

SUPPORTING INFORMATION

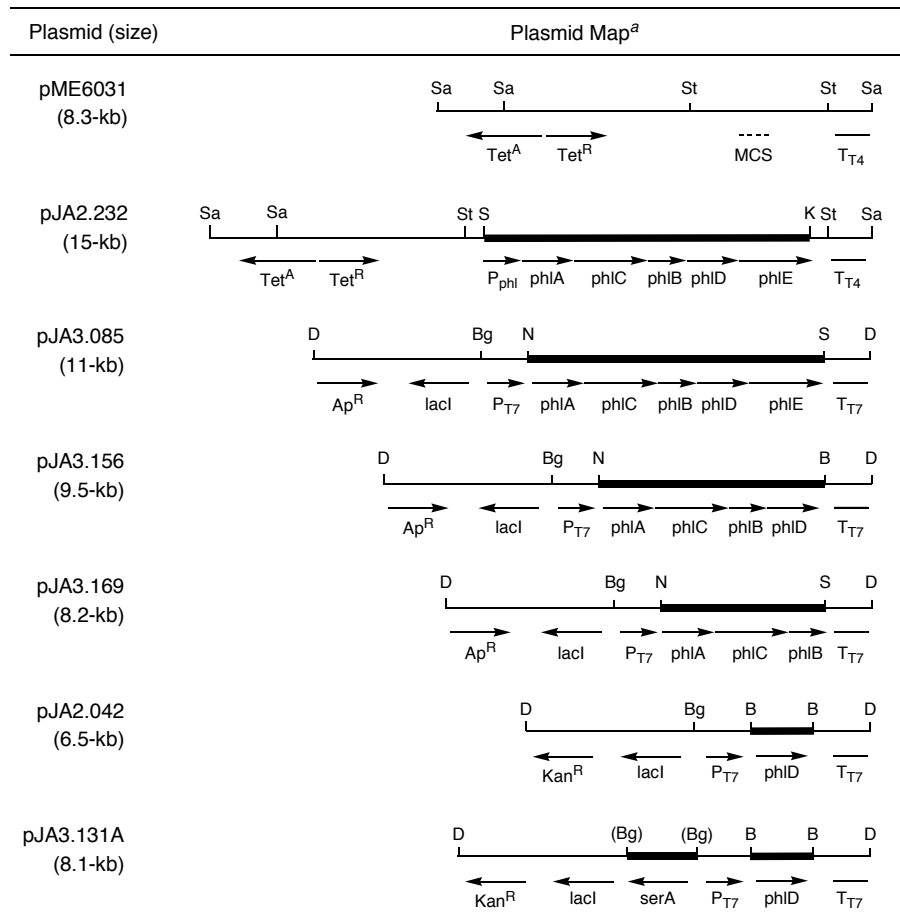
Bacterial Strains and Plasmids. *Escherichia coli* strain DH5 α served as the host strain for all plasmid constructions. *E. coli* BL21(DE3) and *E. coli* JWF1(DE3), respectively, served as the host strains for protein expression in shake-flask and fermentation experiments. *Pseudomonas fluorescens* Pf-5 was obtained from the American Type Culture Collection (ATCC BAA-477). *P. fluorescens* Pf-5 genomic DNA was isolated using the CTAB method.¹ *E. coli* cloning vector pET21a(+) was purchased from Novagen. *E. coli* cloning vector pHis8-3 was provided by Professor Joseph P. Noel (The Salk Institute for Biological Studies, La Jolla, CA).² Shuttle-vector pME6031 was provided by Professor Dieter Haas (Université de Lausanne, Switzerland).³

