

Characterisation of PigC and HapC, the prodigiosin synthetases from *Serratia sp.* and *Hahella chejuensis* with potential for biocatalytic production of anticancer agents

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Supplementary Material

Synthetic Studies: General Methods

All organic solvents used were freshly distilled. Solvents and reagents for anhydrous reactions were dried by conventional methods prior to use. Milli-Q deionised water was used in all chemical reactions and biochemical work. NMR Spectra were recorded in deuteriated solvents (as specified) using a Bruker AM/DPX-400 spectrometer. Chemical Shifts (δ) are quoted in parts per million (ppm) and referenced to residual solvent peaks. Coupling constants (J) are reported in Hertz to the nearest 0.5 Hz.

High resolution mass spectra were obtained on a Waters LCT Premier spectrometer or a Bruker Daltonics FTLCR BioApex II 4.7e spectrometer using electrospray ionisation.

IR spectra were recorded neat on a diamond/ZnSe plate using a Perkin-Elmer Spectrum One FT-IR Universal Attenuated Transmittance Reflectance (ATR) sampling accessory spectrometer with internal referencing.

UV-Vis spectra were recorded on a Varian Cary 100 Bio Spectrophotometer with a Peltier temperature-controlled 6-cell holder using 1 cm path length using either 1 ml quartz cuvettes or polystyrene disposable cuvettes.

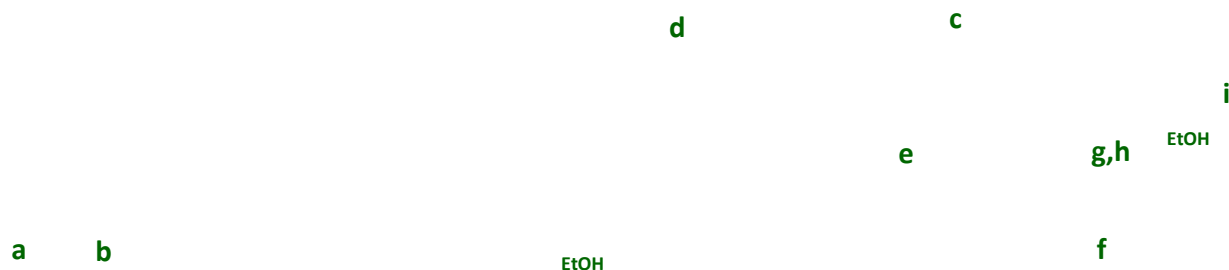
Synthesis

MBC **5** and its derivatives **6-12** were synthesised as previously described¹. 3-Acetyloctanal and MAP **4** were synthesised as previously described.² The hexyl and octyl analogues of MAP were made in the same way except that hexyl and octyl iodide were used in the first step in place of pentyl iodide.

1,2-Dimethyl-3-pentyl-1H-pyrrole **14** (N-Methyl-MAP)

To a solution of 3-acetyloctanal (500 mg, 2.94 mmol) in Et₂O (10 ml) was added a solution of 8 M ethanolic MeNH₂ (1.83 ml, 14.70 mmol) in Et₂O (10 ml). The reaction mixture was stirred at RT for 2 hr and then the solvent was evaporated under reduced pressure carefully so as not to lose the volatile product and the residue was subjected to purification by bulb-to-bulb distillation, to give *N*-methyl MAP **14** (389 mg, 80%) as a colourless oil. R_f 0.62 (acetone:hexanes; 1.5:8.5); ν_{\max} (neat) cm⁻¹ 2956m, 2925s, 2856m; δ_H (400 MHz, CDCl₃) 0.93 (3H, t, J 7.0, CH₃), 1.34-1.40 (4H, m, CH₂CH₂), 1.55 (2H, m, CH₂), 2.15 (3H, s, 2-CH₃), 2.40

(2H, t, J 7.6, 3-CH₂), 3.52 (3H, s, N-CH₃), 5.96 (1H, d, J 2.7, 4-H), 6.50 (1H, d, J 2.7, 5-H); δ_C (100 MHz, CDCl₃) 9.35 and 14.04 (CH₃), 22.58, 26.40, 31.16 and 33.61 (CH₂), 31.78 (N-CH₃), 106.71 (C-4), 119.29 (C-5), 120.37 (C-3), 124.53 (C-2); HRMS, m/z found 165.1517, C₁₁H₁₉N M^+ requires 165.1512.



2-Methyl-3-pentylthiophene **17** and 2-methyl-3-pentylfuran **18**

To a solution of 3-acetyloctanal (500 mg, 2.94 mmol) in THF (10 ml) was added Lawesson's reagent (594 mg, 1.47 mmol) and the reaction mixture stirred at RT for 14 hr. The solvent was then evaporated under reduced pressure and the crude product was filtered through a short silica column eluted with 100% petroleum ether to give a mixture of *thiophene* **17** and *furan* **18** in 5:1 ratio (350 mg, 72%) as a colourless oil. No separation of the two compounds was seen on TLC, R_f 0.69 (acetone:hexanes, 1.5:8.5). ν_{\max} (neat) cm⁻¹ 2958m, 2927s, 2857m, 2854w and 2749w.

For thiophene **17**: δ_H (400 MHz, CDCl₃) 0.94 (3H, t, J 6.6, CH₂CH₃), 1.36-1.42 (4H, m, CH₂CH₂), 1.59 (2H, m, CH₂), 2.39 (3H, s, 2-CH₃), 2.54 (2H, t, J 7.7, 3-CH₂), 6.84 (1H, d, J 5.1, 4-H), 7.01 (1H, d, J 5.1, 5-H); δ_C (100 MHz, CDCl₃) 11.36 and 14.03 (CH₃), 24.71, 28.16, 30.23 and 31.63 (CH₂), 111.51 (C-2), 120.63 (C-4), 128.80 (C-5), 139.57 (C-3); HRMS, m/z found 168.0972, C₁₀H₁₆S M^+ requires 168.0967.

For furan **18**: δ_H (400 MHz, CDCl₃) 0.92 (3H, t, J 6.6, CH₂CH₃), 1.28-1.34 (4H, m, CH₂CH₂), 1.54 (2H, m, CH₂), 2.24 (3H, s, 2-CH₃), 2.35 (2H, t, J 7.7, 3-CH₂), 6.22 (1H, d, J 1.7, 4-H), 7.24 (1H, d, J 1.7, 5-H); δ_C (100 MHz, CDCl₃) 11.41 and 12.82 (CH₃), 22.56, 30.09, 31.47 and 41.37 (CH₂), 119.04 (C-3), 132.27 (C-4), 138.27 (C-5), 146.99 (C-2); HRMS, m/z found 152.1198, C₁₀H₁₆O M^+ requires 152.1196.



Biochemical Studies: Materials and Methods

Growth media, buffers and stock solutions used in this study were made up as follows:

Luria Bertani (LB) medium (per litre): 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 5 g NaCl

Luria Bertani -Sorbitol medium (LB-sorbitol): As above plus 0.25 M sorbitol

Luria Bertani Agar (LBA): As for LB plus 16 g agar

Peptone Glycerol Agar (PGM agar): 5 g Bacto Peptone, 10 ml glycerol, 16 g agar

Resolving gel mix* (8% gel, 10 ml): 2.5 ml 1.5 M Tris (pH 8.8), 2.7 ml 30% acrylamide/bis-acrylamide, 100 µl 10% sodium dodecyl sulphate (SDS), 4.6 ml H₂O, 100 µl ammonium persulfate (APS), 6 µl *N,N,N',N'*-tetramethylethylenediamine (TEMED). Complete polymerization takes about 30 min.

