Proteome analysis of *Pseudomonas putida* F1 genes induced in soil environments.

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Summary

Knowledge of the gene expression dynamics of a single soil bacterial strain contributes to the understanding of its behavior, physiological state, and surrounding microenvironment. Genes expressed in soil environments rather than in laboratory media are considered to particularly relevant. Here, we compared genome-wide gene expression profiles of the bacterium *Pseudomonas putida* F1 inoculated in three different types of non-sterile soils deduced using proteome analysis via sodium dodecyl sulfate-polyacrylamide gel electrophoresis combined with liquid chromatography-tandem mass spectrometry. Proteins commonly detected in all three samples and involved with bacterial growth and fundamental metabolism were excluded. Nine proteins were identified as specifically expressed in soil including an aldehyde dehydrogenase, a nitric oxide dioxygenase, and five proteins encoded by a cluster of metabolism-associated genes. Expression factor analysis revealed that the nitric oxide dioxygenase-coding gene was induced by nitric oxide and the five clustered genes were induced under phosphate starvation. The expression of these genes can be attributed to response to soil environmental stimuli surrounding the F1 cells. These results strongly suggest that our soil metaproteome approach is useful for understanding the autecology and lifestyle of a single bacterial strain in soil environments and allows the prediction of the microenvironment surrounding the bacterial cells.

Introduction

Bacterial cells in natural soil environments respond to physical, chemical, and biological changes. Knowing the gene expression pattern, function, and physiological state of a single bacterial strain is important for the understanding of bacterial ecology. However, it is difficult to directly analyze the gene expression of a single strain in soil environments. Recently, *in vivo* expression technology (IVET) has been developed to assess bacterial gene expression in various environments. IVET is a promoter-trapping technique that selects microbial promoters active in a specific niche (Mahan et al., 1993), offering the advantage of positive selection of genes induced by environmental factors (Rediers et al., 2003) but with the disadvantage of requiring laboratory molecular manipulations. IVET has been adapted for varied use; e.g., *Pseudomonas fluorescens* SBW25 in the rhizosphere (Rainey, 1999), *P. fluorescens* Pf0-1 (Silby and Levy, 2004) and *Burkholderia multivorans* (Nishiyama et al., 2010) in soil, *P. putida* KT2440 in the rhizosphere of different plants (Fernández et al. 2012), *P. syringae* pv. tomato on a tomato leaf (Boch et al., 2002), and *P. stutzeri* A15 in a rice root on agar medium

(Rediers et al., 2003). In addition, Moreno-Forero and van der Meer (2015) reported the genome-wide metatranscriptome analysis of *Sphingomonas wittichii* RW1 grown in sand contaminated with dibenzofurans and obtained evidence for "soil-specific" expressed genes. In comparison, we have previously analyzed the gene expression profile of *P. putida* F1 during incubation in a garden soil contaminated by an aromatic hydrocarbon using a proteome technique, which indicated the feasibility of genome-wide analysis of gene expression of a single strain in soil (Morimoto et al., 2013).

At present, genomes of over 4100 bacterial species have been completely sequenced. Approximately 10% –25% of the open reading frames identified in these genomes represent genes for hypothetical proteins, according to the genome databases in NCBI. To clarify the gene expression and functional data for each completed genome for bacteria from special habitats, general information has been obtained through *in vitro* studies under various conditions. However, this information might differ considerably for bacteria exposed to various environmental factors in the original habitat. For example, it is expected that among the 20% of the 5250 *P. putida* F1 genes that encode hypothetical proteins according the NCBI website, the expression of several might be selectively observed in a soil rather than a laboratory environment.

In this study, we performed differential proteomics analysis using a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) technique combined with liquid chromatography-tandem mass spectrometry (LC–MS/MS) to identify *P. putida* F1 genes induced in soil. The strain F1 was inoculated into three different types of soil and incubated for 3 days, and the proteome datasets were compared. The expression stimuli were identified for the genes that were commonly detected as being induced in the three soil samples but not in laboratory media. Our results suggested that the soil metaproteome approach used in this study was useful for understanding the autecology and lifestyle of a single bacterial strain in a soil environment, and allowed the prediction of the microenvironment surrounding the bacterial cells.

Results and discussion

Overview

To identify *P. putida* F1 genes specifically expressed in soil environments, we compared the proteome datasets obtained from F1 in three different soils. **Figure 1** presents the strategy used to analyze the proteome. The three soil samples (soybean, maize, and forest) were

collected from three fields with different flora in the different areas of Japan. Analysis of the chemical properties showed that the values of C%, N%, C/N, and NO_3^- differed among the soil samples. The viable cell numbers of indigenous bacteria in the soybean, maize, and forest soil samples were approximately 5.0×10^8, 2.5×10^7, and 2.3×10^9 CFU g^{-1}, respectively (**Table S1**), indicating that the forest and maize soil samples contained the highest and lowest number of bacterial cells, respectively.

F1 cells were cultured for 3 days in each of the three different soils, each with three independent biological replicates. During incubation, the viable F1 cell numbers were measured for all inoculated soil samples. After inoculation with 2.7×10^8 F1 cells g^{-1} soil, the number of cells in the soybean, maize and forest samples decreased to 1.9×10^6, 2.1×10^5, and 9.3×10^4, respectively, after 3 days (**Fig. S1**).

For F1 proteome analyses during soil incubation, the indirect extraction method was applied based on the number of identified F1 proteins and the operability of the MS/MS measurements. The bacterial cells were fractionated from the soil samples using Nycodenz density gradient centrifugation (**Fig. S2**) and the bacterial cell proteins were extracted and separated by SDS-PAGE. Only proteins from the forest sample were not well separated, and no bands were observed. The inferiority of the gel image could be attributed to contamination with humic substances and impurities in the protein extract, as the forest sample bacterial layer obtained during cell fractionation had many soil particles and much plant debris, which clearly differed from the other two samples. Next, the gel lanes were cut into 60 strips and in-gel digestion was performed followed by MS/MS identification using more than two unique peptide-filtering criteria and label-free quantification using spectrum counting (Bantscheff et al., 2007). An F1 non-redundant soil proteome dataset was created from the results of three independent experiments for each soil, allowing high coverage of detected proteins among the 5250 known *P. putida* F1 proteins (Gautier et al., 2012).

Proteome analysis of three soil samples

First, we examined the abundance of proteins from *P. putida* strains and *Pseudomonas* spp. among the proteins extracted from the soils inoculated with/without F1 cells using the NCBI database of all completed bacterial genomes to identify bacterial proteins. Few proteins derived from *Pseudomonas* spp. and *P. putida* were identified in the non-inoculated soils whereas in the soils inoculated with the F1 cells, the rates of occurrence of *P. putida* proteins were approximately 60.7%, 47.7%, and 36.7% in the soybean, maize and forest samples,

respectively (**Table S2**). The protein occupancy corresponded with the viable cell numbers of F1 cells in the soil samples after 3 days (**Fig. S1**). Overall, *P. putida* F1 and closely-related species were sparse in the original three soils; almost of the *Pseudomonas*/*P. putida* proteins detected in the F1-inocukated soil samples were confirmed to have derived from the F1 cells.