General Microbiology. All solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). TB medium (1 L) contained Bacto tryptone (12 g), Bacto yeast extract (24 g), glycerol (4 mL), KH₂PO₄ (2.3 g), and K₂HPO₄ (12.5 g). YM broth (1 L) was purchased from Difco and contained peptic digest of animal tissues (5 g), yeast extract (3 g), malt extract (3 g), and D-glucose (10 g). M9 salts (1 L) contained Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NH₄Cl (1 g), and NaCl (0.5 g). M9 medium contained D-glucose (20 g) and MgSO₄ (0.24 g) in 1 L of M9 salts. Except where specifically mentioned, antibiotics were added where appropriate to the following final concentrations: ampicillin (Ap), 50 μ g/mL; kanamycin (Kan), 50 μ g/mL; tetracycline (Tet), 25 μ g/mL. Isopropyl- β -D-thiogalactopyranoside (IPTG) was prepared as a 100 mM stock solution. Solutions of LB medium, TB medium, YM broth, M9 salts, MgSO₄, KH₂PO₄ and K₂HPO₄, and D-glucose were autoclaved individually prior to mixing. Solutions of antibiotics, phloroglucinol, 2-acetylphloroglucinol, 2,4-diacetylphloroglucinol and IPTG were sterilized through 0.22- μ m membranes. Solid medium was prepared by addition of Difco agar to a final concentration of 1.5% (w/v). Unless otherwise stated, cultures of *E. coli* and *P. fluorescens* Pf-5 were grown at 37 °C and 30 °C, respectively, with agitation at 250 rpm.

Standard protocols were used for construction, purification and analysis of plasmid DNA as well as for transformation of *E. coli* strains.¹ Plasmids were transformed into *P. fluorescens* Pf-5 by electroporation.⁴ PCR amplifications were performed by standard methods using *Pfu Turbo* polymerase (Stratagene).¹ DNA fragments longer than 2.8 kb were amplified according to Cheng's PCR protocol using *Tth* (Epicenter) as the main-component non-proofreading DNA polymerase and *Vent* (New England Biolabs) as the fractional-component proofreading polymerase.⁵ Calf intestinal alkaline phosphatase and large fragment (Klenow) of DNA polymerase I were purchased from New England Biolabs. PCR primers were synthesized by the Macromolecular Structure Facility at Michigan State University. DNA sequencing was performed at the Genomic Technology Support Facility at Michigan State University. DNA Clean & Concentrator kit and Gel DNA Recovery kit were purchased from Zymo Research.

Construction of Plasmids. The *P. fluorescens* Pf-5 *phlACBDE* gene cluster sequence was obtained from The Institute for Genomic Research (TIGR) by performing a BLAST search against the *P. fluorescens* Q2-87 gene cluster sequence published on the National Center for Biotechnology Information (NCBI).⁶ The resulting *P. fluorescens* Pf-5 hit sequence was analyzed for open reading frames (ORFs) using Gene Construction Kit™ (Textco, Inc.). Each

ORF was translated in order to identify those proteins with similarities with known PhlA, PhlB, PhlC, PhlD, PhlE, and PhlF sequences in GeneBank using BLAST. The *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, and *phlF* within the *P. fluorescens* Pf-5 genome were thus identified.



^a Restriction enzyme maps of plasmids. Restriction enzyme sites are abbreviated as follows: B = *Bam*HI, Bg = *Bgl*II, D = *Dra*III, K = *Kpn*I, N = *Nhe*I, S = *Sac*I, Sa = *Sac*II, St = *Stu*I. Parentheses indicate that the designed enzyme site has been eliminated. T_{T4} and T_{T7} indicate T₄ and T₇ transcription terminators, respectively. (—) Vector DNA, (—) insert DNA.

pJA2.232. A 6.5-kb fragment encoding the *P. fluorescens* Pf-5 *phlACBDE* cluster including its native promoter and terminator sequences was amplified by long PCR using 5'–CGCTAGAGCTCGCAAATAGTCTCCCTCCA–3' as the forward primer (*Sac*I restriction site is underlined) and 5'–GTGGTACCGGAGTGTTTCAGGGTGACTT–3' as the reverse primer (*Kpn*I restriction site is underlined) from *P. fluorescens* Pf-5 genomic DNA. The PCR product was simultaneously digested with *Sac*I and *Kpn*I and ligated with *Sac*I/*Kpn*I-digested pME6031 to afford the 15-kb plasmid pJA2.232. DNA sequencing of the 6.5-kb insert was consistent with *P. fluorescens* Pf-5 genomic sequences published on TIGR database.

