Supporting Information

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SI Materials and Methods

Materials. [1-^{14}C]phenylacetic acid and L-[U-^{14}C]phenylalanine were purchased from American Radiolabeled Chemicals and GE Healthcare, respectively. L-[U-^{13}C,^{15}N]phenylalanine was obtained from Spectra. Oxygen-^{18}O (normalized, > 97 atom%) and water-^{18}O (normalized with respect to hydrogen, > 97 atom%) were obtained from Campro Scientific. Other chemicals were obtained from Sigma-Aldrich, Merck, Applichem, Roth, or Gerbu. Materials for cloning and expression were purchased from MBI Fermentas, Novagen, Genaxxon, BioScience, biomers.net, or Qiagen.

Molecular Biological Techniques. The genes were amplified by PCR from chromosomal DNA of either *Pseudomonas* sp. strain Y2 or *Escherichia coli* K12. The PCR products were isolated and cloned into the expression vectors pMEKm12 (1), pMAL-c2× (New England Biolabs), pEXP5-CT/TOPO (Invitrogen), or pET16b (Novagen). Recombinant proteins were tagged with maltose-binding protein (mbp), his_6, or his_{10}. All genes of *Pseudomonas* sp. strain Y2 were cloned from the *paa1* cluster; note that this organism harbors two functional *paa* gene clusters (2). An overview of all cloned genes is shown in Table 2.

Bacterial Strains and Growth Conditions. For overproduction of heterologous proteins, transformed *E. coli* DH5α cells (pMEKm12 and pMAL-c2× plasmids) and *E. coli* BL21 (DE3) cells (pET16b and pEXP5-CT/TOPO plasmids) were grown at 37 °C on lysogeny broth medium (3) with 100 μg of ampicillin mL^{-1} and 50 μg of kanamycin mL^{-1} (pMEKm12 plasmids) up to an OD_{578} of 0.6 before induction with 0.5 mM isopropyl-thiogalactopyranoside. The temperature was lowered to 30 °C; after 3 h additional growth, the cells were harvested and stored at −20 °C.

Enzyme Purification. The purifications were performed aerobically at 10 °C. For the preparation of cell extracts for protein purification, one weight part of *E. coli* cells was suspended in one volume part of 20 mM Tris-HCl (pH 8.0) containing 0.1 mg of DNase I mL^{-1} and 100 mM KCl (PaaG overproduction) or 200 mM KCl (PaaABC(D)E, PaaD, PaaZ, PaaH, PaaJ, and PaaF overproduction). The suspension was passed twice through a cooled French pressure cell at 137 MPa and centrifuged (100,000 × *g*) at 4 °C for 1 h. The supernatant (cell extract) was applied directly to an amylose resin column or to a his-trap HP column, respectively. The sources of the enzymes are given in Table S2. Normally, the *Pseudomonas* enzymes were studied. *E. coli* enzymes were chosen when the production of the *Pseudomonas* enzymes was difficult.

Purification of mbp-tagged proteins PaaABC(D)E, PaaD, PaaZ, and PaaH. The cell extract was applied at a flow rate of 2 mL min^{-1} to a 15-mL amylose resin column (New England Biolabs) that had been equilibrated with buffer A [20 mM Tris-HCl (pH 8.0), 200 mM KCl]. After binding of the enzymes, the column was washed with two column volumes of buffer A. The enzymes were eluted with 100% of buffer B [20 mM Tris-HCl (pH 8.0), 200 mM KCl, 10 mM maltose] and stored with 50% (PaaABC(D)E) or 30% (PaaZ, PaaD, PaaH) glycerol (vol/vol).

Purification of his_6-tagged protein PaaG. The cell extract was applied at a flow rate of 1 mL min^{-1} to a 1-mL HisTrap HP column (GE Healthcare) that had been equilibrated with buffer C [20 mM Tris-HCl (pH 8.0) and 100 mM KCl]. After binding of the enzymes, the column was washed with five column volumes of 90% buffer C and 10% buffer D [20 mM Tris-HCl (pH 8.0), 100 mM KCl, and 500 mM imidazole]. The enzyme was eluted with 100% buffer D and stored with 30% glycerol (vol/vol).

