



Title	Proteome analysis of <i>Pseudomonas putida</i> F1 genes induced in soil environments
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## 1 **Supplementary Experimental Procedures**

### 2 *Soil and chemical properties*

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

10 The soil chemical properties measured were pH, total carbon content (C%), total  
11 nitrogen content (N%), carbon and nitrogen ratio (C/N),  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  $\text{PO}_4^{3-}$ . Soil  
12 pH ( $\text{H}_2\text{O}$ ) was measured with a soil-water ratio of 1:2.5. Total C and N levels were  
13 determined using an automatic, highly sensitive N-C analyzer (MT-700, Yanaco New  
14 Sci., Kyoto, Japan), equipped with an MTA-600 autosampler. The  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and  
15  $\text{PO}_4^{3-}$  concentrations in the soil samples were determined using reflectoquant tests with  
16 a RQflex reflectometer (Merck, Darmstadt, Germany), following the manual provided.

17

### 18 *Soil culture conditions of bacterial cells*

19 *P. putida* F1 purchased from the American Type Culture Collection was grown at  
20 30°C for 16 h in mineral salt (MS) medium [18.3 mM  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 11.2 mM  
21  $\text{KH}_2\text{PO}_4$ , 4.8 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.3 mM  $\text{CaCl}_2$ ] (Muñoz et al.,  
22 2007) supplemented with trace elements (14.9  $\mu\text{M}$   $\text{EDTA} \cdot 2\text{Na}$ , 7.2  $\mu\text{M}$   $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  
23 0.35  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.15  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 4.9  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 0.84  $\mu\text{M}$   $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  
24 0.06  $\mu\text{M}$   $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.08  $\mu\text{M}$   $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , and 0.14  $\mu\text{M}$   $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ) and 0.5%  
25 (w/v) glucose as the sole sources of carbon and energy, with vigorous shaking. The cells  
26 were harvested by centrifugation, and washed twice using 0.8% NaCl. Finally, the cell  
27 pellet was resuspended in 0.8% NaCl and diluted to  $1.0 \times 10^9$  CFU  $\text{ml}^{-1}$ . The cell  
28 suspension (5 ml) was inoculated into 50 g of each soil containing 0.5% (w/w) glucose  
29 in a petri dish. The non-inoculated soil samples were used as references. The inoculated  
30 and non-inoculated soil samples were incubated at 30°C for 3 days. The moisture  
31 content (approximately 50%) of the soil samples was gravimetrically controlled during

32 incubation using distilled water (DW). The soil cultures experiments were performed in  
33 three independent biological replicates. Soil samples were taken from three random  
34 locations in a petri dish and then mixed for soil characterization, viable cell count and  
35 proteome analysis.

36

#### 37 *Viable count of bacterial cells in soil*

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

48

#### 49 *Separation of bacterial cells from soil*

50 Bacterial cells were separated from the soil samples using Nycodenz density  
51 gradient centrifugation as previously described (Rickwood et al., 1982, Lindahl and  
52 Bakken 1995, Morimoto et al., 2013). The incubated soil samples (12 g, wet weight)  
53 were suspended in 24 ml 0.8% NaCl and sonicated for 5 min using a VS-F100 sonicator  
54 (AS One, Osaka, Japan). Next, the soil suspension was equally divided into six tubes,  
55 and 6 ml suspension was added to an equal volume of Nycodenz (Axis-Shield PoC AS,  
56 Oslo, Norway) with a 1.3  $\text{g ml}^{-1}$  density, followed by centrifugation at 10,000  $\times g$  for  
57 40 min at 4°C. The bacterial cell layer was carefully collected from the six tubes using a  
58 pipette. The bacteria cells were washed using 0.8% NaCl by centrifugation at 10,000  $\times$   
59  $g$  for 20 min at 4°C to remove the Nycodenz solution.

60

#### 61 *Media and culture conditions*

##### 62 *MS and LB media*

63 *P. putida* F1 was cultured in MS or LB medium. The cultures were incubated at  
64 30°C with vigorous shaking (200 rpm) and the growth was monitored through OD600  
65 measurements. The cultures were harvested by centrifugation at 6,000 × *g* when the  
66 mid-exponential phase (OD600 = 0.3) and the stationary phase (25 h of incubation)  
67 were reached. The pellets were washed twice using 0.8% (w/v) NaCl.

68

#### 69 *Soil extract medium*

70 Soil extract (SE) media were prepared for each soil by suspending 60 g air-dried  
71 soil in 300 ml 3-(N-morpholino)-propanesulfonic acid buffer (10 mM, pH 7) and  
72 shaking at 200 rpm for 1 h (Vilain et al., 2006). The soil suspension was centrifuged at  
73 10,000 × *g* for 20 min at 4°C. The extract was filtered sequentially through 3.0-, 0.45-,  
74 and 0.2-μm mixed cellulose ester-type membrane filters (Advantec, Tokyo, Japan) to  
75 remove soil particles and bacteria cells. The *P. putida* F1 strain was grown at 30°C with  
76 vigorous shaking in the SE media supplemented with 0.5% glucose. The bacteria cells  
77 were harvested at the mid-exponential phase by centrifugation. The pellets were washed  
78 twice using 0.8% (w/v) NaCl.