Table 1 shows the number of F1 proteins detected in the cells from the nine soil samples; twice the number of F1 proteins were detected in the forest versus the soybean samples. To compare the detected proteins among the soil samples, we established three non-redundant proteome datasets, soybean, maize, and forest (**Table S3**) containing 1364, 1273, and 816 proteins respectively, detected in total from all 3 experiments for each category (**Table 1**). A Venn diagram representation of the proteome datasets from the three soil samples is shown in **Fig. 2A**. Overall, 1655 distinct proteins were differentially detected in the soil media among which 674 were detected in all three samples. To identify the proteins induced by soil environments, the proteins involved with bacterial growth and fundamental metabolism were excluded, as were proteins expressed by cultured cells in standard media, identified as follows: the proteome of F1 cells in two growth phase and two standard media cultures (logarithm and stationary phases in minimal salt (MS) and LB media) were analyzed, detecting 1878, 2036, 1547, and 2003 proteins, respectively (**Table S4**), and the MS/LB non-redundant proteome dataset of 2643 proteins was created upon compiling the four datasets (**Table 1**). After excluding the proteins in MS/LB, nine proteins were found to be commonly detected in all three samples **(Fig. 2B**). The 9 genes encoding the identified proteins included a cluster consisting of five genes (Pput_3040–3044). Cluster of Orthologous Groups (COG) categorization assigned five genes as belonging to Metabolism and four remaining genes to unassigned functions. We consider the nine soil-induced genes to represent genes specifically expressed in soil (**Table 2**); these were further characterized using bioinformatics and/or expression analyses.

Of the 5250 *P. putida* F1 proteins, the coverage for the liquid cultures was 29.5%–38.8%, over double that (8.0%–20.1%) obtained for the soil samples. The marked decrease of the detection rate in the soil samples might have resulted from contamination of the extracted proteins with soil impurities and/or a decrease in the proportion of F1 proteins in the extracts owing to the presence of indigenous bacteria.

Characterization of soil-induced genes

Pput_0055

Soil bacterial cell Nycodenz fractionation collects not only F1 but also indigenous bacteria, whose proteins were co-extracted. Therefore, it was possible to detect proteins derived from both indigenous bacteria and F1 cells. Few *Pseudomonas*/*P. putida* proteins were detected in the non-F1-inoculated soil samples, as described above (**Table S2**). To address this issue, we performed proteome analysis of the non-inoculated soil sample using the Mascot program with the *P. putida* F1 protein database. Notably, the Pput_0055 protein was detected in all soil samples regardless of inoculation status (**Fig. 3**), indicating a low possibility of the Pput_0055 gene to represent a soil-induced F1 gene.

Pput_0920

The oligopeptide permease (Opp) represents a periplasmic binding protein-dependent transport system encoded by an operon of five genes (Hiles et al., 1987). Similarly, the dipeptide permease (Dpp) comprises a periplasmic binding protein-dependent transport system with high affinity for dipeptide (Manson et al., 1986). The *opp* operon of *Salmonella typhimurium* (Hiles et al., 1987) and the *dpp* operon of *Escherichia coli* (Abouhamad et al., 1991, Abouhamad and Manson, 1994) are homologous. Both operons contain five cistrons, and the promoter-proximal gene of each encodes an abundant periplasmic or cell-surface peptide-binding protein. The remaining four genes are in the same order in each operon (Abouhamad et al., 1991, Abouhamad and Manson, 1994). The Dpp of F1 is highly homologous to that of *E. coli* and *S. typhimurium* but is composed of eight genes, *dppFDCB* (Pput_0917, 0918, 0919, and 0920) and *dppA* (Pput_0921, 0922, 0923 and 0924) (Ames et al., 1990), which are in the same order as those of *E. coli* and *S. typhimurium*. The Pput_0920 (*dppB*) gene codes for a dipeptide transport system permease protein. The Pput_0917、0918、and 0921 proteins were detected in the liquid proteome but not the soil sample datasets (**Fig. 4**). Since the dipeptide transporter system acts as a DppFDCBA protein complex, its functioning requires all of the component genes to be expressed (Maqbool et al., 2011).Therefore, expression of only a subset of the genes is unlikely in either liquid or soil. Given that all of the proteins are present in the cell, our data suggests that there is also an only low possibility that the Pput_0920 gene represents a soil-induced F1 gene.

Pput_0820

The Pput_0820 gene encoded an aldehyde dehydrogenase and is monocistronic. In F1 genome, 20 genes encode aldehyde dehydrogenase, of which only Pput_0820 gene has no

paralogous. Thus, this gene was not further analyzed owing to lack of information.

Pput_0832

The following differences between soil and liquid media were considered: (i) soil particle interfaces (physical factor), (ii) soil components as nutrition for bacteria (chemical factor), and (iii) direct and/or indirect biological interactions (biological factor). We conducted proteome analysis of the soil-induced proteins after incubating F1 on three soil extracts media to eliminate the physical (solid–liquid and solid–gas interfaces) and biological (F1 versus e.g., ingenious microbes, protozoa, and/or plant cells) factors. Peptides of the five cluster proteins (Pput_3040–3044) were detected in all soil extract media with the exception of Pput_0832 (**Table 3**), indicating that cluster expression is induced by soil components whereas the Pput_0832 gene is induced by either soil particles or biological interactions.

The Pput_0832 gene encodes a nitric oxide (NO) dioxygenase, whose function has not yet been verified in F1. In the nitrogen metabolic pathway, NO can be produced as an intermediate product of the metabolism o NO_2^- to N_2O. However, F1 does not have support the metabolism to produce NO. When F1 cells were grown in MS medium supplemented with NO, unique Pput_0832 protein peptides (15.7 peptides ± 3.2) were detected, whereas no peptides were detected in the absence of NO. Consequently, the expression factor of the Pput_0832 gene was identified to be NO. NO is highly volatile; therefore, the Pput_0832 gene could not be induced in soil extracts, into which NO was not able to diffuse (**Table 3**). In the garden soil that was used in our previous report (Morimoto et al., 2013), approximately 5 unique Pput_0832 peptides were detected in the nonsterile sample, whereas no peptides were detected in the sterile sample, indicating that the NO in the soil samples was derived from the nitrogen metabolism of the indigenous microorganisms. NO has a high toxicity and inhibits the growth of bacteria (Hrabie et al., 1993). Thus, we considered that to survive in soil environments, F1 sense the NO produced via the nitrogen metabolism of other microorganisms, express the Pput_0832 gene, and protects itself by detoxification of the protein (Gardner et al., 1998).

Bioinformatics and expression analyses of soil-induced cluster.

We performed a homology search for the soil-induced cluster using the Gene Cluster Search tool in the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/) (**Fig. 5**). Several bacterial species were found that harbored a

complete cluster of five genes (Type 1 in **Fig. 5**), whereas some species were found with four or only three genes (Type 2–7). All of the bacterial species (> 30% amino acid sequence identity) are affiliated with the phylum *Proteobacteria*, including the subphyla *α*-, *β*-, and *γ-proteobacteria*. Among these, *γ-proteobacteria* were predominant (87%), including many bacteria pathogenic for plants, fish and humans; e.g. *Pseudomonas* spp., *Vibrio* spp., *Xanthomonas* spp. and *Yersinia* spp. Maintenance of the cluster was found to be strain-dependent; it is assumed that the cluster could be spread among bacteria by horizontal transfer.