Purification of his_{10}-tagged proteins PaaJ and PaaF. The cell extract was applied at a flow rate of 1 mL min^{-1} to a 1-mL HisTrap HP column (GE Healthcare) that had been equilibrated with buffer A. After binding of the enzymes, the column was washed with five column volumes of 90% buffer A and 20% buffer E [20 mM Tris-HCl (pH 8.0), 200 mM KCl, and 500 mM imidazole]. The enzymes were eluted with 100% buffer E and stored with 30% glycerol (vol/vol).

Enzyme Measurements. A spectrophotometric assay was used for the measurement of PaaABC(D)E at 30 °C, in which the oxidation of NAPDH was followed spectrophotometrically at 365 nm (ε_{NADPH} = 3,400 M^{-1} cm^{-1}). The reaction mixture (0.3 mL) contained 80 μg PaaABC(D)E, 50 mM Tris/HCl (pH 8.0), and 0.5 mM NADPH and was started by the addition of 0.1 mM phenylacetyl-CoA.

HPLC Analysis. The products of the conversion of phenylacetyl-CoA by enzymes of the phenylacetic acid catabolic pathway were analyzed using reversed-phase HPLC (RP-HPLC) with a C18-E column (LiChrospher 100 RP, end-capped, 5 μm, 125 × 4 mm) (Wicom) combined with optical detection between 200–400 nm by a Waters 996 photodiode array detector. The column was developed at a flow rate of 1 mL min^{-1} by a linear gradient from 2% acetonitrile in 40 mM ammonium acetate (pH 6.8) to 30% acetonitrile in the same buffer within 15 min. The initial reaction mixture (0.25 mL) contained 50 mM Tris-HCl (pH 8.0), 0.5 mM CoA, 1 mM NADPH, 1 mM NADP$^+$, and 0.8 mg of PaaABC(D)E. The reaction was started by the addition of 0.5 mM phenylacetyl-CoA at 30 °C. Further enzymes were added step by step, and respective samples were analyzed.

Determination of Radioactivity. Radioactivity was monitored in RP-HPLC runs using a flow-through solid-state scintillation counter. Radioactive fractions were analyzed by liquid scintillation counting using external standard.

Syntheses. [U-^{13}C]phenylacetic acid was synthesized from 40 mg of L-[U-^{13}C,^{15}N]phenylalanine with 740 kBq L-[U-^{14}C]phenylalanine as a tracer. Phenylalanine was oxidatively deaminated by L-amino acid oxidase to phenylacetic acid as described previously (4).

Phenylacetyl-CoA was synthesized from phenylacetyl succinimide modified after Schachter and Taggart (5) or enzymatically using phenylacetate-CoA ligase as described previously (6). The CoA-esters were quantified roughly by absorption at 260 nm, assuming the same molar extinction coefficient as for CoA (16,400 M^{-1}cm^{-1}). The purity was analyzed by RP-HPLC.

Phenylacetyl succinimide was synthesized using a reaction mixture containing 50 mL dioxane, 10 mmol phenylacetic acid, and 10 mmol N-hydroxysuccinimide that was stirred at 20 °C. A solution of 10 mmol *N,N*-dicyclohexylcarbodiimide in 10 mL dioxane was added slowly over a period of 30 min, and the resulting mixture was stirred for 12 h at 20 °C. The produced N,N-dicyclohexylcarbamide was isolated using a 0.2-μm Teflon filter. Dioxan was evaporated under reduced pressure. Phenylacetyl succinimide was dissolved in boiling chloroform (61 °C) before the addition of diethyl ether. The closed bottle was stored at –20 °C for 12 h. The resulting crystals were isolated with a Teflon filter and were washed with diethyl ether.

Preparation of ^{13}C-Labeled Intermediates. The products of enzymatic conversions of [U-^{13}C]-phenylacetyl-CoA by PaaABC(D)E, PaaG, PaaZ, and PaaJ were prepared by RP-HPLC and analyzed by ^{13}C-NMR. The reactions were started by the addition of labeled substrate. The roman numerals indicate the compounds in Fig. 1*B*.