Stacking gel mix (5% gel, 5 ml): 630 µl 1 M Tris (pH 6.8), 830 µl 30% acrylamide/bis-acrylamide, 50 µl 10% SDS, 3.4 ml H₂O, 50 µl APS, 5 µl TEMED. Complete polymerization takes about 30 min.

5x SDS gel-loading buffer* (10 ml): 125 mM Tris-HCl (pH 6.8), 500 mM Dithiothreitol (DTT), 10% (w/v) SDS, 0.5% Bromophenol blue, 50% (v/v) Glycerol. After DTT was added the solution was stored at 4 °C for at most 7 days.

1xTris-glycine electrolysis buffer: 25 mM Tris-base, 250 mM glycine, 0.1% (w/v) SDS.

Coomassie stain: 0.025% Coomassie brilliant blue (R-250), 40% (v/v) methanol, 7% (v/v) acetic acid

De-stain: 40% (v/v) methanol, 7% (v/v) acetic acid

Lysis buffer (pH 8.0): 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole

Wash buffer (pH 8.0): 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 20% glycerol (v/v)

Elution buffer (pH 8.0): 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole

Storage buffer-A (pH 8.0): 20 mM Tris-HCl (pH 7.0), 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% Glycerol (v/v)

Storage buffer-B (pH 7.0): 20 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% Glycerol (v/v)

Western Blot Transfer Buffer (per Litre): 14.4 g glycine, 3.03 g Tris-base, 150 ml methanol

Western Blot Wash Buffer: 1 ml Tween 20, 1000 ml phosphate-buffered saline (PBS)

Western Blot Blocking Solution: 5% Dried milk in WB-wash buffer

50x TAE (Tris-base + Acetic acid + EDTA) buffer (1000 ml): 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA [pH 8.0]

0.8% Agarose gel: 0.8% Agarose in 1x TAE buffer

10x Agarose gel loading buffer: 2.5 g Orange G, 600 ml 50% (v/v) glycerol

Chemicals and media were purchased from Sigma, Oxoid, BDH, Riedel-de-Haën, Fisons Scientific Equipment Supplies, Boehringer Mannheim, Melford laboratories, BIORAD, and Difco. All media and solutions were made up using deionized water, and sterilized for 20 min at 121 °C [15 p.s.i. (pound per square inch)], except where indicated.

Ampicillin (Amp) was filter-sterilized through 0.22 µm membrane filters from Millipore. The stock solution (100 mg/ml) was stored at 4 °C for no longer than one month and was used at a final working concentration of 100 µg/ml.

The bacterial strains used in this study were as follows:

Strain	Genotype/Phenotype and Construction	Source/Reference
<i>E. coli.</i>		
BL21	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i> <i>λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5</i>	Lab Stock
<i>Serratia</i>		
<i>Serratia</i> 39006	Wild type, Car ⁺ , Pig ⁺	Bycroft <i>et al.</i> ⁵
<i>pigCΔ</i>	In-frame <i>pigCΔ</i> (4910–7508, 877 aa Δ)	Williamson <i>et al.</i> ²

Usual growth temperatures were 37 °C for *E. coli*, 30 °C for *Serratia* strains, unless specified otherwise. Bacterial strains were routinely grown in liquid culture in Luria Bertani (LB) Broth containing appropriate supplements, in glass or plastic universals or conical flasks. Cultures were incubated at the desired temperature overnight. Adequate aeration was achieved by the use of a roller (for glass and plastic universals) at 120 rpm or orbital shakers (for conical flasks) at 300 rpm. The volume of the flask was at least 10 times the volume of the culture, unless otherwise stated. Culturing on solid media was achieved using peptone glycerol media (PGM) agar or LBA plates containing appropriate supplements, unless specified otherwise. Plates were incubated overnight at the desired temperature.

For short term storage (2-4 weeks), all bacterial strains were kept on LBA plates containing appropriate antibiotic or other supplements, sealed with PVC tape, at 4 °C. For long term preservation of all bacterial strains, overnight cultures (grown in LB with appropriate antibiotic or other supplements) were mixed with an equal volume of 50% glycerol and stored at -80 °C.

Plasmids

Plasmids used in this study were as follows. Plasmids were stored in sterile, deionized water at -20 °C.

Plasmids	Description	Source/Reference
pQE80L	6xHis fusion expression vector, Amp ^R	Qiagen

pTM33	pQE80L- <i>pigC</i>	This study
pTM34	pQE80L- <i>hapC</i>	This study
pTM35	pQE80L- <i>pigC</i> E281A	This study
pTM36	pQE80L- <i>pigC</i> R295A	This study
pTM37	pQE80L- <i>pigC</i> H840A	This study
pSRC1-B	pQE80L- <i>redH</i> -	This study
pSRC1-C	pQE80L- <i>redH</i> (duplicate)	This study

3.1.7. Oligonucleotide primers and DNA sequencing

The oligonucleotides used in this study are shown below. Oligonucleotides were synthesized at the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge. Plasmid DNA to be used for sequencing was prepared using the QIAprep Plasmid Kits according to the manufacturer's instructions. 10 μ l of plasmid DNA was prepared in sterile water at a concentration of 100 ng μ l⁻¹ per sequencing reaction. Oligonucleotide primers were prepared in sterile water at a concentration of 10 pM.

Primers	Sequence 5'-3'	Target
NW208	CCTGAATTCGAGGAGACACCCGCCATG	4874-4898 <i>Str coelicolor</i> (EcoR1) - <i>redH</i>
NW209	GTGGGGATCCTCAGTCGGTGGTGGCTC	7499-7525 <i>Str coelicolor</i> (BamH1) - <i>redH</i>
pQEF2	CGTCTTCACCTCGAGAAATC	Forward primer for sequencing of pQE80L-constructs
pQER	GTCATTACTGGATCTATCAACAGG	Reverse primer for sequencing of pQE80L-constructs
NW357 (F)	CGGGCAAGTCACCACAGATAG	5488-5508 <i>Serratia</i> 39006 (used for <i>pigC</i> sequencing)
NW358 (R)	GAATTCGTCTGGCTGGAA	7291-7310 <i>Serratia</i> 39006 (used for <i>pigC</i> sequencing)

DNA was sequenced using the sequencing service at the Department of Biochemistry, University of Cambridge, where ABI model 373/374 DNA analysis systems were used.

Preparation of competent DH5 α cells: LB medium (5 ml) was inoculated with a single colony of freshly grown *E. coli* DH5 α and the culture was allowed to grow at 37 °C for 16 hr. A flask containing 200 ml LB was inoculated with 200 μ l of the primary inoculum. To this flask 3 ml of 1M MgCl₂ was added and the flask was incubated on a shaker at 37 °C to obtain an OD₆₀₀ of 0.4-0.5. Next, the flask was placed on ice for 10 min

and the contents were transferred to centrifuge bottle and spun at 5000 rpm, 4 °C for 15 minutes. The supernatant was discarded and pellet was re-suspended in 60 ml of chilled solution A. The culture was incubated on ice for 20 min and then spun at 5000 rpm, 4 °C for 15 minutes to get the pellet, which was again re-suspended in 12 ml of chilled solution B. An aliquot of this culture was taken in an Eppendorff vial and snap-frozen on dry ice and stored at -80 °C for future use.

Solution A: 0.6 ml 1 M MnCl₂ + 3.0 ml 1M CaCl₂ + 12 ml 50 mM MES buffer + 45 ml SD-H₂O –filter sterilized.

