 40 μL of sequencing-grade modified trypsin (12.5 μg/mL
146 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
147 and washed twice by using 5% formic acid in 70% ACN. Peptide mixtures were used for nano liquid
148 chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS) analysis.
149 NanoLC-ESI-MS/MS analysis was conducted using an LTQ ion-trap MS (Thermo Fisher Scientific, Waltham,
150 MA, USA) coupled with a Paradigm MS2 nano-flow HPLC system (AMR Inc., Tokyo, Japan) and nano-spray
151 electrospray ionization device (Michrom Bioresources Inc., Auburn, CA, USA). The tryptic peptide mixture was
152 loaded onto an L-column2 ODS (Chemicals Evaluation & Research Inst., Tokyo, Japan) packed with
153 C18-modified silica particles (5 μm; pore size, 12 nm). The peptide mixture was separated using a linear
154 gradient of 15–65% buffer B for 40 min, followed by a gradient of 65–95% buffer B for 1 min (buffer A = 2%
155 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
156 min^{-1}. Peptide spectra were recorded over a mass range of m/z 450–1800. MS/MS spectra were acquired in
157 data-dependent scan mode. After the full spectrum scan, one MS/MS spectrum of the most intense single peaks
158 was also collected. Dynamic exclusion features were set as follows: a repeat count of one within every 30 s, an
159 exclusion duration of 180 s, and an exclusion list size of 50. MS/MS data were analyzed using the Mascot
160 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.

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).

Results and Discussion

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. 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 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. 2). For proteome analyses, sampling times of the incubated soil samples were determined to be 6, 12, and 18 days.

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 S1). 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.

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. 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

201 molecular weights (MW). A total of 283 protein spots of the predicted spots and differentially expressed protein
202 spots were excised from the TEB0, T12, E12, and B12 gels, and 247 proteins were identified using more than
203 two unique peptide filtering criteria (Table S2).

204 Close-up images of 2-DE gels are shown in Fig. 4, which shows detected aromatic hydrocarbon degradation
205 proteins. Of the 11 proteins included in the *tod* cluster (*todXFC1C2BADEGIH*), nine proteins (TodA, TodC1,
206 TodC2, TodD, TodE, TodF, TodG, TodH, and TodI) were strongly induced on the T12, E12, and B12 gel
207 compared with TEB0 (Fig. 4A). The spots correspond to TodA on the TEB0 gel were identified not TodA
208 protein, but elongation factor Tu protein (Pput_0473, spots number: 24, 182, 183 in Fig. 3). Of the eight proteins
209 that comprise the propanoate pathway involved in ethylbenzene degradation (Fig. 1), two protein spots (PrpB
210 and PrpF) were detected only on the E12 gel (Fig. 4B). Of the seven proteins comprising the β-ketoadipate
211 pathway involved in benzene degradation (Fig. 1), two protein spots (CatA and CatB) were detected only on the
212 B12 gel (Fig. 4C). On the TEB0 gel the right spot correspond to CatB were 4-aminobutyrate aminotransferase
213 protein (Pput_0229, spots number: 81 in Fig. 3). Although benzene was consumed after three days of incubation
214 (Fig. 2), the key enzymes involved in benzene degradation persisted after 12 days of incubation. These proteins
215 may be induced by small amounts of benzene below the detection limit of GC analysis. Enzymes important for
216 the degradation of toluene, ethylbenzene, and benzene in *P. putida* F1 grown in soil were detected using
217 2-DE/LC-MS/MS analysis. Our results indicate that *P. putida* F1 degrades aromatic hydrocarbons in soil.

218 The 2-DE approach is advantageous because it enables comparison of protein expression profiles and
219 quantification of expression levels (Curreem et al. 2012; Wilkins et al. 1998). However, this approach has some
220 limitations, including that (Curreem et al. 2012; Wilkins et al. 1998): (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.

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. S1). A total of 1373, 1016, and 1389 proteins were identified in E0, E6, and E18 samples, respectively (Table S3). PC values were calculated for the identified proteins based on emPAI (Table S4). 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. 5A and B). 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 (Table S4)

241 (Kasahara et al. 2012). These results indicate that *P. putida* F1 responds to ethylbenzene in soil in a similar
242 manner as in liquid media experiments.