^{13}C-ring 1,2-epoxyphenylacetyl-CoA (III). A reaction mixture (25 mL) containing 0.35 mM [U-^{13}C]phenylacetyl-CoA (^{13}C-PA-CoA) and 27 kBq of [U-^{14}C]phenylacetyl-CoA (^{14}C-PA-CoA), 36 mg of PaaABC(D)E, 100 mM Tris-HCl (pH 8.0), and 2 mM NADPH was stirred for 10 min at 30 °C.

^{13}C-oxepin-CoA (IV). A reaction mixture (15 mL) containing 0.5 mM ^{13}C,^{14}C-PA-CoA, 15 mg of PaaABC(D)E, 0.3 mg of PaaG, 100 mM Tris-HCl (pH 8.0), and 2 mM NADPH was stirred for 10 min at 30 °C.

^{13}C-3-oxo-5,6-dehydrosuberyl-CoA (V). A reaction mixture (10 mL) containing 0.5 mM ^{13}C,^{14}C-PA-CoA, 8 mg of PaaABC(D)E, 0.3 mg of PaaG, 1 mg of PaaZ, 100 mM Tris-HCl (pH 8.0), 1 mM NADPH, and 1 mM NADP$^+$ was stirred for 10 min at 30 °C.

^{13}C-2,3-dehydroadipyl-CoA (VI) and ^{13}C-acetyl-CoA (VII). A reaction mixture (10 mL) containing 0.5 mM ^{13}C,^{14}C-PA-CoA, 13 mg of PaaABC(D)E, 0.2 mg of PaaG, 0.7 mg of PaaZ, 50 mM Tris-HCl (pH 8.0), 0.05 mg of PaaJ, 1 mM NADPH, and 1 mM NADP$^+$ was stirred for 20 min at 20 °C.

The reactions were stopped by adding 1% formic acid to obtain pH 4. The samples were centrifuged (16,000 × *g*) for 10 min at 4 °C. The supernatant was applied to C18-E columns (end-capped, bed size 200 mg, reservoir volume 3 mL) (Phenomenex) that had been equilibrated with two reservoir volumes of 2% methanol in 20 mM ammonium acetate (pH 4.0). The columns were washed with two reservoir volumes of equilibration buffer, and the reaction products were eluted with one reservoir volume of 100% methanol. The methanol solutions were concentrated with a rotary evaporator at low pressure (50 mbar) at 25 °C. The concentrates (0.5–1 mL) were loaded onto a C18-E column using RP-HPLC for separation, as described above. The respective peaks were collected, frozen immediately in liquid nitrogen, lyophilized, and kept frozen (−20 °C). The samples were dissolved in cold MeOD or D_2O (in the case of 3-oxo-5,6-dehydrosuberyl-CoA) directly before NMR measurement.

Stability of the Intermediates. Enzyme-free solutions of different intermediates in water or methanol were incubated at varying pH values and temperatures. HPLC samples were analyzed at different times to test the stability of the intermediates that were analyzed by ^{13}C-NMR. All measured products were stable in MeOH (or H_2O for 3-oxo-5,6-dehydrosuberyl-CoA) at pH 6.8 and 10 °C for more than 6 h. In the case of ring 1,2-epoxyphenylacetyl-CoA, the half-life was roughly 2.5 h in MeOH (pH 6.8) and 2 h in H_2O (pH 4.0–5.0) at 10 °C. The same compound was even more labile in water, under more acidic or more alkaline conditions, and at elevated temperature. In our HPLC assays we observed the release of CoA in the decay process, probably yielding ring 1,2-epoxyphenylacetate which may decompose further to 2-hydroxyphenylacetate.

Derivatization of Ring-1,2-Epoxyphenylacetyl-CoA. A reaction mixture (0.15 mL) containing 0.35 mg of PaaABC(D)E, 3 mM NADPH, and 0.5 mM phenylacetyl-CoA was stirred for 5 min at 20 °C. After incubation, a final concentration of 50 mM N,N-diethyldithiocarbamate (DTC) (50 μL of 200 mM DTC in 100 mM phosphate buffer, pH 7.0) was added to the reaction mixture and incubated for 5 min at 50 °C to test for epoxides (7). We applied 100 μL of the reaction mixture to a C18-E column connected to an RP-HPLC. The separation profile showed a new peak, which was collected and analyzed by mass spectrometry. The detected mass fitted to an epoxide-specific derivatization product of ring 1,2-epoxyphenylacetyl-CoA with DTC (Table 1).