Solution B: 0.12 ml 1M MnCl₂ + 0.6 ml 1M CaCl₂ + 2.4 ml 50 mM MES buffer + 3.6 ml 50% glycerol + 5.25 ml SD-H₂O –filter sterilized.

Polymerase Chain Reaction (PCR) was performed using Bioline BioTaq Polymerase according to manufacturer's directions. PCR (run on a DNAEngine PCR machine) cycling parameters included 5 minutes of initial denaturation at 94 °C, following 30 cycles of denaturation (30 seconds at 94 °C), annealing (30 second at 55 °C) and extension (3 minutes at 72 °C), and a final extension for 3 minutes at 72 °C. The amplified PCR products were analysed by agarose gel electrophoresis. 0.8% (w/v) agarose gel containing ethidium bromide was run at 100V in 1X TAE buffer

Restriction digestion and ligation reactions

The amplified PCR products were purified using a gel extraction kit according to manufacturer's instructions (Qiagen). The PCR products and the pQE80L vector were digested with suitable restriction enzymes 3 hr at 37 °C. The restriction digestion reaction consisted of 28 µl of DNA, 1 µl of each restriction enzyme, 5 µl of 10X BSA (bovine serum albumin) and 5 µl of buffer (as per NEB catalogue 2007). Both the digested vector and PCR products were subjected to agarose gel electrophoresis for DNA analysis. The bands were visualised under UV light. The bands containing the DNA fragments were excised from the gel and purified using the gel extraction kit (Qiagen). The purified restriction enzyme digested PCR products and pQE80L vector were ligated using T4 DNA ligase. The ligation mix comprised of 1 µl T4 DNA ligase, 2 µl 10X ligation buffer, 2 µl pQE80L vector and 2 µl PCR product. This mix was incubated for 2 hr at 25 °C.

Transformation

(a) Heat-Shock Method (for competent *E. coli* DH5α and *E. coli* BL21): The ligation mix was transformed into competent *E. coli* DH5α cells. For each transformation 20 µl of the ligation mix was added to 100 µl of competent DH5α cells and incubated for 45 minutes on ice. Heat shock treatment was given for 2 minutes at 42 °C following immediate transfer to ice for 10 minutes. 1 ml of LB was added to each reaction and incubated for 60 minutes at 37 °C. The cells were harvested by spinning at 4000 rpm for 5 minutes and plated on suitable selective media [LBA with Amp for pQE80L clones] by re-suspending in fresh 50 µl LB. The plates were incubated overnight in 37 °C incubator (DH5α as negative control).

(b) Electroporation Method (for *Serratia* 39006 mutants): A 25 ml culture of *Serratia* 39006 mutants was inoculated from an overnight culture of an OD₆₀₀ of 0.4-0.6 in a conical flask. The culture was incubated on

ice for 30 minutes. Following incubation, the culture was centrifuged at 5000 rpm for 10 minutes at 4 °C. The cell pellet was washed twice in ice cold SD-H₂O and once in ice cold 10% glycerol. After the final wash, the cell pellet was resuspended in 200 µl of 10% glycerol and separated into 40 µl aliquots for immediate use. Each 40 µl aliquot was incubated with 5-50 ng of plasmid DNA for 10 minutes on ice in a Biorad Electroporation cuvette (0.2 cm electrode). The cell and plasmid mixture was subjected to electroporation (Bio-Rad Gene Pulsar; resistance: 200 ohms, capacitance: 25 µFD, voltage: 2.5 kV). Following electroporations, 1 ml of LB was added to the cells and the whole cellular mixture was incubated for 1 hr at 30 °C. Following incubation, the cells were centrifuged and 750 µl of the LB removed. The cells were resuspended in the remaining LB and plated on LBA with appropriate selection.

Screening and selection

Out of the many colonies obtained on the Amp-plates, 20 were selected and subjected to colony PCR using pQEF2 and pQER primers. Three of the colonies showing successful ligation were grown overnight in 5 ml LB-Amp at 37 °C (for *E. coli*) or 30 °C (for *Serratia*). Next morning, 2 ml of the culture was used to extract the DNA using Qiagen's plasmid purification kit. The DNA was resuspended in 80 µl of SD-H₂O. By restriction mapping, a diagnostic restriction digest was carried out with appropriate restriction enzymes to check for positive constructs. Plasmids with correctly sized fragments were selected and sequences confirmed by DNA sequencing.

One-dimensional gel electrophoresis and Western Blotting

Protein samples were separated on 8% 0.75 mm acrylamide gels with Precision Plus Protein™ All Blue Standards (Bio-Rad) using a Bio-Rad Mini Protean system. Gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich) according to the manufacturer's instructions or transferred to a polyvinylidene fluoride membrane using a Bio-Rad Mini Protean transfer cell. Gels were subsequently stained with Coomassie Brilliant Blue (Sigma-Aldrich) to measure the transfer efficiency. Blots were blocked, washed and incubated with anti-his-tag monoclonal antibody (Novagen) before washing and incubation with Antimouse IgG Fc-specific horseradish peroxidase-conjugated secondary antibody (Qiagen). Visualisation was by enhanced chemiluminescence stain as per manufacturer's instructions.

Fig S1 shows the SDS-PAGE gels for the membrane fractions containing 6xHis-PigC and 6xHis-HapC.

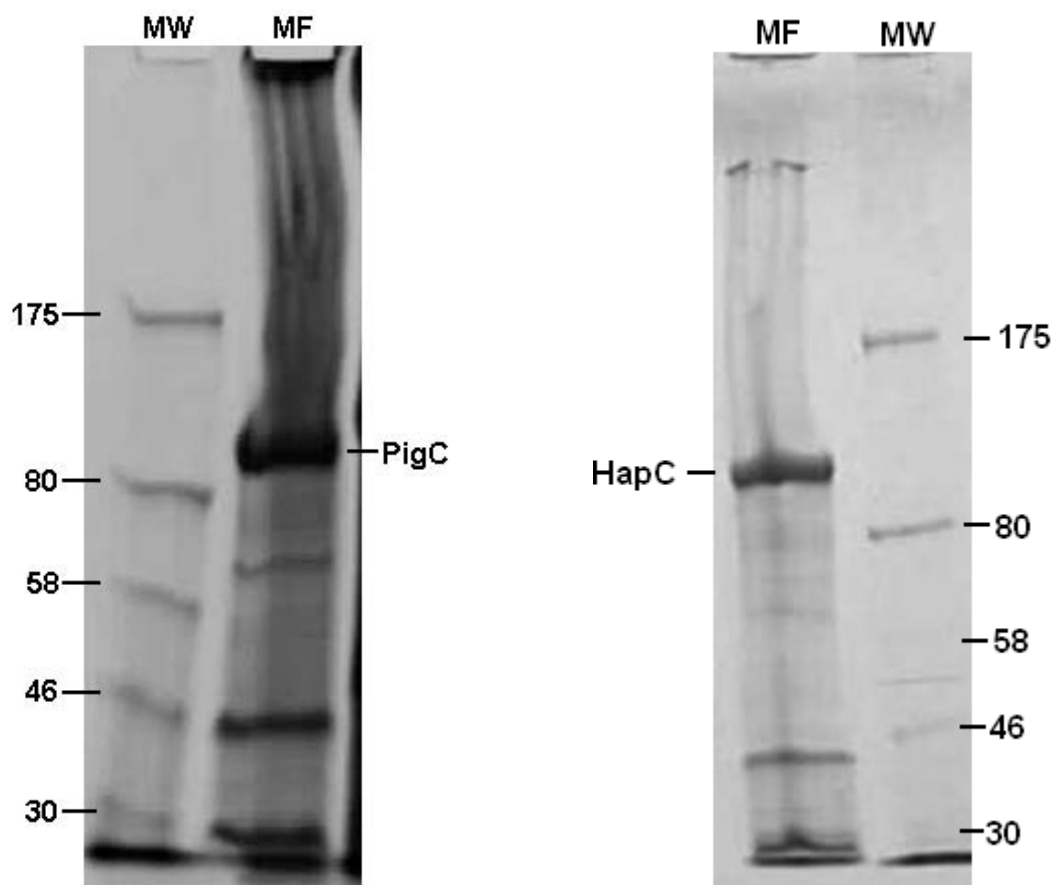


Fig. S1. SDS-PAGE gels of the membrane fractions containing 6xHis-PigC (left) and 6xHis-HapC (right). MF = membrane fraction, MW = molecular weight markers.