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

249 Extracting proteins from soil is difficult because of the presence of humic substances and indigenous
250 materials in the soil (Benndorf et al. 2007; Keiblinger et al. 2012) as well as the adsorption of proteins to soil
251 particles (Ding and Henrichs 2002; Taylor and Williams 2010). To eliminate factors that inhibit protein
252 extraction, we fractionated bacterial cells from soil and extracted cellular proteins. We identified approximately
253 1260 proteins in *P. putida* F1. The number of detected proteins was approximately 70% of the number detected
254 in pure culture (Kasahara et al. 2012). Of these, the proteins involved in the degradation of aromatic
255 hydrocarbons as well as other proteins involved in other bioprocesses were subjected to genome-wide analysis
256 by using bacterial strains in soil. The SDS-PAGE/LC-MS/MS analysis approach allowed the examination of
257 bacterial response to soil environment.

258

259 **Conclusion**

260 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. We 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.

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Legends

Figure 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). (ref. Kasahara et al. 2012)

Figure 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

414 measurements.

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416 Figure 3. 2-DE gel images of cellular proteins obtained from *P. putida* F1 grown in soil of before (day 0; TEB0)

417 and after addition (day 12; T12, E12 and B12) of the toluene, ethylbenzene, and benzene. Arrows indicate

418 protein spots analyzed by LC-MS/MS. Proteins (100 μg) were loaded on an 18-cm strip with a linear gradient of

419 pH 4–7 for IEF, following electrophoresis of 12.5% SDS-PAGE and silver staining.

420

421 Figure 4. The close-up images of the identified aromatic hydrocarbons degradation proteins in TEB0, T12, E12,

422 and B12 gels. (A) Protein spot involved in the *tod* cluster. (B) Protein spots involved in the propanoate pathway.

423 (C) Protein spots involved in the *β*-ketoadipate pathway.

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425 Figure 5. Protein content (PC) of proteins involved in the aromatic hydrocarbon degradation in E0, E6, and E18

426 samples. (A) Proteins involved in the *tod* cluster. (B) Proteins involved in the propanoate pathway.

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Supporting Information

Figure S1. SDS-PAGE image of the extracted proteins from soil amended with ethylbenzene (E0, E6, and E18 samples). Twenty-five μg proteins per a lane were loaded on 12.5% SDS-PAGE. The gels were stained with CBB.

Table S1. Viable cell number of *P. putida* F1 in soil during incubation for 18 days.

Table S2. Proteins identified on the 2-DE gels in TEB0, T12, E12, and B12 samples.

Table S3. Proteins identified on the 1-D SDS-PAGE/LC-MS/MS in E0, E6, and E18 samples.

Table S4. Exponentially modified protein abundance index (emPAI) and protein content (PC) of the identified proteins in E0, E6, and E18 samples.