^{18}O-Labeling of Ring-1,2-Epoxyphenylacetyl-CoA. Labeling assays (2.5 mL) were performed in a 7.5-mL closed tube with roughly 50% $^{18}O_2$/50% ^{16}O (vol/vol) gas phase or with 50% $H_2{}^{18}O$/50% $H_2{}^{16}O$ (vol/vol). Phenylacetyl-CoA was converted with 3 mM NADPH and 50 mM Tris-HCl (pH 8.0) by 4.8 mg PaaABC(D)E. Assay mixtures were stirred at room temperature for 5 min. The enzymatic reactions were stopped by adding 1% formic acid to obtain pH 4.5. Denatured protein was removed by centrifugation. The supernatant was purified by RP-HPLC and analyzed by mass spectrometry. Forty-three percent ^{18}O-labeled (MH^+ = 904.164) and 57% unlabeled product (MH^+ = 902.158) was detected when 50% $^{18}O_2$ was present in the enzymatic assay (Table 1). No ^{18}O labeled product (MH^+ = 904.164) was observed in the presence of 50% $H_2{}^{18}O$.

Protein-Analyzing Methods. Enzyme fractions were analyzed by SDS-12.5% PAGE (8). Proteins were visualized by Coomassie brilliant blue R-250 staining. Protein concentrations were determined by the Bradford method (9) with BSA as a standard.

Computational Analysis. The BLASTP searches were performed via the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) (10, 11) in November 2009. The amino acid sequences of PaaA and PaaC were used as queries for BLASTP searches against assembled bacterial genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Unfinished bacterial genome projects were excluded from the BLASTP search. Occurrence and similarity of PaaA and PaaC were analyzed in 640 completely sequenced bacterial genomes. Archaea were analyzed separately. The percentage of organisms that contained homologs for both key enzymes was 16.3% (104 of 640 bacterial genomes). Homologs taken into account had an expectation value $<e^{-67}$ for PaaA or $<e^{-11}$ for PaaC, respectively.

Amino acid sequences were aligned using CLUSTALW (12) implemented within BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Mass Spectrometry. Fractions of RP-HPLC runs were injected at 5 μL/min with a syringe pump into the nanoelectrospray interface of a Fourier transform ion cyclotron resonance mass spectrometer (Thermo Fisher Scientific). Measurements covered 600–1,200 *m/z* at a resolution of 100,000. The detected masses are listed in Table 1.

^{13}C-NMR Analysis. ^{13}C-NMR spectra were measured at 10 °C using a Bruker DRX 500 spectrometer with a ^{13}C^{1}H dual probe head. Two-dimensional INADEQUATE experiments were performed according to standard Bruker software. Typically, 1,000–20,000 scans were accumulated for 1D ^{13}C NMR spectra with a repetition delay of 5 s. The free induction decays were multiplied with a mild Gaussian function before Fourier transformation. The chemical shift was adjusted to the solvent signal of MeOD or referenced to an external standard (when the solvent was D_2O). Because of the ^{13}C-enrichment of the [U-^{13}C]phenylacetyl-CoA precursor, the ^{13}C NMR spectra of the products were dominated by the signals caused by the ^{13}C-enriched positions. Signals caused by the unlabeled CoA-ester residue were not detected directly. However, the presence of the thio-ester moiety was gleaned from the down-field shifts of the carbonyl-CoA atoms (180–200 ppm). The multiple ^{13}C-labeling also was conducive to scalar couplings between adjacent ^{13}C atoms. The chemical shifts, as well as the multiplicities and the coupling constants, are listed in Table 2. ^{13}C–^{13}C couplings also were detected in 2D INADEQUATE experiments, which identified pairs of adjacent ^{13}C atoms. In combination with the mass data, the NMR chemical shifts, couplings, predictions, and correlations provided solid evidence for the proposed structures.

