SUPPLEMENTAL MATERIALS AND METHODS

Strains, plasmids and reagents

All strains, plasmids and oligonucleotides used in this study are provided in the Supplementary Materials, Tables S1 and S2, respectively. Oligonucleotide primers were obtained from Integrated DNA Technologies, Coralville, IA. DNA sequencing was performed on an ABI 3730 Sequencer (Indiana Molecular Biology Institute, Bloomington, IN). Plasmids were introduced into *E. coli* via transformation with standard chemically competent cell preparations and into *A. tumefaciens* via either electroporation (1) or conjugation (2). The *E. coli* strains used for plasmid DNA transformation or conjugation of plasmids were grown in LB broth (Difco Bacto tryptone at 10 g liter^{-1}, Difco yeast extract at 10 g liter^{-1}, and NaCl at 5 g liter^{-1}, pH 7.2) with or without 1.5% (w v^{-1}) agar. Unless noted otherwise, the *A. tumefaciens* strains were grown on either LB or AT minimal medium (3) supplemented with 0.5% (w v^{-1}) glucose and 15 mM ammonium sulfate (ATGN). To prevent the accumulation of iron oxide precipitate, the $FeSO_4$ prescribed in the original AT recipe was omitted, with no adverse growth effect. However, for biofilm cultures, 22 μM $FeSO_4 \cdot 7H_2O$ was added to ATGN medium immediately before inoculation. For *sacB* counter-selection, 5% (w v^{-1}) sucrose (Suc) replaced glucose as the sole carbon source (ATSN). Chemicals, antibiotics, and culture media were obtained from Fisher Scientific and Sigma-Aldrich. Monapterin was purchased from Schircks Laboratories and H_2-folate was from Sigma-Aldrich. Other oxidized pterin derivatives were synthesized. When required, appropriate antibiotics were added to the medium as follows: for *E. coli*, 100 μg ml^{-1} ampicillin, 50 μg ml^{-1} gentamicin (Gm), and 50 μg ml^{-1} kanamycin (Km); and for *A. tumefaciens*, 300 μg ml^{-1}

Gm and 300 μg ml^{-1} Km. 400 μM Isopropyl-β-D-thiogalactopyranoside (IPTG) was used when required. For Congo Red plates, the dye was dissolved in methanol at 20 mg/ml, and passed through 0.2 μm syringe filters immediately before use to remove aggregates. 500 μl filtered Congo Red was added per 100 ml (final ~100 μg/ml) to generate ATGN-CR agar medium.

For Congo Red staining *A. tumefaciens* cultures were grown to mid-exponential phase, normalized to an OD_{600} of 0.5, and spotted (5 μl) onto ATGN-CR with or without IPTG. Spots were dried, and photographs taken after 48 hours of growth at 28°C.

Controlled expression plasmids. For plasmid-borne expression analysis, wild type coding sequences were cloned into the $LacI^{Q}$ encoding, IPTG-inducible expression vector pSRKGm (4). Coding sequences were PCR amplified from *A. tumefaciens* C58 genomic DNA (and that from other bacterial species as indicated), using the corresponding primers for each gene (Table S2) and the Phusion polymerase (NEB). Amplicons were ligated into pGEM-T Easy (Promega), confirmed by sequencing, excised by restriction enzyme cleavage, and ligated with appropriately cleaved pSRKGm. Plasmid derivatives harboring the correct inserts were verified by restriction digestion and sequencing prior to electroporation into competent *A. tumefaciens* cells.

Site-specific mutagenesis. A modified version of the QuikChange mutagenesis protocol (Stratagene) was used to engineer site-specific mutations in *pruA* and *dcpA*. Briefly, two complementary primers were designed with the desired base pair change/s flanked by ~15 bp of unmodified wild type sequence on each side. The primers were

used in a reaction (16 cycles) in a thermal cycler using Phusion Polymerase (NEB) to generate nicked plasmid derivatives with modified sequences. Proper amplification was checked via gel electrophoresis, 1 μl of the methylation-dependent restriction endonuclease Dpn I was added to digest unmodified/methylated wild type plasmid, and the digested mixture was transformed into competent *E. coli*. Plasmid clones were isolated and sequenced to ensure presence of desired mutation.