Plasmid construction and site-directed mutagenesis, Protein expression and purification, Microtiter plate assay and HPLC

These are described in the Experimental Procedures part of the paper. The results are shown in the paper and Figs. S2-3.

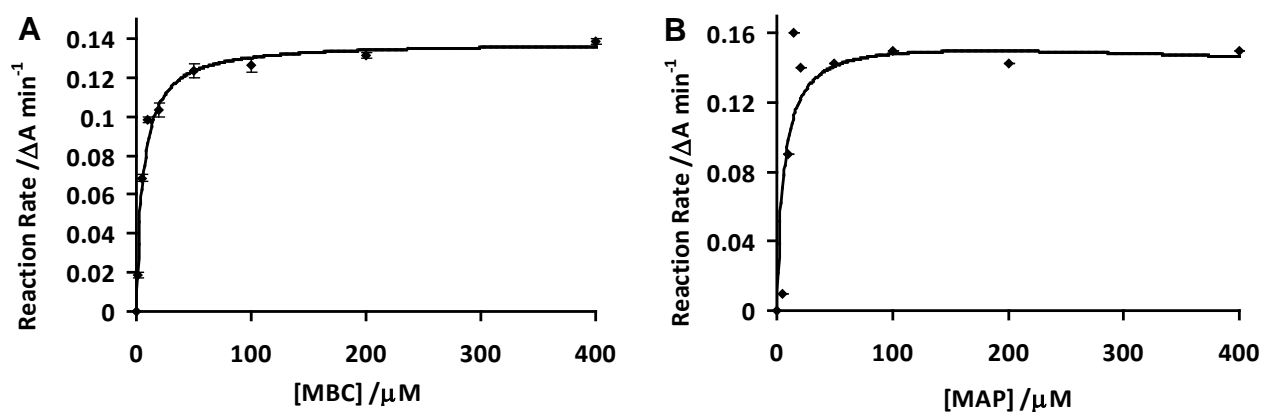


Fig. S2. Assays of HapC. (A) Rate of reaction with [MAP] = 100 μM and varying [MBC]; the line is the Michaelis Menten curve for $K_M = 6 \mu\text{M}$ and $V_{\text{max}} = 0.138$. (B) Rate of reaction with [MBC] = 100 μM and varying [MAP]; the line is the Michaelis Menten curve with substrate inhibition according to Eq. 1 (below) with $K_{\text{MBC}} = 6 \mu\text{M}$, $K_{\text{MAP}} = 13 \mu\text{M}$, $k_1 = k_2 = 0.33$. and $K_I = 150 \mu\text{M}$.

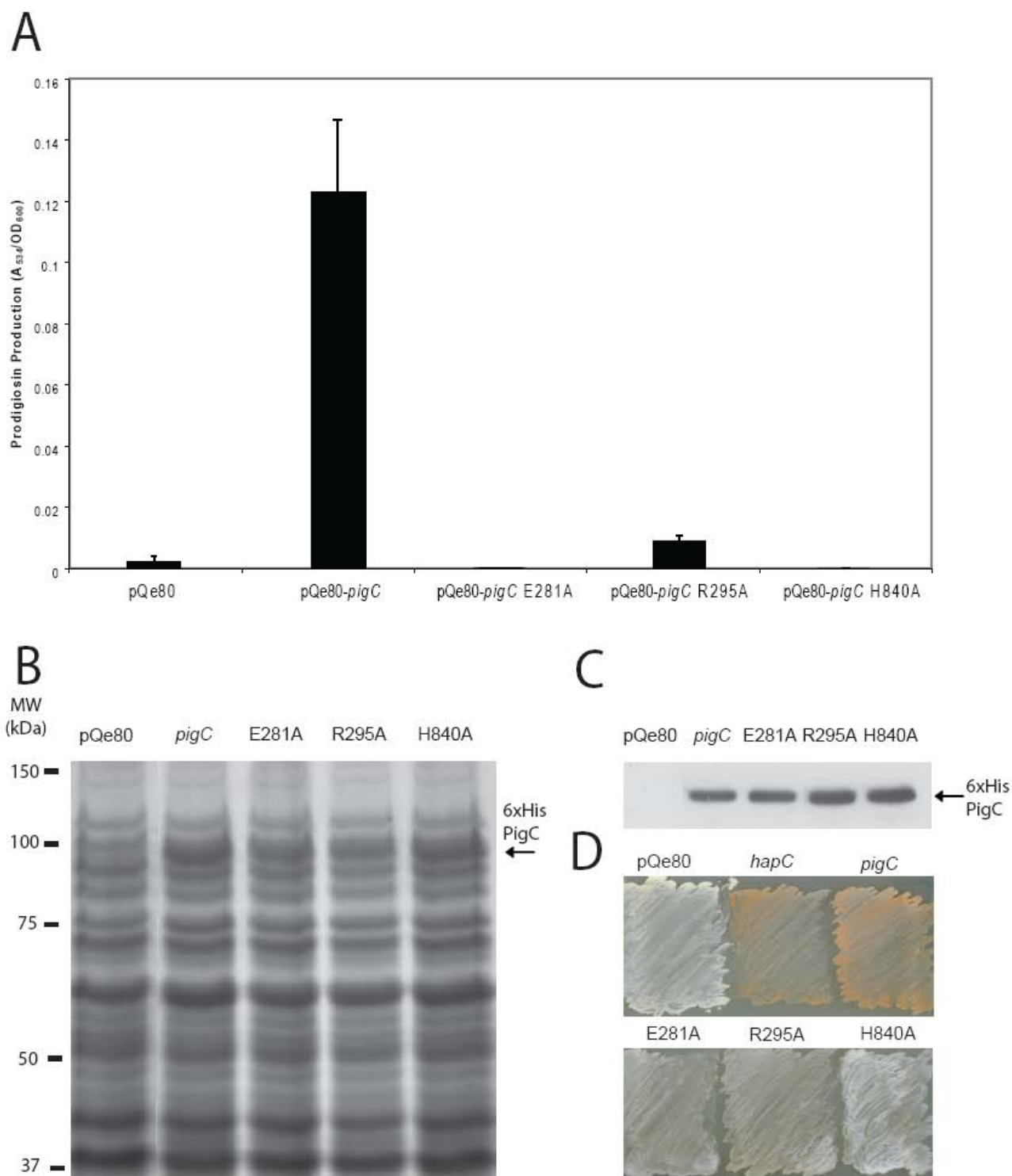


Figure S3. Reduction or elimination of prodigiosin production by site-directed mutagenesis of E281, R295 and H840 of PigC. (A) Pigment production by *Serratia* 39006 Δ *pigC* transformed with pQe80-L and mutant constructs. (B) Total cell lysates of each culture after 16 h analysed by SDS-PAGE. (C) Western blotting performed with anti-his-tag antibodies. Blot visualised by ECL. (D) Pigment phenotype of *Serratia* 39006 Δ *pigC* pQe80-L, pQe80-L-*pigC*, pQe80-L-*hapC*, pTM35 (E281A), pTM36 (R295A) and pTM37 (H840A) on LB agar + ampicillin.