pJA3.085. A 5.5-kb fragment encoding the *phlACBDE* genes was amplified by long PCR from pJA2.232 using 5'–GGGCGCGCTAGCATGAACGTGAAAAGATAGG–3' as the forward primer (*Nhe*I restriction site is underlined, *phlA* start codon in boldface) and 5'–CCAGAGAGCTCGAACAGCAGGGTCTCGTACA–3' as the reverse primer (*Sac*I

restriction site is underlined). The PCR product was simultaneously digested with *NheI* and *SacI* and then ligated with *NheI/SacI*-digested pET21a(+) to afford the 11-kb plasmid pJA3.085.

pJA3.156. A 4.1-kb fragment encoding the *phlACBD* genes was amplified by long PCR from pJA2.232 using 5'–GGGCGCGCTAGCATGAACGTGAAAAAGATAGG–3' as the forward primer (*NheI* restriction site is underlined, *phlA* start codon in boldface) and 5'–AATAGGATCCAGCCCGGCAACGTCAGGTG–3' as the reverse primer (*BamHI* restriction sites are underlined). The PCR product was sequentially digested with *NheI* and *BamHI* and then ligated with *NheI/BamHI*-digested pET21a(+) to afford the 9.5-kb plasmid pJA3.156.

pJA3.169. A 2.8-kb fragment encoding the *phlACB* genes was amplified by long PCR from pJA2.232 using 5'–GGGCGCGCTAGCATGAACGTGAAAAAGATAGG–3' as the forward primer (*NheI* restriction site is underlined, *phlA* start codon in boldface) and 5'–GAGACGAGCTCATGGCTAACTTCAAGGTGC–3' as the reverse primer (*SacI* restriction sites are underlined). The PCR product was simultaneously digested with *NheI* and *SacI* and then ligated with *NheI/SacI*-digested pET21a(+) to afford the 8.2-kb plasmid pJA3.169.

pJA2.042. A 1.1-kb fragment containing the *phlD* gene was amplified by PCR from *P. fluorescens* Pf-5 genomic DNA using *Pfu Turbo* as the DNA polymerase, 5'–CGCGGATCCCCCTTAACTTGTGGCTTAC–3' as the forward primer, and 5'–AATAGGATCCAGCCCGGCAACGTCAGGTG–3' as the reverse primer (*BamHI* restriction sites are underlined). The PCR product was digested with *BamHI* and ligated with *BamHI*-digested pHis8-3 vector to afford the 6.5-kb plasmid pJA2.042.

pJA3.131A. A 1.6-kb *serA* locus was excised from plasmid pRC1.55B⁷ by digestion with *SmaI* and ligated to the 6.5-kb plasmid pJA2.042, which had been previously treated with *BglII*, Klenow fragment, and CIAP. In the resulting 8.1-kb plasmid pJA3.131A, the *serA* and *phlD* genes are divergently transcribed.