To identify a soil component that induced the expression of genes of the cluster, F1 was incubated in the following nine media: the original MS medium, $MgSO_4$-, $(NH_4)_2SO_4$-, SO_4^{2-} [$MgSO_4$ and $(NH_4)_2SO_4$]-, $CaCl_2$-, PO_4^{3-} (Na_2HPO_4 and KH_2PO_4)-, and trace element-free MS media, 0.8%NaCl, and DW. The proteome data for the cluster genes are shown in **Table 4**. The peptides of all five genes were detected only after incubation in the PO_4^{3-}-free MS medium, 0.8%NaCl or DW (**Table 4**), suggesting that the expression factor was likely a deficiency of phosphate. To verify the expression of the five genes under the above conditions, RT-PCR analysis was performed using RNA samples from F1 incubated in MS medium and DW. In DW, all of the cluster genes were expressed to a much higher extent than in MS medium (**Fig. 6**). After incubation in MS medium, the intensity of all RT-PCR bands showed that the genes were only marginally expressed. During proteome analysis in MS/LB media, these proteins would likely be below the detection level of the MS/MS technique. In the preset study, when the bacterial cells were retrieved from the soil samples via Nycodenz density gradient centrifugation, F1 cells were exposed to solutions without phosphate for approximately 1 hour. We thus examined the influence of Nycodenz treatment on the expression of the cluster in the garden soil as determined by the proteome data of the soil protein samples obtained using extraction methods with/without Nycodenz treatment (Morimoto et al., 2013). We detected the cluster proteins in the samples obtained using both extraction methods, indicating that the expression of the genes was not affected by the artificial processing. Consequently, these cluster genes were determined to represent one of the phosphate starvation response systems; however, the function of the cluster is unknown yet.

Since phosphorus is an essential element for bacteria, they express a variety of genes for survival under conditions of phosphate starvation. Among the phosphate starvation response systems, the two-component system, *phoR–phoB*, and the phosphate transport system genes,

phoU–pstBACS, have been extensively studied (Santos-Beneit, 2015). These systems work by efficiently uptaking phosphate into the cell under phosphate limitation conditions. Comparison of the component genes, *phoR–phoB* (Pput_5228 and 5229) and *phoU–pstBACS* (Pput_5233, and Pput_2167 and 5234, Pput_2166 and 5235, Pput_2165 and 5236, and Pput_2164 and 5237) with our proteome datasets from the soil extracts and MS/LB media showed that the peptide numbers in the phosphate-deficient soil extracts were larger than in the MS/LB media (**Table S4 and S5**). This shows that the phosphate transport system is functional in these bacteria and that the cluster represents a novel phosphate starvation response system. In addition, a two-component system (Pput_3038 and 3039) belonging to the Omp family is located downstream of the cluster. However, the proteome datasets suggest that expression of this two-component system might less relevant to phosphate conditions. Additional study is needed to clarify cluster function as well as its relationship with the two-component (Pput_3038 and 3039) and the phosphate transport system.

Approximately 20% of the genes in the F1 genome encode for hypothetical proteins or have unknown functions. Among these, it was expected that some might only be expressed in a soil environment. In this study, we identified seven soil-induced genes. The result indicated that the proteome analysis approach is useful and effective in obtaining genome-wide information on soil bacteria. The number of proteins detected in the cells from soil samples comprised less than half those detected from liquid media; thus, many proteins might remain that were not detected in the soil samples. The indirect extraction method using the Nycodenz density gradient, which was used to separate bacterial cells from soil prior to cell lysis, theoretically removes soil factors that might inhibit protein extraction. Some of the data obtained might be explained by the incomplete cell separation achieved by Nycodenz. A drawback of indirect extraction is the challenge of collecting cells from low bacterial biomass in soil. In our previous experiments (Morimoto et al., 2013), the required bacterial cell numbers exceeded 1×10^7 CFU g^{-1}. Therefore, in this study 3 days were considered to be a maximum incubation period for soil samples. The most serious drawback of this method is contamination of protein extracts with humic substances and impurities. Empirically, when soil contaminants are present at a high level, a protein extraction kit cannot be used. Contaminants can interfere with SDS-PAGE (e.g. causing in-gel digestion after gel loading) and can clog columns and tubes of MS/MS instruments. However, for a soil metaproteomics study, it is ideal to obtain a large number and a more diverse set of bacterial proteins. Thus, to fundamentally understand the metabolic states of the bacterial cells in soil, higher protein

purification and concentration levels should be obtained in future studies.

P. putida F1 soil-induced genes were identified and characterized using our metaproteome analysis approach. The advantage of this method, unlike the IVET method, is that the generation of many mutants is not required. Furthermore, non-sterile environmental samples, which have a high diversity and large numbers of bacteria, can be used, under conditions similar to natural environments. This makes it possible to analyze bacteria cells in diverse environments (e.g., soil, hydrosphere, plants, animals), including biological interactions. Overcoming the drawback of soil metaproteome analysis might lead to the identification of considerably more soil-induced and soil endemic genes. In addition, this method is useful not only for a single bacterial strain but for a mixture of two or several strains, which might be developed for a study of bacterial networks in soil. In the future, *in situ* soil proteomics will likely provide a deeper understanding of bacterial *in situ* functioning, lifestyle and autecology in natural habitats.

Conclusion

Two issues of long-standing importance in microbial ecology relate to the physiological and metabolic state of bacteria in the natural environment and the profile of genes that are expressed in a given environment. For a strain isolated from the environment, it is important to know which genes are expressed in the soil environment (i.e., the original habitat) to predict the microenvironment of the bacterial cell from its gene expression kinetics. We identified soil-induced genes using a comprehensive proteome-wide analysis of *P. putida* F1 inoculated into different soils. The data obtained were useful in elucidating the interactions between the bacterium and the environment and in the discovery of novel functional genes in the bacterial genome.

Conflict of interest: The authors declare no conflict of interest.

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Figure legends

Fig. 1. Strategy used for the proteome analysis of *P. putida* F1 in three different soil samples. *P. putida* F1 was grown at 30°C for 16 h in mineral salt medium supplemented with trace elements and 0.5% (w/v) glucose. The cell pellet harvested by centrifugation was resuspended in 0.8% NaCl and diluted to 1.0×10^9 CFU ml^{-1}. The cell suspension (5 ml) was inoculated into 50 g of each soil containing 0.5% (w/w) glucose in a petri dish. Non-inoculated soil

samples were used as references. The soil samples were incubated at 30°C for 3 days. The moisture content (approximately 50%) of the soil samples was gravimetrically controlled during incubation using distilled water (DW). Three biological replicates per culture condition were used.

Fig. 2. Venn diagram showing the total number of identified proteins (A) in the three soil samples, and (B) those not included in MS/LB media. The numbers of proteins detected with at least two unique peptides are indicated in each category.

Fig. 3. Comparison of the number of unique peptides of the *P. putida* F1 proteins encoded by the genes commonly expressed in the three soil samples with and without *P. putida* F1 inoculation. Bar colors: orange, soybean; green, maize; and red, forest.

Fig. 4. Number of peptides detected for the eight proteins encoded by the genes of a dipeptide transport system operon in the three soil samples (S, soybean, M, maize, and F, forest) and in MS/LB media.

Fig. 5. Homology analysis of the gene cluster commonly expressed in the three soils using Gene Cluster Search in KEGG SSDB (http://www.kegg.jp/ssdb-bin/ssdb_gclust?org_gene=ppf:Pput_3040). The number of strains in each group is indicated. White arrows show gene deletions in Pput.

Fig. 6. RT-PCR analysis of the five genes, Pput_3040–3044 genes, commonly expressed in the three soils. Total RNA obtained from F1 cells cultured to the mid-exponential phase in MS medium and then incubated for 2 h at 30°C in MS medium and distilled water (DW) was reverse transcribed. The *rpoB* gene was used as a control.

Supporting information

Table S1. Soil chemical properties and numbers of CFUs in the three samples.

Table S2. Number of detected proteins of the *P. putida* strains and *Pseudomonas* spp. among the proteins extracted from the three soil samples inoculated with/without *P. putida* F1 cells.

Table S3. Proteins identified using 1-D SDS–PAGE/LC–MS/MS in soybean, maize, and forest soil samples.