Kinetic parameters for PigC with analogues of MBC

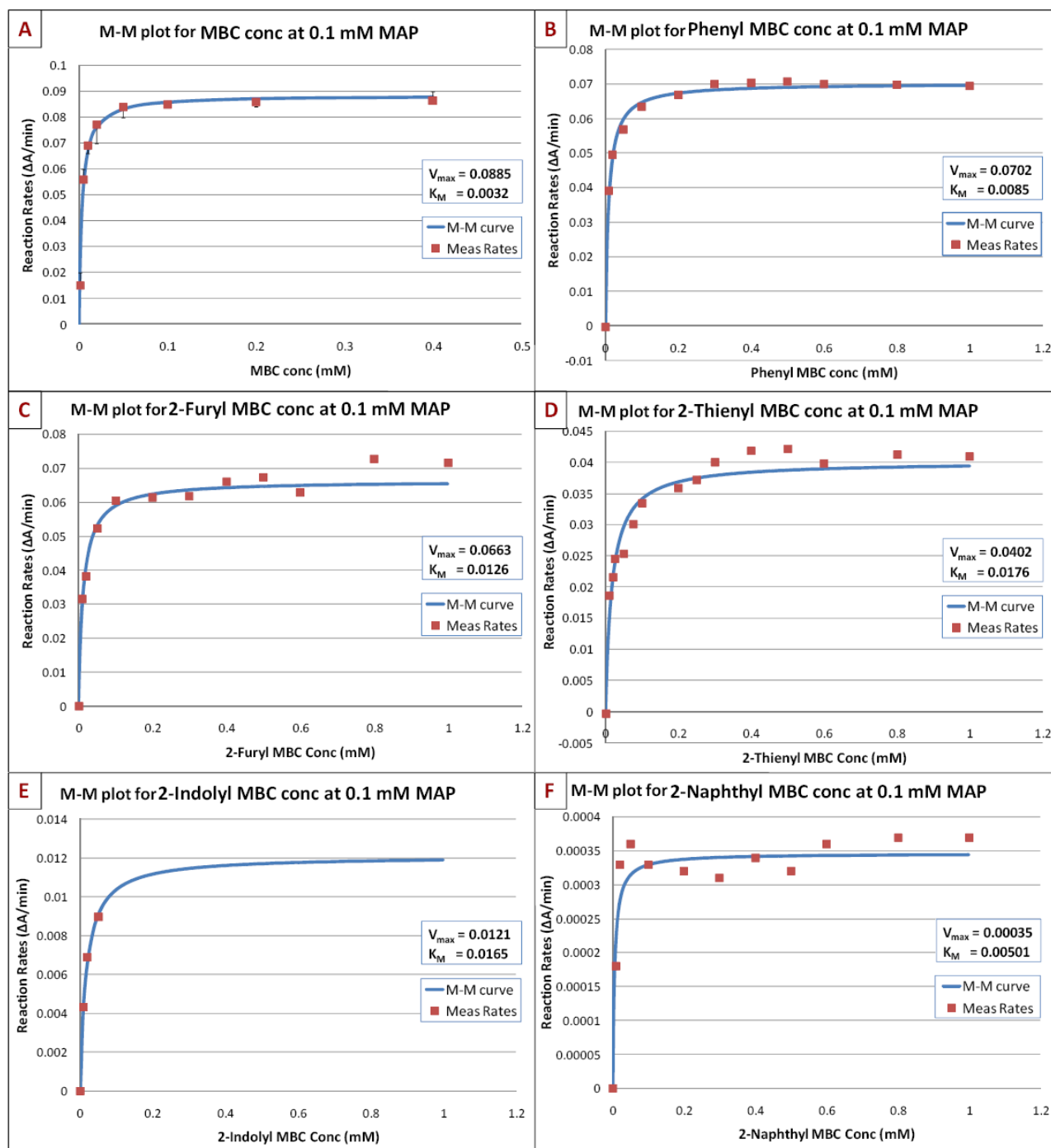


Fig. S4. Michaelis–Menten plots of rates of PigC–catalyzed reactions at a fixed MAP concentration of 0.1 mM in response to changes in concentration of (A) MBC **4**, (B) phenyl MBC **7**, (C) 2–furyl MBC **8**, (D) 2–thienyl MBC **9**, (E) 2–indolyl MBC **10**, and (F) 2–naphthyl MBC **11**. The reaction rates obtained for 2–indolyl MBC **10** beyond 0.1 mM were disregarded for plotting the Michaelis–Menten curve because of its incomplete solubility at higher concentrations under the conditions of assay.