1. Lu SE, Scholz-Schroeder BK, Gross DC (2002) Construction of pMEKm12, an expression vector for protein production in *Pseudomonas syringae*. *FEMS Microbiol Lett* 210: 115–121.
2. Bartolomé-Martín D, et al. (2004) Characterization of a second functional gene cluster for the catabolism of phenylacetic acid in *Pseudomonas* sp. strain Y2. *Gene* 341: 167–179.
3. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
4. Ismail W, et al. (2003) Functional genomics by NMR spectroscopy. Phenylacetate catabolism in *Escherichia coli*. *Eur J Biochem* 270:3047–3054.
5. Schachter D, Taggart JV (1953) Benzoyl coenzyme A and hippurate synthesis. *J Biol Chem* 203:925–934.
6. Fuchs G, Mohamed M el-S (1993) Purification and characterization of phenylacetate-coenzyme A ligase from a denitrifying *Pseudomonas* sp., an enzyme involved in the anaerobic degradation of phenylacetate. *Arch Microbiol* 159:554–562.
7. Dupard-Julien CL, Kandlakunta B, Uppu RM (2007) Determination of epoxides by high-performance liquid chromatography following derivatization with N,N-diethyldithiocarbamate. *Anal Bioanal Chem* 387:1027–1032.
8. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
9. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
10. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410.
11. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2009) GenBank. *Nucleic Acids Res* 37(Database issue):D26–D31.
12. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.

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ECH    104 VNGYALGGGC ELAMMCDIIY AG-EKAQFGQ PEILLGTIPG AGGTQRLTRA 152
PaaF    99 VNGYALGAGC ELALLCDVVV AG-ENARFGL PEITLGIMPG AGGTQRLIRS 147
PaaG   106 VNGVAAGAGA TLALGGDIVI AA-RSAKFVM AFSKLGLIPD CGGTWLLPRV 154
pECI   119 LNGPAIGLSA ALVALCDIVY SINDKVYLLY PFANLGLITE GGTTVSLPLK 168
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Fig. S1. Partial sequence alignment of PaaG and PaaF from *Escherichia coli* K12 with rat enoyl-CoA hydratase (ECH) and peroxisomal Δ^3,Δ^2-enoyl-CoA isomerase (pECI) from *Saccharomyces cerevisiae*. Indicated in light gray are the conserved catalytic Glu residues responsible for the incorporation or elimination of water. Shown in dark gray are the catalytic Glu158 residue of pECI and the proposed catalytic Asp144 of PaaG.

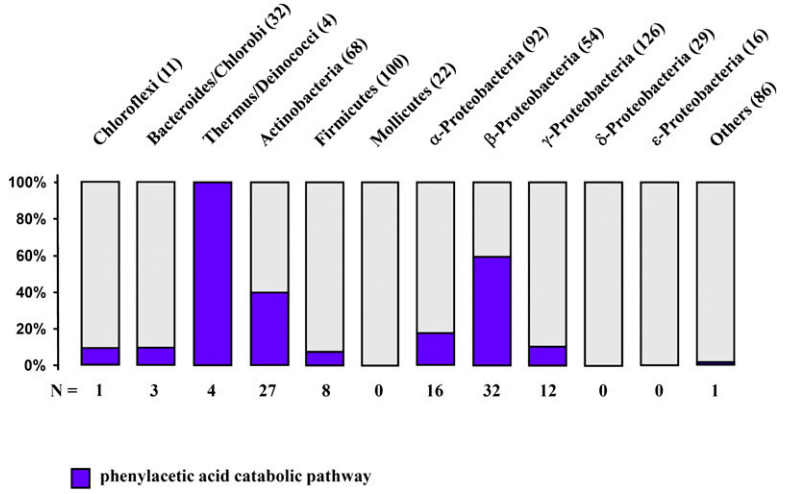

Fig. S2. Bacteria within selected phylogenetic groups that harbor the key genes *paaA* and *paaC* for the aerobic phenylacetate catabolic pathway. The total number of bacterial species whose genomes have been sequenced completely is shown in parentheses after the name of the phylogenetic group. The number (*N*) beneath each column displays the number of species that contain the key genes for phenylacetic acid catabolism.