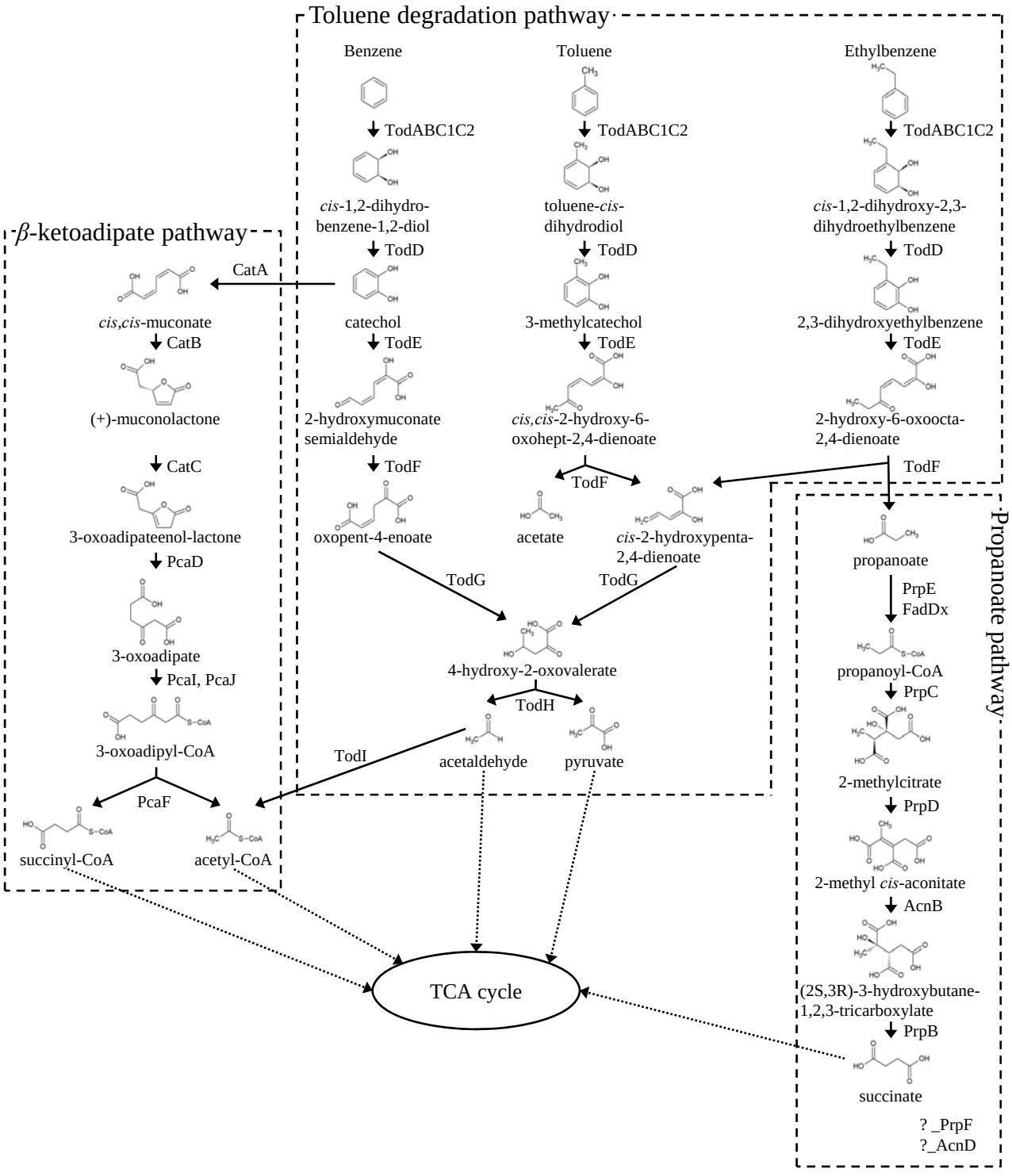

Fig. 1 Morimoto H. et al