Construction of in-frame markerless deletions. In-frame deletions of indicated genes were constructed using a previously described method (5). 500-750 bp of sequence upstream (P1 and P2) and downstream (P3 and P4) of the gene to be deleted were amplified using Phusion polymerase (NEB). Care was taken to leave extreme 5' and 3' ends of genes intact, and to retain any potential translational coupling between linked genes. Primers P2 and P3 were designed with 5' sequences (lowercase in Table S2 15-20 bp) with reverse complementarity to each other's 3' proximal sequence. The complementary sequence on these two primers facilitated *s*plicing by *o*verlapping *e*xtension (SOE) of the two PCR products, as described (5). Both flanking sequences were amplified and gel purified. The two purified products were then used as both templates and primers in a second PCR reaction, generating the final spliced product, which was ligated into pGEM-T easy (Promega) and sequenced to ensure proper splicing. The deletion construct was then excised using restriction enzymes and ligated into the suicide vector pNPTS138 cleaved with compatible restriction enzymes. The pNPTS138 plasmid confers Km resistance (Km^R) and sucrose sensitivity (Suc^S). Derivatives of pNPTS138 were introduced into *A.*

tumefaciens C58 by mating with pNPTS138 containing S17-λpir *E. coli.* The ColE1 origin of pNPTS138 does not replicate in *A. tumefaciens*, necessitating single-crossover integration into the chromosome to obtain Km^R transformants. Plasmid integration was confirmed by patching onto ATGN-Km and ATSN to identify Suc^S derivatives. Excision of the integrated plasmid was then mediated by growing overnight cultures of Suc^S Kan^R derivatives and plating dilutions onto ATSN. Plasmid excision was confirmed by patching Suc^R clones onto ATSN and ATGN-Km to identify Kan^S derivatives. Correct deletion of the target gene was confirmed via diagnostic PCR using primers (P5/P6) flanking the deletion site.

Allelic replacement for site-specific base substitutions. The QuikChange procedure mentioned above was utilized to engineer desired mutation/s in PruA. This construct was then ligated into the suicide vector pNPTS138 and transferred into C58 *A. tumefaciens* via mating. Single-crossover integration and subsequent excision was mediated as mentioned above. The presence of the desired mutation was confirmed via PCR and DNA sequencing.

Growth and analysis of static biofilms. *A. tumefaciens* biofilms were grown and analyzed similar to as described previously (6). In summary, trimmed PVC coverslips were placed vertically in 12-well polystyrene culture plates (Corning Inc.) and UV sterilized. Mid-to-late exponential phase culture were then sub-cultured into ATGN (with or without added IPTG, 400 μM) to an OD of 0.05 and statically incubated at room temperature for approximately 48 h. For crystal violet (CV) visualization, coverslips

were rinsed with ddH_20, stained with 0.1% (w v^{-1}) CV, and rinsed once more with ddH_20. CV stained biomass was then quantified by submerging coverslips in 1 ml of 33% acetic acid to solubilize CV. Quantification was performed by reading absorbance of soluble CV at 600 nm (A_{600}) on a Biotek Synergy HT microplate reader. Values were normalized for planktonic growth by dividing the CV A_{600} by the OD_{600} of the remaining culture in the biofilm well.

UPP production assays. The UPP was visualized using wheat germ agglutinnin (WGA) labeled with Alexa Fluor 594 (Invitrogen). *A. tumefaciens* were grown in ATGN to an OD_{600} of 0.5-0.8. 1 ml of culture was centrifuged (7,000 x g), washed twice with ATGN, and pelleted cells were resuspended in 100 uL ATGN. Following resuspension, 1 μl of labeled WGA stock solution (1 mg ml^{-1}) was added to 100 μl of the suspension and incubated for 20-40 min. Bacteria were collected by centrifugation (7,000 x g) and washed twice with ATGN. 1 μL of labeled cell suspension was placed on a concave microscope slide containing an agarose pad (1%, 200 μL). A coverslip was applied and samples were observed by fluorescence microscopy (Nikon E800) using a 100× oil immersion objective and NIS-Elements software.