Table S4. Proteins identified using 1-D SDS–PAGE/LC–MS/MS in MS and LB media.

Table S5. List of the proteins identified in the three soil extracts media.

Fig. S1. Cell density of *P. putida* F1 during incubation in the soil samples for 3 days. Approximately 1 g soil sample was serially diluted in sterilized water. The soil suspension was inoculated onto an LB ager (1.5%) plate containing 100 μg ml^{-1} ampicillin. CFUs were counted in the samples after incubation at 30°C for 24 h. Five replicate plates were prepared for each samples.

Fig. S2. Fractionation of soil bacterial cells from the three soil samples using Nycodenz density gradient centrifugation. The incubated soil samples (12 g, wet weight) were suspended in 24 ml 0.8% NaCl and sonicated for 5 min. The soil suspensions (6 ml) were added to an equal volume of Nycodenz with a 1.3 g ml^{-1} density, followed by centrifugation at 10,000 × *g* for 40 min at 4°C. The bacterial cell layer is indicated by the blue line.

Supplementary Experimental Procedures

Soil and chemical properties

Soil samples were collected from three different regions, a soybean field at a private organic farm in Saitama, Japan (soybean soil sample), a maize field at the National Agricultural Research Center for the Kyushu Okinawa Region in Miyazaki, Japan (maize soil sample), and a sub-boreal forest at Tomakomai Experimental Forest at Hokkaido University in Hokkaido, Japan (forest soil sample). The soil samples were collected from the top 10 cm of each field. Plant material was carefully removed by hand. The soil samples were stored at 4°C until use.

The soil chemical properties measured were pH, total carbon content (C%), total nitrogen content (N%), carbon and nitrogen ratio (C/N), NH^{4+}, NO^{3-}, and PO_4^{3-}. Soil pH (H_2O) was measured with a soil-water ratio of 1:2.5. Total C and N levels were determined using an automatic, highly sensitive N-C analyzer (MT-700, Yanaco New Sci., Kyoto, Japan), equipped with an MTA-600 autosampler. The NH^{4+}, NO^{3-}, and PO_4^{3-} concentrations in the soil samples were determined using reflectoquant tests with a RQflex reflectometer (Merck, Darmstadt, Germany), following the manual provided.

Soil culture conditions of bacterial cells

P. putida F1 purchased from the American Type Culture Collection was grown at 30°C for 16 h in mineral salt (MS) medium [18.3 mM $Na_2HPO_4 \cdot 12H_2O$, 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 $CaCl_2$] (Muňoz et al., 2007) supplemented with trace elements (14.9 μM EDTA·2Na, 7.2 μM $FeSO_4 \cdot 7H_2O$, 0.35 μM $ZnSO_4 \cdot 7\ H_2O$, 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 0.5% (w/v) glucose as the sole sources of carbon and energy, with vigorous shaking. The cells were harvested by centrifugation, and washed twice using 0.8% NaCl. Finally, the cell pellet was resuspended in 0.8% NaCl and diluted to 1.0×10^9 CFU ml^{-1}. The cell suspension (5 ml) was inoculated into 50 g of each soil containing 0.5% (w/w) glucose in a petri dish. The non-inoculated soil samples were used as references. The inoculated and non-inoculated soil samples were incubated at 30°C for 3 days. The moisture content (approximately 50%) of the soil samples was gravimetrically controlled during incubation using distilled water (DW). The soil cultures experiments were performed in three independent biological replicates. Soil samples were taken from three random locations in a petri dish and then mixed for soil characterization,

viable cell count and proteome analysis.

Viable count of bacterial cells in soil

Viable cell numbers of indigenous bacteria and *P. putida* F1 in soil were determined using the dilution plate method. Soil samples were taken prior to the inoculation of F1cells to count indigenous bacteria and at 1 h (as day 0) and, 1, 2, and 3 days after the inoculation to count F1 cells. Approximately 1 g soil sample was serially diluted. The soil suspensions were inoculated onto a nutrient broth (Beckton Dickinson, Bedford, MA, USA) agar (1.5%) plate for indigenous bacteria and onto an Luria broth ager (LB; 0.5% yeast extract, 1.0% tryptone, 0.5% NaCl and 1.5% agar) plate containing ampicillin at 100 μg ml^{-1} for the F1strain. Viable cells (CFU g^{-1}) were counted in the samples after incubation of indigenous bacteria and the F1 strain at 30°C for 7 days and 24 h, respectively. Five replicate plates were prepared for each sample.

Separation of bacterial cells from soil

Bacterial cells were separated from the soil samples using Nycodenz density gradient centrifugation as previously described (Rickwood et al., 1982, Lindahl and Bakken 1995, Morimoto et al., 2013). The incubated soil samples (12 g, wet weight) were suspended in 24 ml 0.8% NaCl and sonicated for 5 min using a VS-F100 sonicator (AS One, Osaka, Japan). Next, the soil suspension was equally divided into six tubes, and 6 ml suspension was added to an equal volume of Nycodenz (Axis-Shield PoC AS, Oslo, Norway) with a 1.3 g ml^{-1} density, followed by centrifugation at 10,000 × *g* for 40 min at 4°C. The bacterial cell layer was carefully collected from the six tubes using a pipette. The bacteria cells were washed using 0.8% NaCl by centrifugation at 10,000 × *g* for 20 min at 4°C to remove the Nycodenz solution.

Media and culture conditions

MS and LB media

P. putida F1 was cultured in MS or LB medium. The cultures were incubated at 30°C with vigorous shaking (200 rpm) and the growth was monitored through OD600 measurements. The cultures were harvested by centrifugation at 6,000 × *g* when the mid-exponential phase (OD600 = 0.3) and the stationary phase (25 h of incubation) were reached. The pellets were washed twice using 0.8% (w/v) NaCl.

Soil extract medium

Soil extract (SE) media were prepared for each soil by suspending 60 g air-dried soil in 300 ml 3-(N-morpholino)-propanesulfonic acid buffer (10 mM, pH 7) and shaking at 200 rpm for 1 h (Vilain et al., 2006). The soil suspension was centrifuged at 10,000 × *g* for 20 min at 4°C.The extract was filtered sequentially through 3.0-, 0.45-, and 0.2-μm mixed cellulose ester-type membrane filters (Advantec, Tokyo, Japan) to remove soil particles and bacteria cells. The *P. putida* F1 stain was grown at 30°C with vigorous shaking in the SE media supplemented with 0.5% glucose. The bacteria cells were harvested at the mid-exponential phase by centrifugation. The pellets were washed twice using 0.8% (w/v) NaCl.

Extraction of bacterial proteins.

Protein extraction from the soil bacterial pellets was performed using a modified protocol described by Wang *et al* (2006). A soil pellet was washed sequentially using 1 ml 10% trichloroacetic acid/acetone, 0.1 M ammonium acetate/80% methanol, and 80% acetone in a 2-ml microtube. After the sample was dried by evaporation to remove the residual acetone, 0.5 ml SDS buffer [30% sucrose, 2% SDS, 0.1 M Tris-Cl (pH 8.0), and 5% *β*-mercaptoethanol] and 0.5 ml phenol (pH 8.0) were added and the tube was shaken for 30 min. After centrifugation at 8,000 × *g* for 10 min, the upper phenol phase was transferred to a fresh tube. The SDS-phenol extraction step was repeated twice. To wash the phenol phase (1 ml), an equal volume of 1 mM Tris-HCl (pH 8.0) was added, and the mixture was shaken for 10 min and centrifuged at 8,000 × *g* for 10 min; this washing step was repeated twice. The phenol phase (0.8 ml) was added to one-third volume of 100% ethanol and two volumes of 100% isopropanol. The solution was mixed thoroughly and stored at −20°C overnight to precipitate the proteins. The phenol solution was centrifuged at 12,000 × *g* for 15 minutes at 4°C. The protein pellet was washed once using 2.0 ml 0.1 M ammonium acetate/methanol and once using 2.0 ml 80% acetone and then air-dried. Finally, the protein was dissolved in a UTC buffer [7 M urea, 2 M tiourea, 2% CHAPS, and 0.1 M Tris-HCl (pH 6.8)]. The F1 cell pellets that were harvested from the liquid cultures were lysed using the ReadyPrep Protein Extraction Kit (Total Protein) (Bio-Rad Laboratories, Hercules, CA, USA).