Kinetic parameters for HapC with analogues of MBC

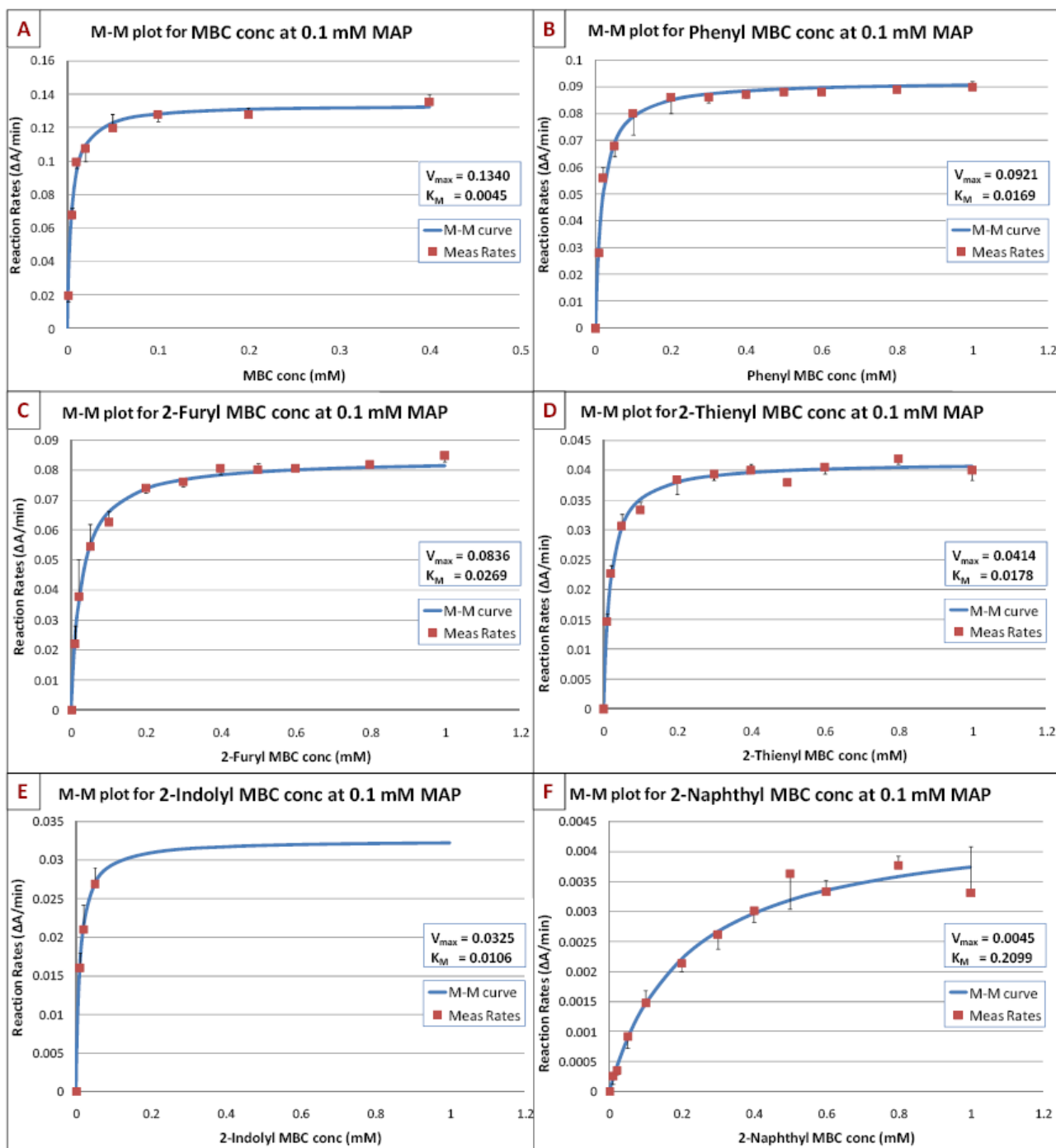


Fig. S5. Michaelis–Menten plots of rates of HapC-catalyzed reactions at a fixed MAP concentration of 0.1 mM in response to changes in concentration of (A) MBC **4**, (B) phenyl MBC **7**, (C) 2-furyl MBC **8**, (D) 2-thienyl MBC **9**, (E) 2-indolyl MBC **10**, and (F) 2-naphthyl MBC **11**. The reaction rates obtained for 2-indolyl MBC **10** beyond 0.1 mM were disregarded for plotting the Michaelis–Menten curve because of its incomplete solubility at higher concentrations under the conditions of assay.

Kinetic Models

In Figs. 2B and 2D the fitted line assumes the following kinetic model

for which the steady-state rate equation has the form

Eq. 1

Although parameters could be found that gave a good fit at $[MBC] = 100 \mu\text{M}$, the same parameters did not give a good fit at different concentrations of MBC. In order to make a single set of parameters give a reasonable fit at all concentrations of MBC, the following kinetic scheme was adopted

Eq. 2

for which the steady-state rate equation has the form

Fig. S6 shows the best fit of this equation to the data for several different concentrations of MBC.

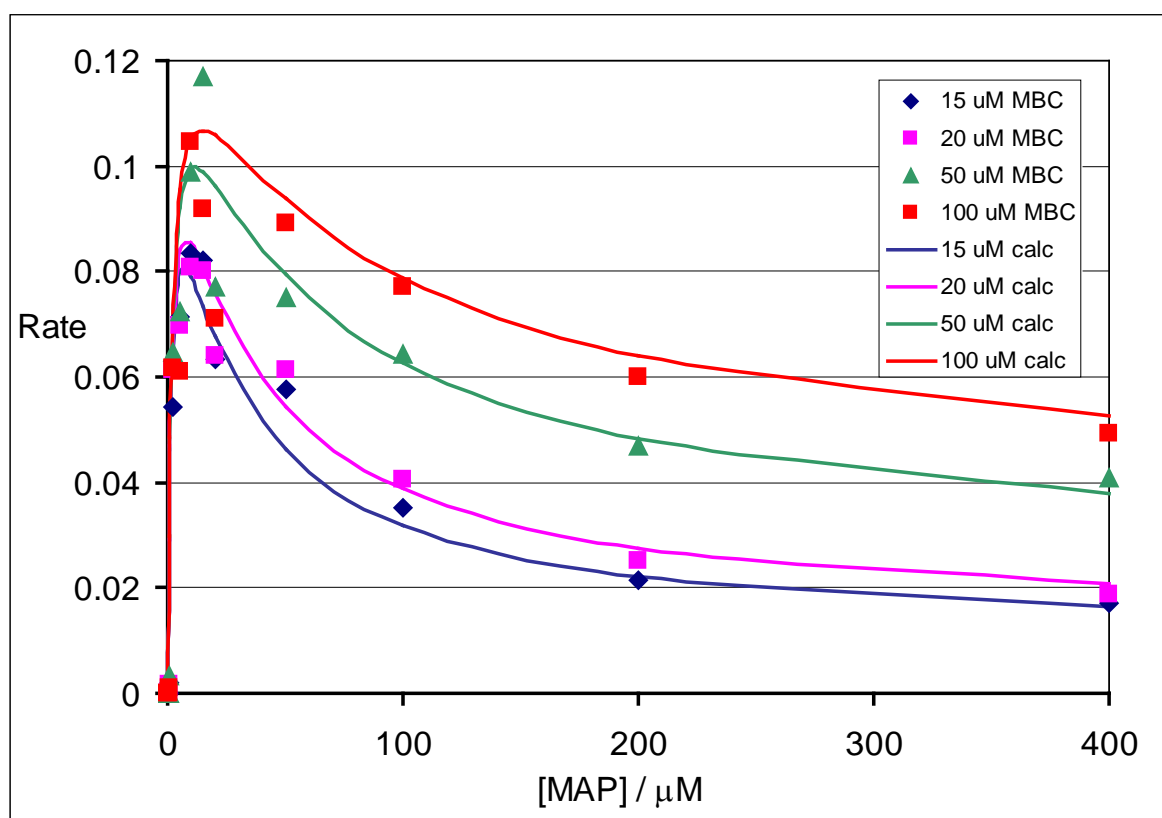
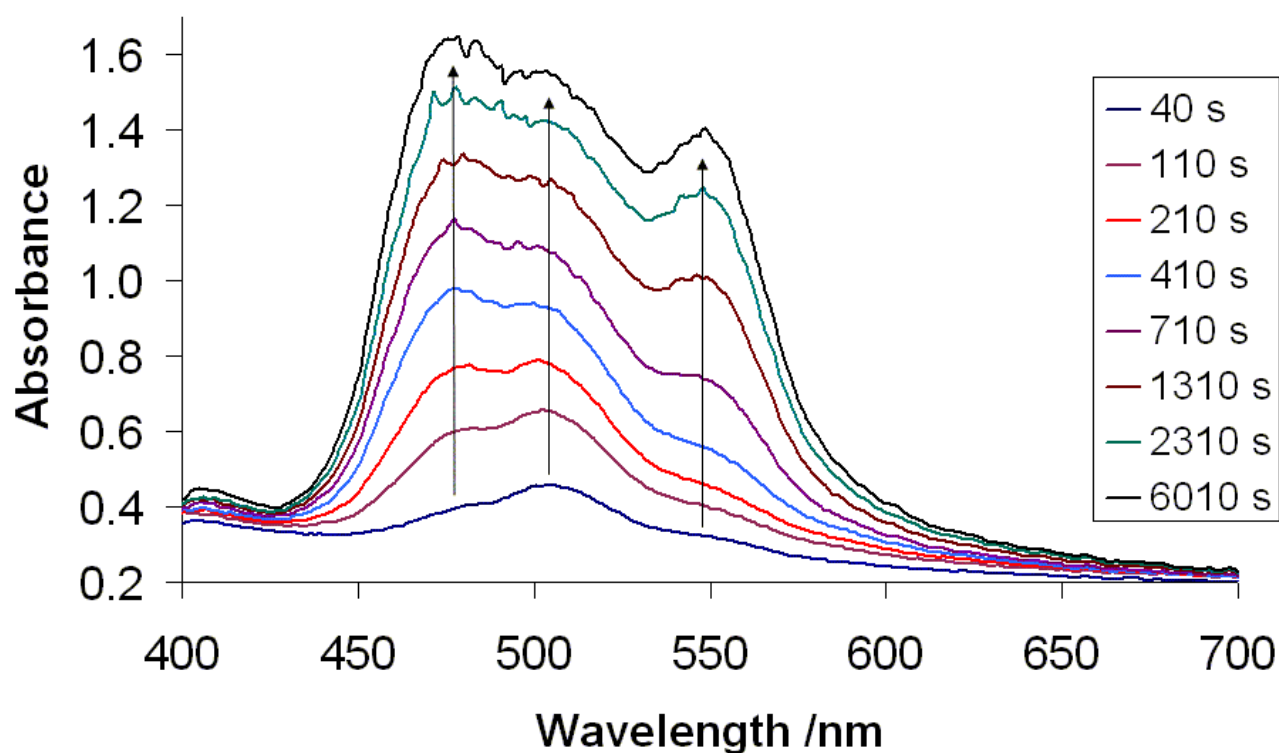