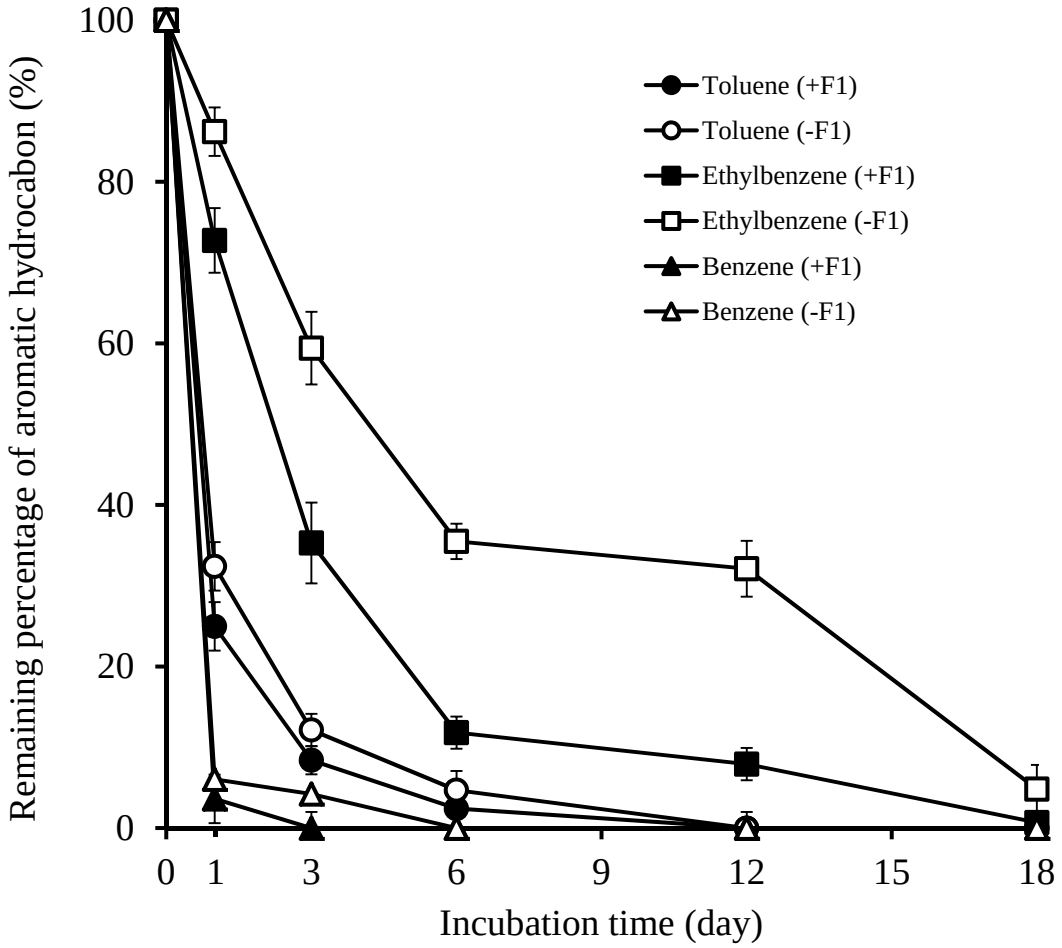

Fig. 2 Morimoto H. et al

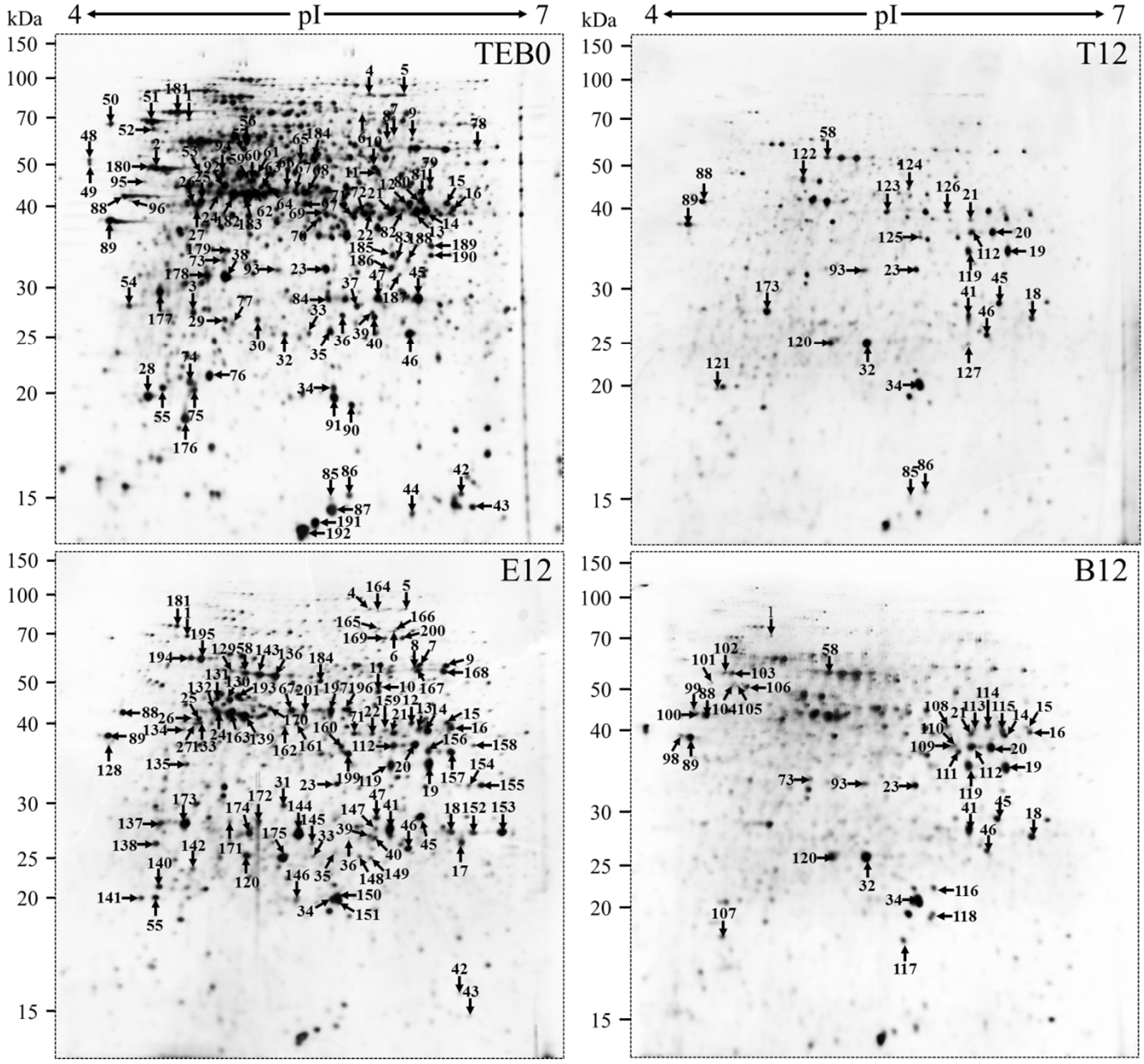