LC-MS/MS analysis of c-di-GMP levels. Cultures of *A. tumefaciens* derivatives were grown in ATGN medium (plus 400 μM IPTG if needed) at 28°C to stationary phase (>OD 1.0). Cultures of *E. coli* DH5α derivatives were grown in LB (plus 400 μM IPTG if needed) at 37°C to an OD_{600} of 0.5-0.8. Culture densities were normalized by collecting cells by centrifugation, adjusting the volume so that all samples had the same cell

density once resuspended in extraction buffer. Culture aliquots were spun for 5 min at 10,000 x g at 4 ^{o}C and the pellet was immediately resuspended in 250 μl extraction buffer (methanol/acetonitrile/dH_2O 40:40:20 + 0.1 N formic acid cooled at -20^{o}C) by vigorous vortexing and pipetting. The extractions were incubated at -20^{o}C for 30 min, followed by transfer to a new microfuge tube on ice. Cell debris were removed by centrifugation at 10,000 x g 3 min., and 200 □l of supernatant was transferred into a new tube on ice. Samples were neutralized within 1 h of preparation by adding 4 □l of 15% NH_4HCO_3 per 100 μl of sample, aimed to set a pH of 7 ~ 7.5. If sample c-di-GMP concentrations were anticipated to exceed the highest value on standard curve, the samples were diluted 1:10 before measuring.

Prior to analysis, the sample was subjected to vacuum centrifugation to remove the extraction buffer and resuspended in an equal volume of water. Ten μL of each sample was then analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a Quattro Premier XE mass spectrometer (Waters Corporation) coupled with an Acquity Ultra Performance LC system (Waters Corporation). Detection and quantification of c-di-GMP was performed as previously described (7). To calculate the c-di-GMP concentration, chemically synthesized c-di-GMP (Axxora) was dissolved in water at concentrations of 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, and 1.9 nM and analyzed using LC-MS/MS to generate a standard curve.

Chemical synthesis of methylated pterins. 6-Methoxymethylpterin was prepared from 6-(bromomethyl)-2,4-pteridinediamine hydrobromide as previously described (8). This compound was converted into 6-methoxymethylpterin as described (9). In order to

chemically synthesize 2'-O-methylmonapterin, 1-*O*-Methyl-2,-3-*O*-isopropylidene-α-D-xylopyranoside was prepared from 1-*O*-methyl-β-D-xylopyranoside (Sigma) by treatment with 2-methyloxypropene in dimethylformamide as previously described (10). This material was methylated with methyl iodide in the presence of silver oxide to produce 1,4-*O*-dimethyl-2,-3-*O*-isopropylidene-α-D-xylopyranoside as described (10). The resulting sugar was de-protected by heating for 10 min in 1 M HCl. The sample was then neutralized with an equal amount of base and condensed with triaminopyrimidine. The desired pterin was purified by preparative thin-layer chromatography (TLC) and was readily identified by intense blue fluorescence on the TLC plate. After purification, the resulting 2'-O-methylmonapterin was analyzed by HPLC and LC-MS/MS as described above.

Extraction and analysis of pterins. Lyophilized cells (200 mg) from *A. tumefaciens* log-phase cultures were resuspended in 1 mL 50% methanol. The suspension was incubated at 100 °C for 10 min, followed by centrifugation (14,000 × g, 5 min) to pellet insoluble cell debris. The resulting soluble extract was concentrated by evaporation under a stream of nitrogen gas and applied to a Dowex 50W-8X-H+ column (1 x 5 mm). The column was washed with water (500 µl) and the pterins were eluted with 6 M aqueous ammonia (500 µl). After removal of the solvent by evaporation with stream of nitrogen gas, the sample was dissolved in 100 µl of water for high-pressure liquid chromatography (HPLC) analysis.

A Shimadzu HPLC system with a Pursuit XRs 5 C18 (Agilent, 250 x 4.6 mm, 2,6 um particle size) reverse phase column equipped with a photodiode array detector

(PDA) and a fluorescence detector was utilized for initial identification of pterins. The elution profile consisted of 5 min at 95% buffer A (25 mM sodium acetate pH 6.0, 0.02% sodium azide) and 5% buffer B (methanol), followed by a linear gradient from 5% to 50% buffer B over 25 min at 1 mL/min. The pterins were detected by excitation at 356 nm and emission at 450 nm. The pterin-containing peak eluting at 12.5 min from *A. tumefaciens* C58 cells was collected, concentrated to dryness, and resuspended in 50 µl of water for LC-MS/MS analysis.