Shake-flask Cultivation of *E. coli* Constructs. Inoculants of *E. coli* strains were started by introducing a single colony from an agar plate into 100 mL of LB medium containing the appropriate antibiotic. Cultures were grown for 12 h. A 10 mL aliquot of this culture was transferred to 0.5 L of TB medium containing the appropriate antibiotic. The 0.5 L culture was grown at 37 °C with agitation at 250 rpm to OD₆₀₀ = 1.0–1.2. Protein expression was then induced by addition of IPTG to a final concentration of 0.5 mM. The cultures were shaken at 23 °C and 250 rpm for 4 h. The cells were chilled on ice for 2 min, harvested by centrifugation (1500 g, 4 °C, 4 min), and gently resuspended in 0.5 L of M9 medium containing the appropriate antibiotic. Experiments were initiated (t = 0) by addition of IPTG to a final concentration of 0.5 mM. Solutions of phloroglucinol (10 mg/mL in H₂O), 2-acetylphloroglucinol (5 mg/mL in H₂O:EtOH, 3:1, v/v), and 2,4-diacetylphloroglucinol (5 mg/mL, H₂O:EtOH 1:2, v/v) were added to cultures to afford a final concentration of 100 mg/L. Samples (15 mL) of culture broth were removed at 24 h intervals and centrifuged (4300 g, 4 °C, 6 min). The resulting cell-free supernatant was frozen at –20 °C pending analysis. Product yields were determined by gas chromatography after derivatization (see below).

Shake-flask Cultivation of *P. fluorescens* Pf-5 Constructs. *P. fluorescens* Pf-5 inoculants were started by introducing a single colony from an agar plate into 100 mL of YM medium containing tetracycline. Cultures were grown for 24 h at 30 °C with agitation at 250 rpm. A 10 mL aliquot of this culture was transferred to 1 L of YM medium containing tetracycline to initiate ($t = 0$) shake-flask experiments. The 1 L culture was grown at 30 °C with agitation at 250 rpm while monitoring cell growth at OD_{600} . Samples (25 mL) of culture broth were removed at 12 h intervals and centrifuged (4300 g , 4 °C, 6 min). The resulting cell-free supernatant was frozen at -20 °C pending analysis. Product yields were determined by gas chromatography after derivatization (see below).

Fed-batch Fermentations. Fed-batch fermentation was performed in a 2.0-L working capacity B. Braun M2 culture vessel under glucose-rich conditions as previously described.⁸ Utilities were supplied by a B. Braun Biostat MD controlled by a DCU-3. Data acquisition utilized a Dell Optiplex GX200 personal computer (PC) equipped with a B. Braun MFCS/Win software (v2.0). Temperature, pH, and dissolved oxygen (D.O.) were controlled with PID control loops. Temperature was maintained at 36 °C. Addition of concentrated NH_4OH or 2 N H_2SO_4 was used to maintain pH at 7.0. D.O. was monitored using a Mettler-Toledo 12 mm sterilizable O_2 sensor fitted with an Ingold A-type O_2 permeable membrane and maintained at 20% of air saturation. Antifoam (Sigma 204) was added as needed. The initial glucose concentration in the fermentation medium was 30 g/L. Glucose concentration ranged from 10-30 g/L over the course of the fermentation. Inoculants were prepared by introducing a single colony of JWF1(DE3)/pJA3.131A into 5 mL of M9 medium. Culture were grown at 37 °C with agitation at 250 rpm for 24 h and then transferred to 100 mL of M9 medium, grown at 37 °C for an additional 12 h to $OD_{600} = 1.0-2.5$, and then transferred into the fermentor vessel. Fermentation experiments were thus initiated ($t = 0$). Cell densities were determined by dilution of fermentation broth with water (1:1000) followed by measurements of absorption at 600 nm (OD_{600}). IPTG was added to a final concentration of 25 mg/L at 6 h intervals. Samples (15-25 mL) of fermentation broth were removed at 6 h intervals and centrifuged (4300 g , 4 °C, 6 min). The resulting cell-free supernatant was frozen at -20 °C pending analysis. Product yields were determined by gas chromatography after derivatization (see below).