Fig. 3 Morimoto H. et al

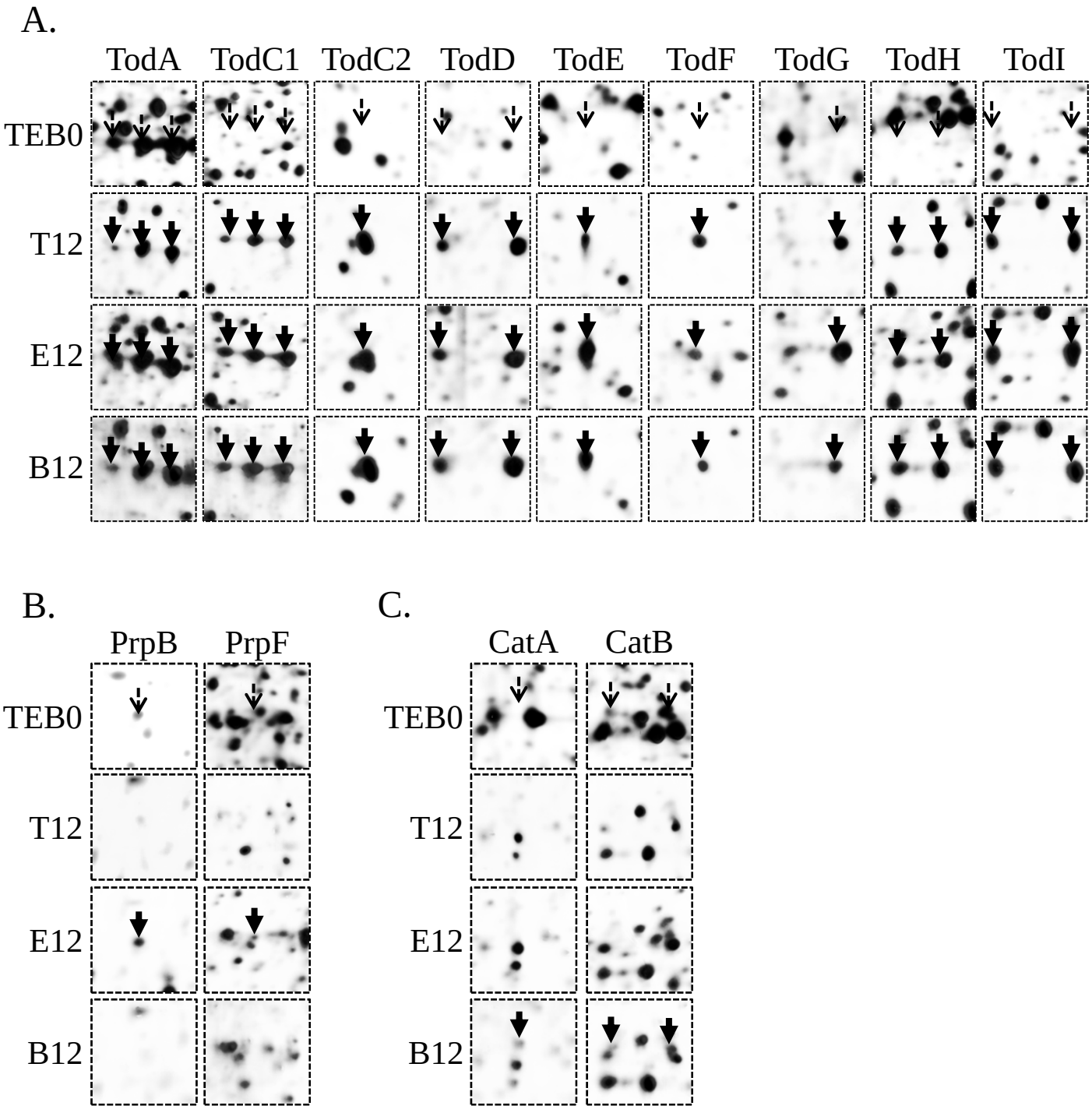