The protein concentrations of all samples were measured using the Protein Assay Kit (Bio-Rad Laboratories).

555 *Trypsin in-gel proteolysis and nanoLC-MS/MS analysis*

556 Proteome analysis was performed as previously described (Kasahara et al., 2012).
557 Proteins (50 μg) were separated using 12.5% SDS–PAGE and stained using Coomassie
558 brilliant blue. The gel lanes were cut into 60 strips of ~1 mm. The gel strips were completely
559 de-stained using 30% acetonitrile (ACN) in 25 mM NH_4HCO_3, reduced using 10 mM
560 dithiothreitol, and alkylated using 55 mM iodoacetamide. After the gel strips were completely
561 dried, the proteins were digested using 40 μl sequencing-grade modified trypsin (12.5 ng μl^{-1}
562 in 50 mM NH_4HCO_3) at 37°C overnight. The digested peptides were extracted once using 25
563 mM NH_4HCO_3 in 60% ACN and twice using 5% formic acid in 70% ACN.

564 Nano-liquid chromatography-electrospray ionization-tandem mass spectrometry
565 (nanoLC–ESI–MS/MS) analysis of the peptide mixtures was performed using an LTQ
566 ion-trap MS (Thermo Fisher Scientific, Yokohama, Japan) coupled with a multidimensional
567 HPLC Paradigm MS2 (AMR Inc., Tokyo, Japan) and a nano-spray electrospray ionization
568 device (Michrom Bioresources Inc., Aubum, CA, USA). The tryptic peptides were loaded
569 onto an L-column2 ODS (Chemicals Evaluation & Research Inst., Tokyo, Japan) packed with
570 C18 modified silica particles (5 μm, 12-nm pore size) and separated by a linear gradient of
571 15–65% buffer B for 40 min, followed by a gradient of 65–95% buffer B for 1 min (buffer B:
572 90% methanol and 0.1% formic acid in H_2O) at a flow rate of 1 μl min^{-1}. Peptide spectra were
573 recorded in a mass range of m/z 450–1,800. MS/MS spectra were acquired in a
574 data-dependent scan mode. After completing the full spectrum scan, the MS/MS spectra of the
575 most intense individual peaks were also collected. The dynamic exclusion features were set as
576 follows: a repeat count of one within 30 s, exclusion duration of 180 s, and an exclusion list
577 size of 50.

578

579 *Protein identification*

580 The MS/MS data obtained were searched against a database using Mascot ver. 2.4
581 (Matrix Science, London, UK), on an in-house server to identify proteins. The protein
582 databases used were the *P. putida* F1 (NC_009512) sequence and all completed bacterial
583 genomes (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/all.faa.tar.gz) in NCBI. The search
584 parameters were set as follows: tryptic digest with a maximum of two missed cleavage sites;
585 fixed modifications, carbamidomethyl cysteine; variable modifications, methionine oxidation;
586 peptide masses, monoisotopic, positive charge (+1, +2, +3) of the peptide; and mass tolerance
587 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 8.9%. Proteins were identified with more than two unique peptide-filtering criteria.

Response to NO

The NO-releasing compound used was 1-Hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12) (Dojindo, Kumamoto, Japan). Stock solutions (10 mM) were freshly prepared in 0.1 M NaOH. The F1 strain was cultured in MS medium supplemented with the trace elements and 0.5% glucose containing 0.1 mM NOC12. The medium without NOC12 was used as a control. The cultures were incubated at 30°C with vigorous shaking and harvested at the mid-exponential phase (OD600 = 0.3). The pellets were washed using 0.8% NaCl, and lysed using the ReadyPrep Protein Extraction Kit (Total Protein) (Bio-Rad Laboratories).

Expression factor analysis for the cluster of soil-induced genes

Culture condition

The F1 strain was cultured at 30°C with vigorous shaking to the mid-exponential phase (OD600 = 0.3) and centrifuged at 6,000 × *g* for 5 min. The cell pellet was resuspended and transferred to the original MS medium, modified MS media without $MgSO_4$, $(NH_4)_2SO_4$, SO_4^{2-} [$MgSO_4$ and $(NH_4)_2SO_4$], $CaCl_2$, PO_4^{3-} (KH_2PO_4 and Na_2HPO_4), or trace element, respectively, 0.8% NaCl, and sterile DW. The cell suspensions were incubated for 2 h at 30°C, and the cells were harvested by centrifugation. The cell pellets were used for proteome analysis and reverse transcription PCR (RT-PCR) experiments.

Proteome analysis

After separating the F1 cellular proteins (50 μg) using 12.5% SDS-PAGE and staining with Coomassie, the gel was cut into seven and four strips corresponding to the protein ranges of 20–30 and 40–55 kDa, respectively.

RT–PCR analysis

Total RNA from F1 cells incubated in MS medium or DW was extracted using Isogen II (Nippon Gene Co., Ltd., Tokyo, Japan), with the addition of a DNase treatment step, using 10 U DNase (TaKaRa Bio, Otsu, Japan) for 30 min at 37°C. RT-PCR was performed with the

RNA samples using the SuperScript III First-Strand Synthesis System (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. The following gene sequences were amplified using specific sets of forward and reverse primers: Pput_3040 (160 bp), 5'-TTGGACCAGGCAGGCAGC-3' and 5'-TCAAGGGTTCAGGTGTGC-3'; Pput_3041 (158 bp), 5'-CTGGAGCTGGCTGAACAG-3' and 5'-TCGATGACATGTTCGCGCC-3'; Pput_3042 (156 bp), 5'- GTCAGCCTGGACAGCTAC-3' and 5'-GTGGCCGTACTCCTCTTC-3'; Pput_3043 (150 bp), 5'-GCAGCGTTACACCTACCG-3' and 5'-GCGCGTTCGGCGAACAGC-3' and Pput_3044 (154 bp), 5'-GATGTGCAGCATTACCTG-3' and 5'-GGTTACCCGTGAAACAGC-3'. DNA-directed RNA polymerase subunit beta (*rpoB*, Pput_0480) was used as a control, with the primers 5'-CCGGACGTCATGGATGTG-3' and 5'-CTCCAGGGCAGCATTGCC-3'. The RT-PCR products were separated and visualized using 2.0% agarose gel electrophoresis. The experiment was repeated three times.