Fig. S6. Data for the rate of prodigiosin formation (in units of $\Delta A \text{ min}^{-1}$) vs [MAP] by PigC at various different concentrations of MBC. The curves are calculated using Eq. 2 and the following parameters: $K_{\text{MBC}} = 4 \mu\text{M}$, $K_{\text{MAP}} = K_{\text{I1}} = 3.5 \mu\text{M}$, $K_{\text{I2}} = 100 \mu\text{M}$, $k_1 = k_2 = 0.27 \text{ min}^{-1}$, $k_3 = 0.09 \text{ min}^{-1}$.

UV/Visible Spectra during the production of N-Methylprodigiosin from N-Methyl-MAP

(a)



(b)

Fig. S7. (a) Timecourse for the production of N-Methylprodigiosin from N-Methyl-MAP **14**. The spectra shown were taken after the time intervals shown at the right. Initially the peak near 500 nm is the major one but after long time periods there are three similar sized peaks near 480, 500 and 550 nm. The arrows show the direction of growth of the peaks with time. (b) The four rotamers of the N-methylprodigiosin; it seems likely that the *Z,E*- and *E,E*-rotamers are more stable than the *Z,Z*- and *E,Z*-rotamers due to steric hinderance of the N-methyl group in the latter two.

Alignment of Sequences

The following sequences were aligned using the NCBI Cobalt alignment tool (<http://www.ncbi.nlm.nih.gov/tools/cobalt>): CAH55631, PigC from *Serratia sp.*; ABB69075, HapC from *Hahella chejuensis*; RedH from *Streptomyces coelicolor* A3(2)]; Q9K0I2, phosphoenolpyruvate synthase from *Neisseria meningitidis*, residues 1-355 (ATP-binding domain) and 360-475 (phosphoryl-transfer domain); P22983, pyruvate, phosphate dikinase from *Clostridium symbiosum*, residues 1-357 (ATP-binding domain) and 392-520 (phosphoryl-transfer domain). Coloured in red are the residues in the ATP-binding domain of PigC that were mutated (Glu-281 and Arg-295) and coloured in blue is the histidine of the phosphoryl-transfer domain that is phosphorylated (His-840).

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CAH55631.1 MN-QPLV-----VEIS--GDKALEHHHLGGKGYSLNNL
ABB69075.1 MTYDSHI-----VEVS--GQQNSNAQSLGGKASSLNTL
NP_733678.1 MD--THVSDTTTPAGAGEGPAGPVTDPAAGAACVVTLGAGGPTGGHRGELGGKGRRLAEL
Q9K0I2.1 MA-DNYV-----IWFEN--LRMTDVERVGGKKNASLGEM
P22983.1 MA--KWV-----YKFEEG--NASMRNLLGGKGCNLAEM
*                                     *** *
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CAH55631.1 IHA----GLPVPSAFCVTAQAYQQF-----IEEV-----VPGAELTD-----
ABB69075.1 AAA----GFPVPRAYCLTVDAYASF-----LRES----GLDQWISGLDQ-----
NP_733678.1 SAA----GLPVPPAFCLTTALFDAY-----LRET----GIAAEAAGADP-----
Q9K0I2.1 ISQLTEKGVVRVPGGFATTAAYRAF-----LAHN----GLSERISAALA-----
P22983.1 TIL----GMPVPPAFCLTTALFDAY-----LRET----GIAAEAAGADP-----
* * *
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CAH55631.1 -----GDLIA---VRDAILHADIPDSLKQAIGD-----AYQHLG-----
ABB69075.1 -----QDASAFQTQIRQRIEETQLPSSLRQAIIG-----AYKDIG-----
NP_733678.1 -----RTLREIRILGTRMPASIADAVLD-----AYGSMGR-----
Q9K0I2.1 KLDVEDVAELARVVGKEIRQWILDTPFPEQLDAEIEA-----AWNKMVA-----
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P22983.1 PLLVSVRSGARASMPGMMDTILNLGLNDVAVEGFAKKTGNPRFAYDSYRRFIQMYSDVVM
*

CAH55631.1 -----HDTTIAVRSSALDEDEDGQRQSFA-----GQYETYLVHVGKSEAVL
ABB69075.1 -----AER-VAVRSSAISEDSEEHSA-----GQYDTYLVHENEETLV
NP_733678.1 --PR-----VAVRSSGLREDSAAQSFA-----GQHDTVLDVCGDEDVL
Q9K0I2.1 -----DAGGADISVAVRSSATAEDLPDASFA-----GQOETFLLNINGLDNVK
P22983.1 EVPKSHFEKI IDAMKEEKGVHFD TDLTADDLKELAEKFKAVYKEAMNGEEFPQEPKDQLM
*

CAH55631.1 HKVQACWASLWAERAAQYRHE-----SASHSAIAVILQVMVDAD-----AAGVMF
ABB69075.1 DCVKRCWGSFWTERAHAYEGRQDQRRRSSDADAPMQGIAVVIQAMIDAD-----AAGVLF
NP_733678.1 DAVLRCWASLWSDRATVYRDTDAP-----DALAVVVQEMIHTD-----VSGVMF
Q9K0I2.1 EAMHHVFASLYNDRAISYRVHK-----GFEHDI VALSAGVQRMVRSDSG---ASGVMF
P22983.1 GAVKAVFRSWDNPRAIVYRRMN-----DIPGDW-GTAVNVQTMVFGNKGETSGTGVA
* ** * * * *

CAH55631.1 TQDPLSGSTDKVVIDSCWGLGEGVVSGQVTTDSFTLTKATGE-----LCDQQIRHK---P
ABB69075.1 TADPLNGDPQRTVIESCWGLGEGVVSGQVTTDTFVIDNQKME-----LLEQTLREK---P
NP_733678.1 TVDPVNPRLVVEACQGLGEGVLSGQVSSDFFVVDDEKLE-----VVEERVRYK---V
Q9K0I2.1 TLDTESGYDQVVFVTSSYGLGENVVQAVNPDEFYVFKPTLKAGKPAILRKTMTGSKHIKM
P22983.1 TRNPSTGEKGIYGEYLINAQGEDVAVGVRTPQPITQL-----
* ** * *