Table S1. Overview of all proteins involved in phenylacetic acid degradation of *Escherichia coli* K12 and their proposed function

Protein	Function	Molecular mass (kDa)	E.C. number
PaaZ	Oxepin-CoA hydrolase/3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase	73	4.2.1.119/1.2.1.1x
PaaA	Subunit of ring 1,2-phenylacetyl-CoA epoxidase	35.5	1.14.13.x
PaaB	Subunit of ring 1,2-phenylacetyl-CoA epoxidase	10.9	1.14.13.x
PaaC	Subunit of ring 1,2-phenylacetyl-CoA epoxidase	27.9	1.14.13.x
PaaD	Possible subunit of ring-1,2-phenylacetyl-CoA epoxidase	18.5	1.14.13.x
PaaE	Subunit of ring 1,2-phenylacetyl-CoA epoxidase	39.3	1.14.13.x
PaaF	2,3-dehydroadipyl-CoA hydratase	27.2	4.2.1.17
PaaG	1,2-epoxyphenylacetyl-CoA isomerase	28.4	5.3.3.x
PaaH	3-hydroxyadipyl-CoA dehydrogenase	51.7	1.1.1.35
PaaI	Thioesterase	14.9	3.1.2.2x
PaaJ	3-oxoadipyl-CoA/3-oxo-5,6-dehydrosuberyl-CoA thiolase	42.2	2.3.1.16
PaaK	Phenylacetate-CoA ligase	49	6.2.1.30
PaaX	Transcriptional repressor	35.3	
PaaY	Acetyltransferase	21.3	

Table S2. Primers and plasmids used in this study

Gene(s)		Primer	Restriction sites	Plasmid	Tag
paaABCDE	Forward	5′-CCG ***GAA TTC*** TAC GCA CAA CTG GTT GAG AC-3′	Eco R1	pMEKm12	mbp
(*Pseudomonas* sp. str. Y2)	Reverse	5′-CCC ***AAG CTT*** TCA AAT CTG GTC GAA GTC GAG-3′	HindIII		
paaD	Forward	5′- CCG ***GAA TTC*** CGA CCT GGT GAG CTG AT-3′	Eco R1	pMEKm12	mbp
(*Pseudomonas* sp. str. Y2)	Reverse	5′- CCC ***AAG CTT*** TCA GAT GCA CTT GAA ATA GTC GA-3′	HindIII		
paaG	Forward	5′- ATG ACC TTC GAG CAC ATC CTG TT -3′	–	pEXP5-CT /TOPO	his_6
(*Pseudomonas* sp. str. Y2)	Reverse	5′- GCG TCC TTT GAA GAC GGG CGT -3′	–		
paaZ	Forward	5′- G T***GA AAC ATT TC***G AGA GTA GCG ATG CAG-3′	XmnI	pMAL-c2X	mbp
(*Escherichia coli* K12)	Reverse	5′- GGG CA***T CTA GA***C GCC GTT GCC CTT C-3′	XbaI		
paaF	Forward	5′- CGT C***CA TAT G***CC TCA TAC CCT TAG CGT CG-3′	NdeI	pET16b	his_{10}
(*Pseudomonas* sp. str. Y2)	Reverse	5′- CTA T***AA GCT T***GA TGG GAG TCA GGG CCG GTG -3′	HindIII		
paaH	Forward	5′- GG T***GA ACG GGT TC***T AGC ATG ATG ATA AAT GTG-3′	XmnI	pMAL-c2X	mbp
(*Escherichia coli* K12)	Reverse	5′- GTT C*TC TAG A*AT TGC ATG GGC ATT TTG-3′	XbaI		
paaJ	Forward	5′- CAG GAG AAC G***CA TAT G***AA TGA AGC CCT TAT C-3′	NdeI	pET16b	his_{10}
(*Pseudomonas* sp. str. Y2)	Reverse	5′- GG***A AGC TT***G CCC CTG GTG TTT GAG -3′	HindIII		

The source of chromosomal DNA is shown beneath each gene. Introduced restriction sites are shown in bold italics. Produced proteins were tagged with mbp or with histidines (his_6 or his_{10}).