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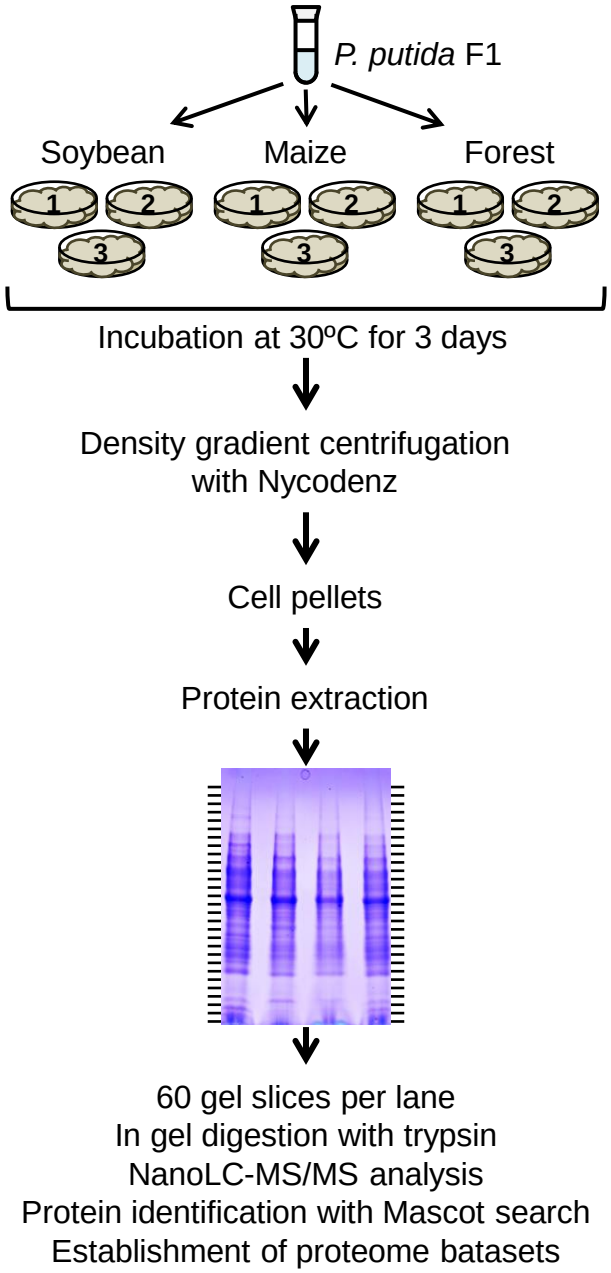

Fig. 1. Strategy used for the proteome analysis of *P. putida* F1 in three different soil samples. *P. putida* F1 was grown at 30°C for 16 h in mineral salt medium supplemented with trace elements and 0.5% (w/v) glucose. The cell pellet harvested by centrifugation was resuspended in 0.8% NaCl and diluted to 1.0×10^9 CFU ml^{-1}. The cell suspension (5 ml) was inoculated into 50 g of each soil containing 0.5% (w/w) glucose in a petri dish. Non-inoculated soil samples were used as references. The soil samples were incubated at 30°C for 3 days. The moisture content (approximately 50%) of the soil samples was gravimetrically controlled during incubation using distilled water (DW). Three biological replicates per culture condition were used.

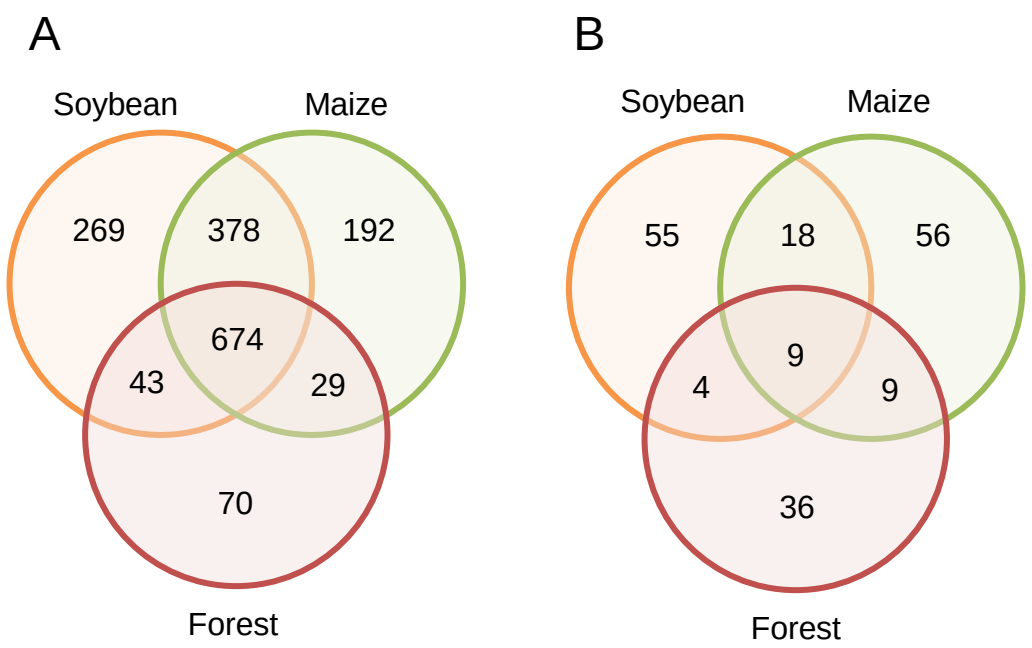

Fig. 2. Venn diagram showing the total number of identified proteins (A) in the three soil samples, and (B) those not included in MS/LB media. The numbers of proteins detected with at least two unique peptides are indicated in each category.

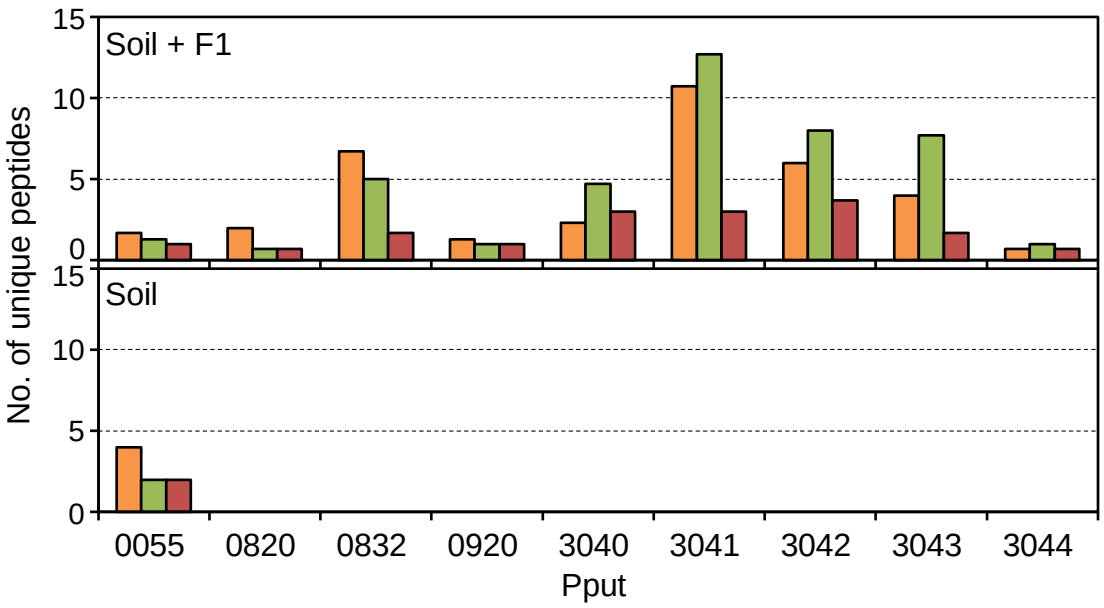

Fig. 3. Comparison of the number of unique peptides of the *P. putida* F1 proteins encoded by the genes commonly expressed in the three soil samples with and without *P. putida* F1 inoculation. Bar colors: orange, soybean; green, maize; and red, forest.