79

#### 80 *Extraction of bacterial proteins.*

81 Protein extraction from the soil bacterial pellets was performed using a modified  
82 protocol described by Wang *et al* (2006). A soil pellet was washed sequentially using 1  
83 ml 10% trichloroacetic acid/acetone, 0.1 M ammonium acetate/80% methanol, and 80%  
84 acetone in a 2-ml microtube. After the sample was dried by evaporation to remove the  
85 residual acetone, 0.5 ml SDS buffer [30% sucrose, 2% SDS, 0.1 M Tris-Cl (pH 8.0), and  
86 5% β-mercaptoethanol] and 0.5 ml phenol (pH 8.0) were added and the tube was shaken  
87 for 30 min. After centrifugation at 8,000 × *g* for 10 min, the upper phenol phase was  
88 transferred to a fresh tube. The SDS-phenol extraction step was repeated twice. To wash  
89 the phenol phase (1 ml), an equal volume of 1 mM Tris-HCl (pH 8.0) was added, and  
90 the mixture was shaken for 10 min and centrifuged at 8,000 × *g* for 10 min; this  
91 washing step was repeated twice. The phenol phase (0.8 ml) was added to one-third  
92 volume of 100% ethanol and two volumes of 100% isopropanol. The solution was  
93 mixed thoroughly and stored at -20°C overnight to precipitate the proteins. The phenol

94 solution was centrifuged at  $12,000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ . The protein pellet was  
95 washed once using 2.0 ml 0.1 M ammonium acetate/methanol and once using 2.0 ml  
96 80% acetone and then air-dried. Finally, the protein was dissolved in a UTC buffer [7 M  
97 urea, 2 M thiourea, 2% CHAPS, and 0.1 M Tris-HCl (pH 6.8)]. The F1 cell pellets that  
98 were harvested from the liquid cultures were lysed using the ReadyPrep Protein  
99 Extraction Kit (Total Protein) (Bio-Rad Laboratories, Hercules, CA, USA).

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

102

### 103 *Trypsin in-gel proteolysis and nanoLC-MS/MS analysis*

104 Proteome analysis was performed as previously described (Kasahara et al., 2012).  
105 Proteins (50  $\mu\text{g}$ ) were separated using 12.5% SDS-PAGE and stained using Coomassie  
106 brilliant blue. The gel lanes were cut into 60 strips of  $\sim 1$  mm. The gel strips were  
107 completely de-stained using 30% acetonitrile (ACN) in 25 mM  $\text{NH}_4\text{HCO}_3$ , reduced  
108 using 10 mM dithiothreitol, and alkylated using 55 mM iodoacetamide. After the gel  
109 strips were completely dried, the proteins were digested using 40  $\mu\text{l}$  sequencing-grade  
110 modified trypsin (12.5  $\text{ng } \mu\text{l}^{-1}$  in 50 mM  $\text{NH}_4\text{HCO}_3$ ) at  $37^{\circ}\text{C}$  overnight. The digested  
111 peptides were extracted once using 25 mM  $\text{NH}_4\text{HCO}_3$  in 60% ACN and twice using 5%  
112 formic acid in 70% ACN.

113 Nano-liquid chromatography-electrospray ionization-tandem mass spectrometry  
114 (nanoLC-ESI-MS/MS) analysis of the peptide mixtures was performed using an LTQ  
115 ion-trap MS (Thermo Fisher Scientific, Yokohama, Japan) coupled with a  
116 multidimensional HPLC Paradigm MS2 (AMR Inc., Tokyo, Japan) and a nano-spray  
117 electrospray ionization device (Michrom Bioresources Inc., Auburn, CA, USA). The  
118 tryptic peptides were loaded onto an L-column2 ODS (Chemicals Evaluation &  
119 Research Inst., Tokyo, Japan) packed with C18 modified silica particles (5  $\mu\text{m}$ , 12-nm  
120 pore size) and separated by a linear gradient of 15–65% buffer B for 40 min, followed  
121 by a gradient of 65–95% buffer B for 1 min (buffer B: 90% methanol and 0.1% formic  
122 acid in  $\text{H}_2\text{O}$ ) at a flow rate of  $1 \mu\text{l min}^{-1}$ . Peptide spectra were recorded in a mass range  
123 of  $m/z$  450–1,800. MS/MS spectra were acquired in a data-dependent scan mode. After  
124 completing the full spectrum scan, the MS/MS spectra of the most intense individual

125 peaks were also collected. The dynamic exclusion features were set as follows: a repeat  
126 count of one within 30 s, exclusion duration of 180 s, and an exclusion list size of 50.