Gas Chromatography. Gas chromatography was performed on an Agilent 6890N instrument equipped with an HP-5 capillary column (30.0 m x 0.25 mm x 0.25 μm). The injector and FID detector temperatures were set to 250 °C and 300 °C, respectively. Gas flow through the column was set at 1.8 mL/min (24 psi). The oven initial temperature was held at 120 °C for 3 min, increased to 210 °C at a rate of 15 °C/min, and held at the final temperature for 5 min. Injected sample volume was 2 μL . Product yields were determined based on response factors established for authentic samples relative to dodecane as the internal standard after derivatization with bis(trimethylsilyl)trifluoroacetamide. Response factors of 0.867, 0.697, and 0.675 were measured for phloroglucinol, 2-acetylphloroglucinol, and 2,4-diacetylphloroglucinol, respectively. In a typical procedure, cell-free broth samples were extracted three times with equal volumes of EtOAc:AcOH (100:1, v/v). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated to dryness. The crude residue was treated with pyridine (0.9 mL, 11.1 mmol), bis(trimethylsilyl)trifluoroacetamide (1 mL, 3.76 mmol), and dodecane (0.1 mL, 44.0 mM solution in pyridine). The reaction was stirred at rt for 2 h and then analyzed by gas chromatography.

Purification of PhlD from *E. coli* BL21(DE3)/pJA2.042. Protein concentrations were determined using the Bradford dye-binding method.⁹ Protein assay solution was purchased from Bio-Rad. Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin. Protein purity was assessed by SDS-PAGE. *E. coli* BL21(DE3)/pJA2.042 was grown and PhlD expressed in TB medium as described above. Cells were harvested by centrifugation (4000 g, 4 °C, 8 min). The wet cell paste (2.5 g) was resuspended in buffer (5 mL; NaH₂PO₄, 50 mM, pH 8.0; NaCl, 300 mM) followed by disruption using two passes through a French pressure cell (SLC Aminco) at 16000 psi. Cellular debris was removed from lysate by centrifugation (48000 g, 4 °C, 30 min). PhlD was purified from the resulting cell-free lysate by affinity chromatography using Ni-NTA resins (Qiagen). The clarified cell lysate (4 mL) was treated with 50% (w/v) Ni-NTA slurry (1 mL) and mixed by gentle shaking for 1 h at 4 °C. The mixture was loaded onto a polypropylene column. PhlD was eluted using a gradient of 10-100 mM imidazole in resuspension buffer. Fractions containing pure PhlD were pooled, dialyzed using potassium phosphate buffer (50 mM, pH 7.0), frozen in liquid N₂ and stored at -80 °C.

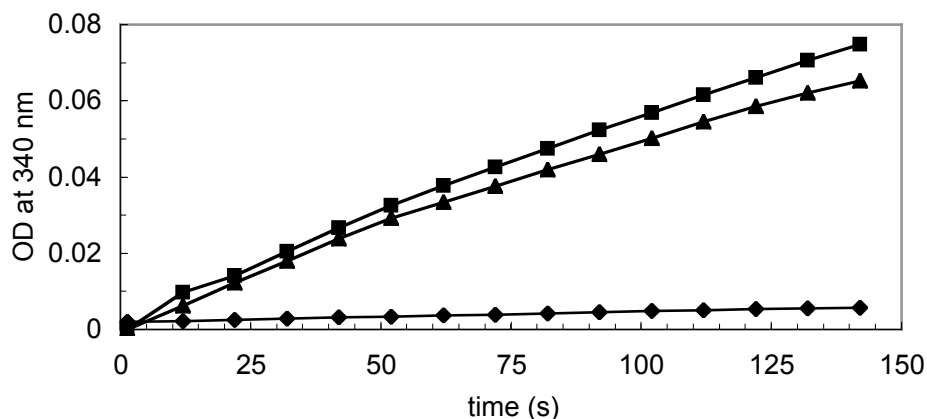


Figure 1. PhlD activity for various substrates monitored by UV at 340 nm. 0.024 mg purified PhlD was employed under the above-described conditions. PhlD activities were 0 for 0.2 mM acetylCoA (diamond), 0.2 U/mg for 0.2 mM malonylCoA (triangle), and 0.2 U/mg for 0.2 mM acetylCoA and malonylCoA (square).