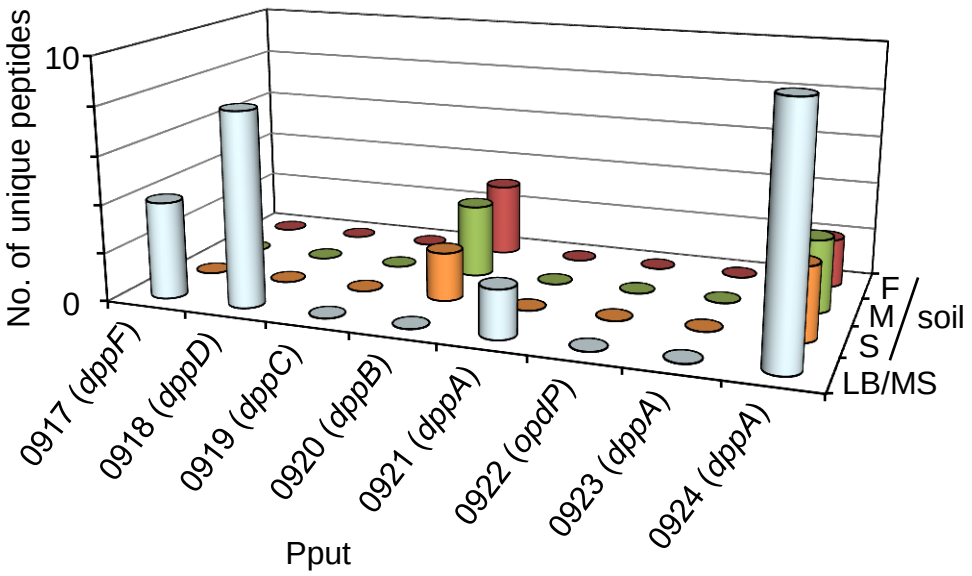

Fig. 4. Number of peptides detected for the eight proteins encoded by the genes of a dipeptide transport system operon in the three soil samples (S, soybean, M, maize, and F, forest) and in MS/LB media.

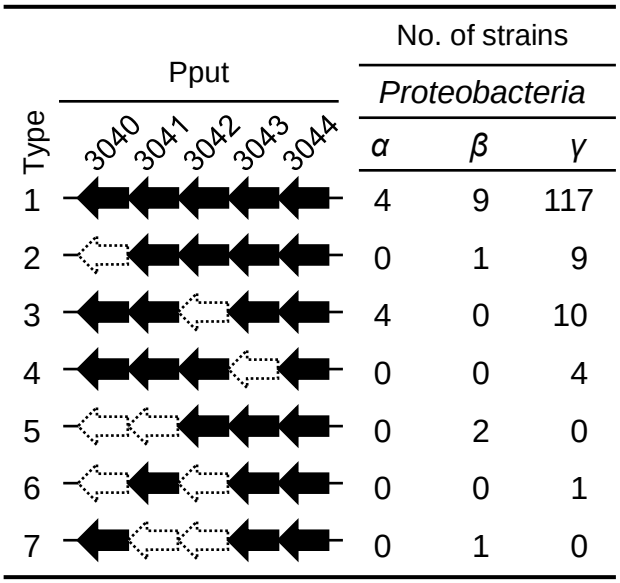

Fig. 5. Homology analysis of the gene cluster commonly expressed in the three soils using Gene Cluster Search in KEGG SSDB (http://www.genome.jp/). The number of strains in each group is indicated. White arrows show gene deletions in Pput.

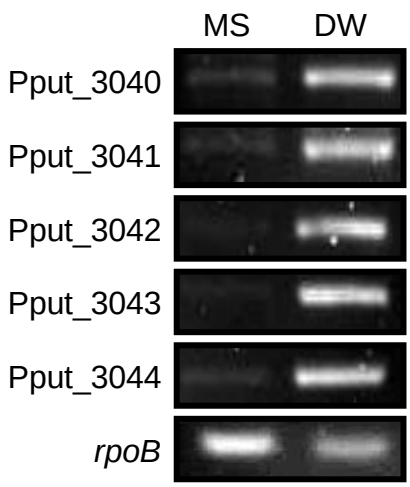

Fig. 6. RT-PCR analysis of the five genes, Pput_3040–3044 genes, commonly expressed in the three soils. Total RNA obtained from F1 cells cultured to the mid-exponential phase in MS medium and then incubated for 2 h at 30° C in MS medium and distilled water (DW) was reverse transcribed. The *rpoB* gene was used as a control.

Table 1

Table 1. The number of detected proteins of P. putida F1 in three soil samples and two liquid media.

Number of detected proteins							
Soil culture					Liquid culture		
	Soybean	Maize	Forest				
Exp. #1	1053	1039	626		MS	log	1878
Exp. #2	915	855	450			sta	2036
Exp. #3	863	727	421		LB	log	1547
						sta	2003
No. of nr protein	1364	1273	816				2643

Abbreviations: Exp, experiment; MS, mineral salt;.nr, nonredundant; log, logarithmic phase; sta, stationary phase.

Table 2

Table 2. List of P. putida F1 genes commonly expressed in three soils.

Locus tag	Length (nt)	MW (kDa)	pI	Functional annotation	Localization (SOSUI)[a]
Pput_0055	1998	70.5	6.4	Heavy metal translocating P-type ATPase	Inner membrane
Pput_0820	477	17.5	6.0	Aldehyde dehydrogenase	Cytoplasmic
Pput_0832	1179	43.3	5.4	Nitric oxide dioxygenase	Cytoplasmic
Pput_0920	1011	37.0	7.9	Dipeptide transport system permease	Inner membrane
Pput_3040	642	23.5	8.7	Hypothetical protein	Periplasm
Pput_3041	807	29.4	9.4	Short chain dehydrogenase	Cytoplasmic
Pput_3042	681	25.3	5.0	Hypothetical protein	Cytoplasmic
Pput_3043	1470	53.1	5.5	AMP-dependent synthetase and ligase	Cytoplasmic
Pput_3044	681	24.8	6.1	Hypothetical protein	Inner membrane

[a] http://bp.nuap.nagoya-u.ac.jp/sosui (Hirokawa et al., 1998)

Table 3

Table 3. The number of peptide of the six proteins encoded by the genes commonly expressed in three soils in incubating in each of three soil extract media.

	Number of unique peptides[a]		
Locus tag	Soybean	Maize	Forest
Pput_0832	0 ± 0.0	0 ± 0.0	0 ± 0.0
Pput_3040	3.3 ± 0.6	5.0 ± 0.0	11.3 ± 4.0
Pput_3041	9.0 ± 3.5	15.0 ± 1.5	18.7 ± 0.6
Pput_3042	5.7 ± 1.2	8.7 ± 0.6	10.3 ± 1.2
Pput_3043	3.3 ± 2.5	7.0 ± 3.0	12.7 ± 4.0
Pput_3044	2.0 ± 1.0	2.7 ± 0.6	3.7 ± 0.6

[a]The values are the means and standard deviations of three independent experiments.

Table 4

Table 4. The number of peptides of the proteins encoded by the genes commonly expressed in three soils under condition incubated in various of media..