127

### 128 *Protein identification*

129 The MS/MS data obtained were searched against a database using Mascot ver. 2.4  
130 (Matrix Science, London, UK), on an in-house server to identify proteins. The protein  
131 databases used were the *P. putida* F1 (NC\_009512) sequence and all completed  
132 bacterial genomes (<ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/all.faa.tar.gz>) in NCBI.  
133 The search parameters were set as follows: tryptic digest with a maximum of two  
134 missed cleavage sites; fixed modifications, carbamidomethyl cysteine; variable  
135 modifications, methionine oxidation; peptide masses, monoisotopic, positive charge (+1,  
136 +2, +3) of the peptide; and mass tolerance of 1.2 Da for the precursor ion and 0.8 Da for  
137 product ions. To assess false-positive identifications, an automatic decoy search was  
138 performed against a randomized database with a default significance threshold of  $P <$   
139 0.05; the false discovery rate at the identity threshold was below 8.9%. Proteins were  
140 identified with more than two unique peptide-filtering criteria.

141

### 142 *Response to NO*

143 The NO-releasing compound used was  
144 1-Hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12) (Dojindo,  
145 Kumamoto, Japan). Stock solutions (10 mM) were freshly prepared in 0.1 M NaOH.  
146 The F1 strain was cultured in MS medium supplemented with the trace elements and  
147 0.5% glucose containing 0.1 mM NOC12. The medium without NOC12 was used as a  
148 control. The cultures were incubated at 30°C with vigorous shaking and harvested at the  
149 mid-exponential phase ( $OD_{600} = 0.3$ ). The pellets were washed using 0.8% NaCl, and  
150 lysed using the ReadyPrep Protein Extraction Kit (Total Protein) (Bio-Rad  
151 Laboratories).

152

### 153 *Expression factor analysis for the cluster of soil-induced genes*

#### 154 *Culture condition*

155 The F1 strain was cultured at 30°C with vigorous shaking to the mid-exponential

156 phase (OD600 = 0.3) and centrifuged at  $6,000 \times g$  for 5 min. The cell pellet was  
157 resuspended and transferred to the original MS medium, modified MS media without  
158  $\text{MgSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{SO}_4^{2-}$  [ $\text{MgSO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$ ],  $\text{CaCl}_2$ ,  $\text{PO}_4^{3-}$  ( $\text{KH}_2\text{PO}_4$  and  
159  $\text{Na}_2\text{HPO}_4$ ), or trace element, respectively, 0.8% NaCl, and sterile DW. The cell  
160 suspensions were incubated for 2 h at  $30^\circ\text{C}$ , and the cells were harvested by  
161 centrifugation. The cell pellets were used for proteome analysis and reverse  
162 transcription PCR (RT-PCR) experiments.

163

#### 164 *Proteome analysis*

165 After separating the F1 cellular proteins (50  $\mu\text{g}$ ) using 12.5% SDS-PAGE and  
166 staining with Coomassie, the gel was cut into seven and four strips corresponding to the  
167 protein ranges of 20–30 and 40–55 kDa, respectively.

168

#### 169 *RT-PCR analysis*

170 Total RNA from F1 cells incubated in MS medium or DW was extracted using  
171 Isogen II (Nippon Gene Co., Ltd., Tokyo, Japan), with the addition of a DNase  
172 treatment step, using 10 U DNase (TaKaRa Bio, Otsu, Japan) for 30 min at  $37^\circ\text{C}$ .  
173 RT-PCR was performed with the RNA samples using the SuperScript III First-Strand  
174 Synthesis System (Life Technologies, Tokyo, Japan) according to the manufacturer's  
175 instructions. The following gene sequences were amplified using specific sets of  
176 forward and reverse primers: Pput\_3040 (160 bp), 5'-TTGGACCAGGCAGGCAGC-3'  
177 and 5'-TCAAGGGTTCAGGTGTGC-3'; Pput\_3041 (158 bp),  
178 5'-CTGGAGCTGGCTGAACAG-3' and 5'-TCGATGACATGTTCGCGCC-3';  
179 Pput\_3042 (156 bp), 5'-GTCAGCCTGGACAGCTAC-3' and  
180 5'-GTGGCCGTACTCCTCTTC-3'; Pput\_3043 (150 bp),  
181 5'-GCAGCGTTACACCTACCG-3' and 5'-GCGCGTTCGGCGAACAGC-3' and  
182 Pput\_3044 (154 bp), 5'-GATGTGCAGCATTACCTG-3' and  
183 5'-GGTTACCCGTGAAACAGC-3'. DNA-directed RNA polymerase subunit beta  
184 (*rpoB*, Pput\_0480) was used as a control, with the primers  
185 5'-CCGGACGTCATGGATGTG-3' and 5'-CTCCAGGGCAGCATTGCC-3'. The  
186 RT-PCR products were separated and visualized using 2.0% agarose gel electrophoresis.

187 The experiment was repeated three times.

188

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