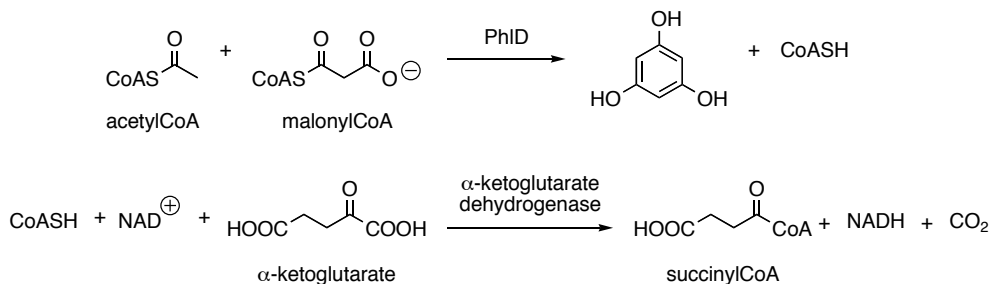


Figure 2. The continuous assay used for determining PhlD activity.

Enzyme Activity Assays. A continuous assay with ketoglutaric acid dehydrogenase as the coupling enzyme measured the amount of CoASH generated during PhlD-catalyzed formation of phloroglucinol from malonylCoA (Figures 1 and 2).¹⁰ CoASH-dependent oxidation of α -ketoglutarate was accompanied by the reduction of NAD⁺ to NADH. The rate of NADH ($\epsilon = 6220 \text{ M}^{-1}\text{cm}^{-1}$) formation was monitored at 340 nm. Assay solutions contained fixed

concentrations of α -ketoglutarate (2 mM), NAD^+ (0.30 mM), and ketoglutaric acid dehydrogenase (0.1-0.3 U) in buffer (KH_2PO_4 , 50 mM, pH 7.0). Concentrations of malonylCoA were varied (5-200 μM). NADH formation was monitored at 22 °C for 200 s. One unit of PhlD activity was defined as the formation of 1 μmol of CoASH per min at 22 °C. Kinetic parameters of PhlD for CoASH formation were derived using a Hanes plot.

pH Optimum for PhlD Enzyme Activity. The pH dependence of PhlD-catalyzed phloroglucinol production from malonylCoA was examined using the enzyme activity assay described above. NADH formation was monitored at 22 °C for 200 s for assay solutions containing α -ketoglutarate (2 mM), NAD^+ (0.30 mM), α -ketoglutarate dehydrogenase (0.1-0.3 U) and malonylCoA (200 μM) in buffer (KH_2PO_4 , 50 mM, pH 5.5-9.0). Assays were initiated by adding purified PhlD. Initial and final pH measurements were used to confirm that the pH of assay solutions remained stable. PhlD specific activity reached a maximum value of 0.2 U/mg at pH 7.0 (Figure 3).

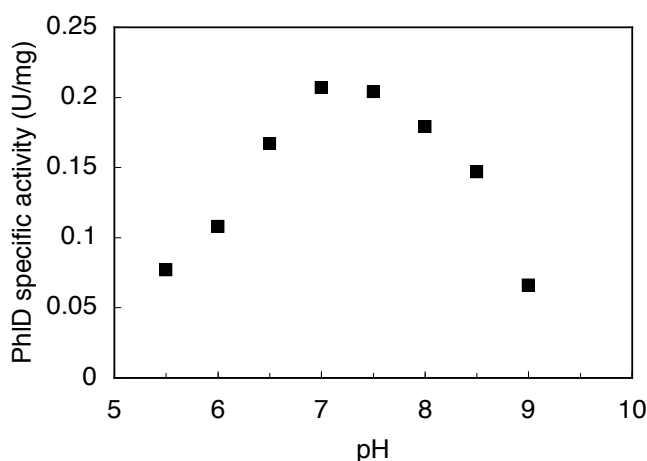


Figure 3. Determination of the pH optimum for phloroglucinol-synthesizing PhlD activity.

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