	Number of unique peptides								
	free MS medium								
Locus tag	MS[a]	$MgSO_4$	$(HN_4)_2SO_4$	SO_4^{2-b}	$CaCl_2$	PO_4^{3-c}	TE	DW[a]	0.8% NaCl
Pput_3040	0 ± 0	0	0	0	0	4	0	5.5 ± 1.8	3
Pput_3041	0 ± 0	0	0	0	0	2	0	6.3 ± 2.8	4
Pput_3042	0 ± 0	0	0	0	0	3	0	5.5 ± 2.0	5
Pput_3043	0 ± 0	0	0	0	0	1	0	2.3 ± 1.1	4
Pput_3044	0 ± 0	0	0	0	0	2	0	4.0 ± 2.5	2
RpoB[d]	13.3 ± 3.8	17	18	7	22	22	19	18.0 ± 5.6	16
SucC[e]	14.3 ± 7.9	9	14	8	13	13	10	18.0 ± 7.5	9

[a]The values are the means and standard deviations of three independent expreriments. [b]$MgSO_4$ and $(HN_4)_2SO_4$, [c]KH_2PO_4 and Na_2HPO_4, [d]DNA-directed RNA polymerase subunit beta, rpoB gene, [e]Succinyl-CoA synthetase subunit beta, sucC gene, Abbreviations: MS, mineral salt; TE, trace element; DW, distilled water.

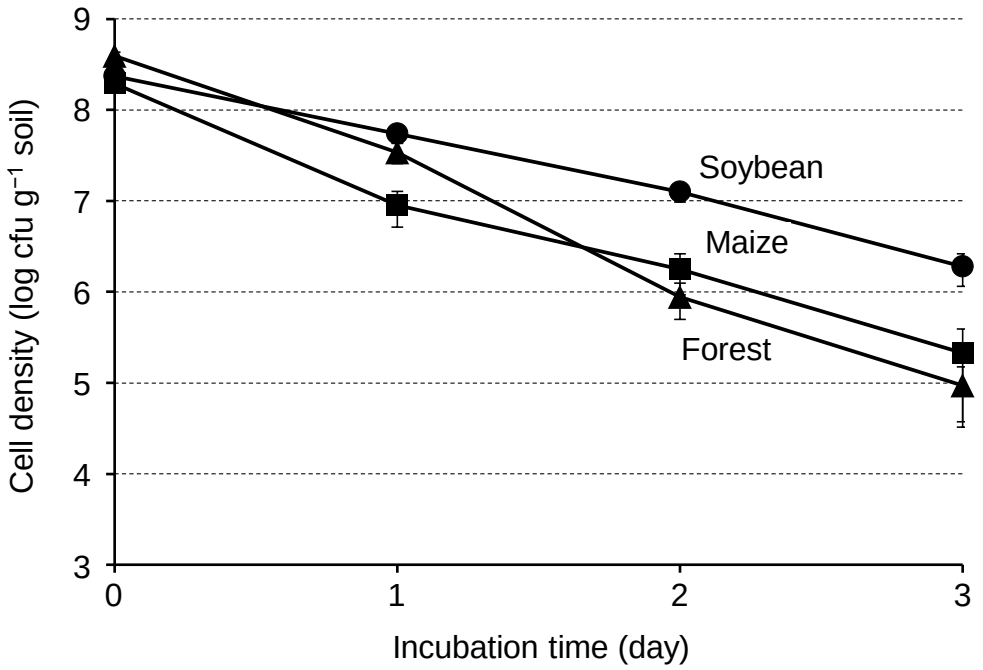

Fig. S1. Cell density of *P. putida* F1 during incubation in the soil samples for 3 days. Approximately 1 g soil sample was serially diluted in sterilized water. The soil suspension was inoculated onto an LB ager (1.5%) plate containing 100 μg ml^{-1} ampicillin. CFUs were counted in the samples after incubation at 30°C for 24 h. Five replicate plates were prepared for each samples.

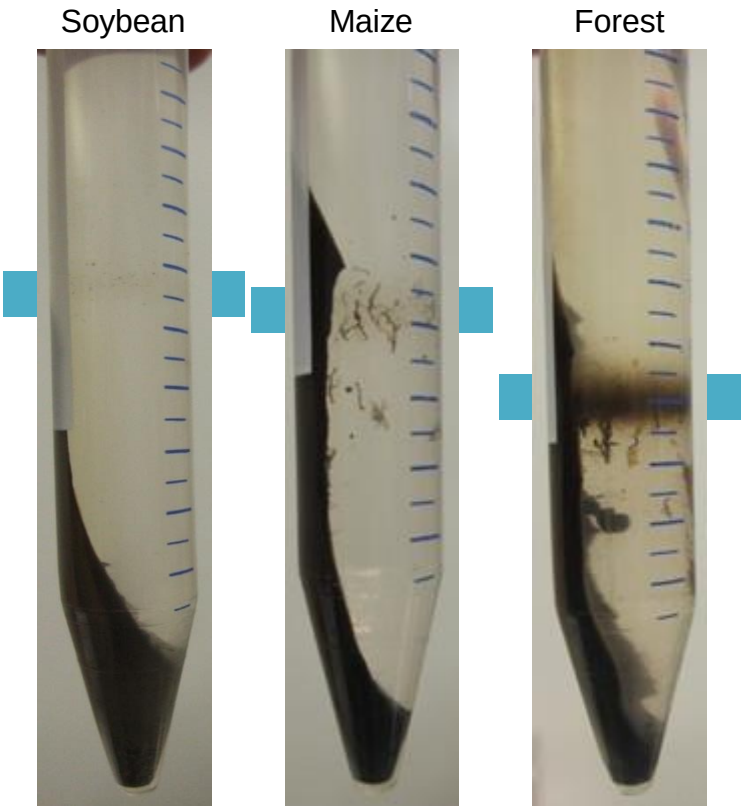

Fig. S2. Fractionation of soil bacterial cells from the three soil samples using Nycodenz density gradient centrifugation. The incubated soil samples (12 g, wet weight) were suspended in 24 ml 0.8% NaCl and sonicated for 5 min. The soil suspensions (6 ml) were added to an equal volume of Nycodenz with a 1.3 g ml^{-1} density, followed by centrifugation at 10,000 × *g* for 40 min at 4°C. The bacterial cell layer is indicated by the blue line.

Table S1

Table S1. Soil chemical properties and numbers of CFUs in the three samples.

Soil sample	pH	C%	N%	C/N	NO_3^- (µg g^{-1} soil)	NH_4^+(µg g^{-1} soil)	PO_4^{3-} (µg g^{-1} soil)	CFU[a]
Soybean	5.3	2.73	0.26	10.5	18.8 ± 2.5	< 3[b]	< 0.2[b]	5.0 ± 0.3 x 10^8
Maize	5.3	4.72	0.38	12.4	24.2 ± 2.3	< 3[b]	< 0.2[b]	2.5 ± 0.8 x 10^7
Forest	5.2	12.62	0.83	15.2	45.8 ± 3.7	< 3[b]	< 0.2[b]	2.3 ± 0.4 x 10^9

[a]The values are the means and standard deviations of five replicate plates. [b]Below the delection limit using reflectoquant tests and RQ flex reflectometer. Abbreviations: C%, total carbon content; N%, total nitrogen content.

Table S2

Table S2. The number of detected protein of P. putida strains and Pseudomonas spp. among the proteins extracted from the three soil samples inoculated with/without P. putida F1 cell.

	No. of detected proteins (%)					
	Soybean		Maize		Forest	
F1 strain	-	+	-	+	-	+
Pseudomonas putida	2 (0.2)	634 (60.7)	1 (0.2)	396 (47.7)	0 (0.0)	230 (36.7)
Psudomonas spp.	14 (1.2)	70 (6.7)	1 (0.2)	61 (7.3)	2 (0.5)	47 (7.5)
Other bacteria	1044 98.6)	340 (32.6)	559 (99.6)	373 (44.9)	401 (99.5)	349 (55.8)
Total	1156 (100)	1044 (100)	561 (100)	830 (100)	403 (100)	626 (100)