Fig. 4 Morimoto H. et al

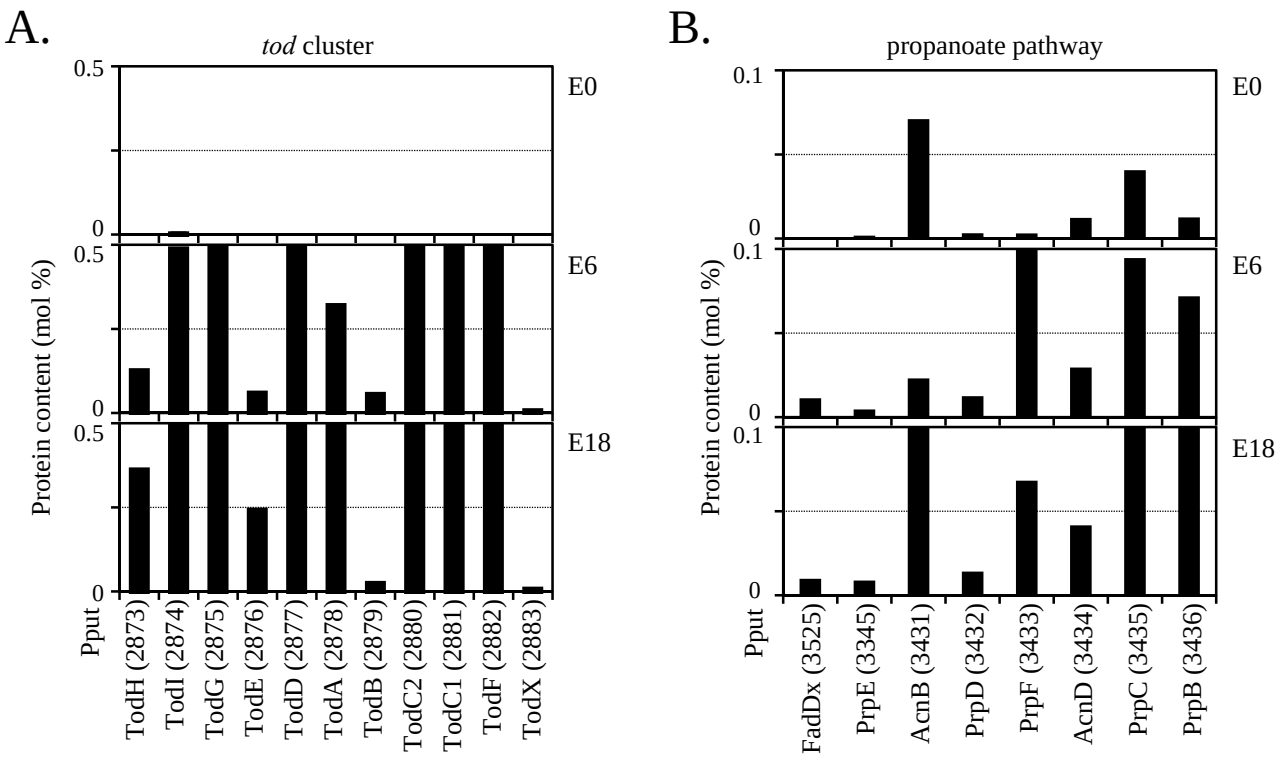

Fig. 5 Morimoto H. et al