CAH55631.1 NYCQRDEHGL-VTLLQTPEAKRDLPSLTPAQLQQLVTLARQAQLIYSTELEDI EWAVK--D
ABB69075.1 LMSTRAENGG-VCLRKTAENKIRAPTLNENEALALAGYANAIRSHYGREMDI EWALK--D
NP_733678.1 TKCAPLEPGR-IGMTKVDAAARSVPCLTHDQLREL GALAVRIRDLYGSEQDI EWGVR--D
Q9K0I2.1 IFTDKAEAGKSVTNVDVPEEDRNRFSITDEEITELAHYALTI EKHYGRPMDI EWGRDGLD
P22983.1 -----ENDMPDCYKQFMDLAMKLEKHFRDMQDM EFTIE--E
* * *

CAH55631.1 DKVWLLQAR PVT-TSAKTANVIYANPWESDPA---AKEG
ABB69075.1 GKIWILQAR P PITVTP TNSDNRLFADADESNSY---IRDN
NP_733678.1 GVHFLFQTR P ITTRPAAPAAPSGALSPYVAPQPEPVLNG
Q9K0I2.1 GKLYILQAR PETVKSQEEG NR-----NLRRFAING
P22983.1 GKLYFLQTR NGK-RTAPAAL-----QIACDL---VDEG
* *

CAH55631.1 | AFFSRMDTGEIVTGLMTPLGLSFCQFYQKHIHGPAIKTMGLADISHWQI
ABB69075.1 | ALFSRMDTGEIVTGLMTPLGLSFCRFYQHNHVGPAIKTMGLLDIGDSQH
NP_733678.1 | TLWSRMDIGEIVFVGLMTPLGLS FARYYQRNVHTDCAGALGVRDTGEADL
* * * * * * * * * * * * * * * * * *

CAH55631.1 | YMGYIQGYVYLNISGSAYMLRQC P PTRNEMKFTTRYATDEIDFKDYKNPYGAGVQGWYD
ABB69075.1 | YMGYLQGHVYLNISASAMLLTQC P PTRDALKFTKRYSTEEVDLNFYTNPYGKAPQGLQYL
NP_733678.1 | HMGFYQGHVYLNISYSSYLLAQCLP TRDQRHFTSRFVSEEVLDLADYENPFGTFFPGMEDL
* * * * * * * * * * * * * * * * * *

CAH55631.1 | KSCWYWLKQQVRNMRSAARTVEQMIALRQDETTRFLGLDLTAMTLQQLDQELQRIDRFFL
ABB69075.1 | KSAGYWTLYQVRNLVTAERTVRDMI SLRKRETERFLKLDLSAMSLGELNAELQRIDRYFL
NP_733678.1 | LSTVHWLQHTAREMTQMKSRSQQMVDARLYEFDRARGLDLTRMSRRELHGELHRDLAWFH
* * * * * * * * * * * * * * * * * *

CAH55631.1 | DSCAAYMPFFLQSFALYDALAQACERHIKD-GKGLQNR IKASMNLR TIEVTLGI IKLVA
ABB69075.1 | ESCAAYMPFFLQSFALYDALAE LCEKWLGD TGKGLQNR IKASLSNLR TIEVTRGVCDLAA
NP_733678.1 | DMHIGYMPYYINAFAYGLLTEL CARWLGS DGVGLQNRVKTDMSSLRTVESAKEVWSVAQ
* * * * * * * * * * * * * * * * * *

CAH55631.1 | TVNQO TELKALFEQHRADELVTLLPVHDI SRAFWQGD FDFLVEFGSRGRQEFDL S IPRW
ABB69075.1 | SVQRSSHRLRQLVLETPL EQLAQALQNDDEGARFWNND FMAFLRDY GARGRQEFELS IPRW
NP_733678.1 | AAKNDPAVLR I IKDEPLEDIARLLREDPAGQRFWDRHMEPFLRANGTRGHQEMEITHPRW
* * * * * * * * * * * * * * * * * *

CAH55631.1 | RDDPSYLLQVMKMYLQHPVDLHKKLRETELLRQDSEALFSAMSWSGRFLKLTLIKLYGM

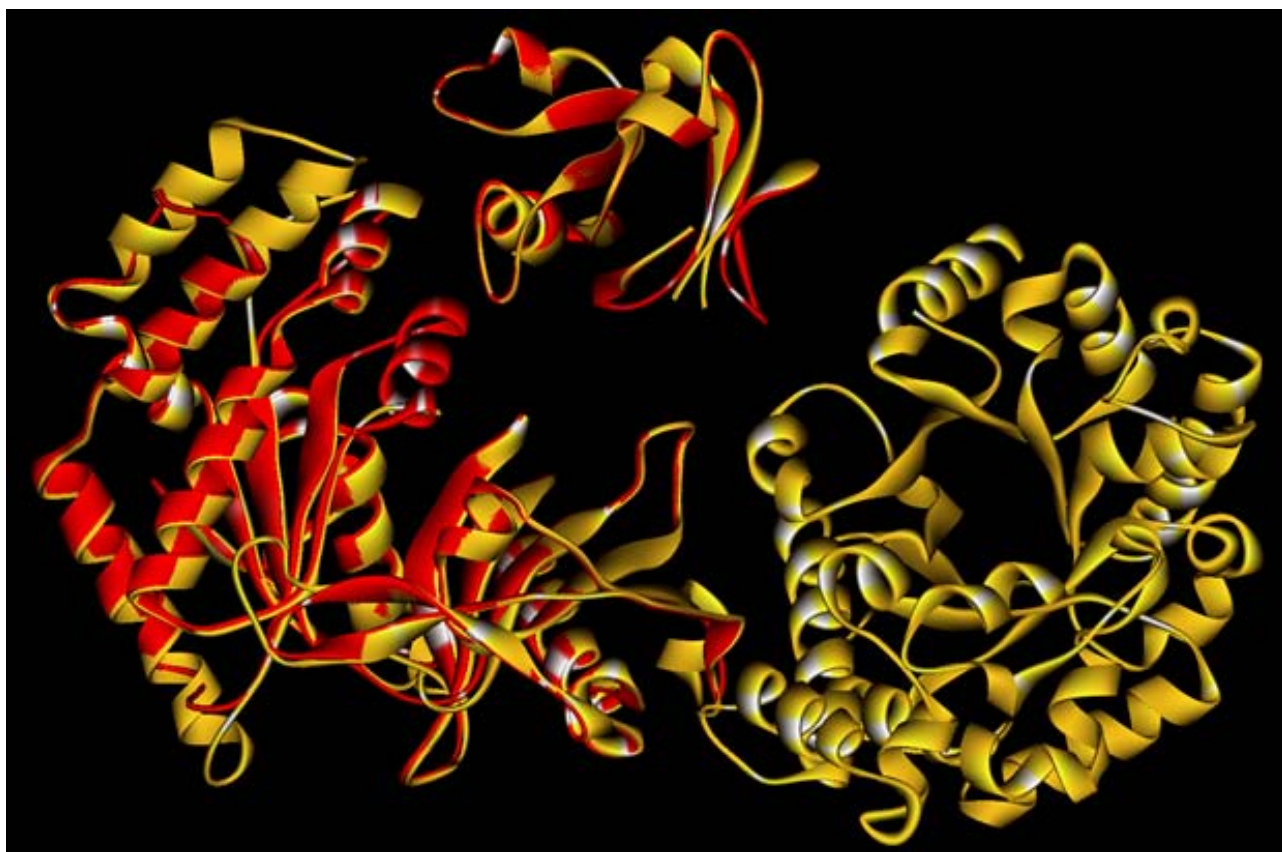


Fig. S8. Overlay of the structure of PEPS from *N. meningitidis* (2OLS) in yellow, with PHYRE-predicted structures of the N-terminal ATP-binding domain and the C-terminal phosphoryl-transfer domain of PigC in red.

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