An AB Sciex 3200 Q TRAP mass spectrometer system attached to an Agilent 1200 Series liquid chromatograph with a Zorbax Eclipse XDB-C18 (Agilent, 4.6 × 50 mm, 1.8 µm particle size) column was used for identification of the unknown pterin. The elution profile consisted of a 10 min linear gradient from 95% solvent A (25 mM ammonium acetate) and 5% solvent B (methanol) to 35% solvent A and 65% solvent B at 0.5 mL/min. MS data were acquired in both positive mode and negative mode with electrospray ionization (ESI) at 4500 V and -4500 V, respectively, and a temperature of 400 °C. The curtain gas was set at 35, ion source gas 1 was 60, and ion source gas 2 was 50. For collision-induced fragmentation of the target ion (MS/MS) in negative mode (m/z=266), the collision energy was -30 V. Analyst software (Applied Biosystems/MDS SCIEX) was used for system operation and data processing. Chemically synthesized methylated pterins were utilized for comparison.

Purification and enzymatic assay of a His_6-PruA protein. The *pruA* coding sequence was PCR amplified and fused downstream of and in frame with the His_6-tag coding sequence in pET-15b cleaved at the *Nde*I and *Bam*HI restriction sites. The

resulting plasmid pNF034 was transformed into chemically competent *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) and the cells were grown at 37°C with shaking in 200 mL LB broth supplemented with 100 µg/mL ampicillin. When the culture reached an OD_{600} of 1.0, recombinant protein expression was induced by the addition of 28 mM lactose, followed by an additional 4 hrs incubation. , Cells were harvested by centrifugation (5,000 rpm, 20 min) and stored at -20°C. For purification, 2 g (half of above culture) of cells containing His_6-PruA tag were thawed on ice, resuspended in 5 mL extraction buffer (50 mM TES, 10 mM $MgCl_2$,10 mM DTT, pH 7.0) and lysed by sonication. After centrifugation (10,000 rpm, 10 min) much of the overexpressed PruA was insoluble, but some remained soluble based on SDS-PAGE analysis. Therefore, the supernatant was loaded onto a column containing 3 mL TALON Metal Affinity Resin (Clontech) equilibrated in binding buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10% glycerol, 10 mM imidazole, pH 8). Step gradient elution was carried out with 4 mL of binding buffer preparations containing 50 mM imidazole, 250 mM imidazole, 500 mM imidazole, and 1 M imidazole. PruA was present in the 250 mM imidazole fraction based on SDS-PAGE analysis and MALDI-MS of the excised band. Purified protein was dialyzed into 50 mM Na_2HPO_4 + 100 mM NaCl + 10% glycerol, pH 8. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard and the enzyme was aliquoted and stored at -80°C for future use.

The H_6-PruA preparation was tested for enzymatic activity with dihydromonapterin (H_2MPt) for pteridine reductase assays. Monapterin at 1 mM in 1M NaOH was mixed with ~5 mg zinc dust (11). After incubation at room temperature for 2 hrs, the mixture was centrifuged to remove excess zinc dust. The solution was adjusted

to neutral pH with HCl for subsequent enzyme assays. The concentration of the dihydromonapterin was determined at pH 1 using an extinction coefficient of 4470 M^{-1} cm^{-1} at 362 nm (12). PruA pteridine reductase activity was determined spectrophotometrically by measuring NADPH consumption in the presence of various pterin and folate substrates and using previously determined extinction coefficients for the NADPH-pterin oxidation-reduction pair (13). 1 mL assays were performed at room temperature in reaction buffer (100 mM KH_2PO_4, pH 6 with 100 µM NADPH, 50 µM pterin substrate) and 5.25 µg PruA. Reductase activities were measured using a continuous assay initiated with the addition of PruA and monitoring the decrease in absorbance at 340 nm over 3 min. Control assays lacking PruA were carried out to correct for non-enzymatic oxidation of NADPH

Supplemental Material and Methods References

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