

**SUPPLEMENTARY INFORMATION**

**One-Pot Synthesis of Adipic Acid from Guaiacol in *Escherichia coli***

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## Supplementary Materials

### General Materials and Methods

*E. coli* BL21(DE3) and *E. coli* DH5 $\alpha$  cells were obtained from Dr Louise Horsfall and Dr Janice Bramham (University of Edinburgh), respectively. The strains were stored as 1:1 v/v LB:glycerol stocks at  $-80$  °C and used as required. *E. coli* Lemo21(DE3) competent cells were purchased from New England Biolabs.

Proton nuclear magnetic resonance spectra ( $^1\text{H}$  NMR) were recorded using a Bruker AVA400, AVA500, Pro500 or AVA600 NMR spectrometer at the specified frequency at 298 K. Proton chemical shifts are expressed in parts per million (ppm,  $\delta$  scale) and are referenced to residual protium in the NMR solvent (DMSO- $d_6$  = 2.50 ppm;  $\text{CDCl}_3$  = 7.26 ppm). Carbon nuclear magnetic resonance spectra ( $^{13}\text{C}$  NMR) were recorded using a Bruker AVA400, AVA500, Pro500 or AVA600 NMR spectrometer at the specified frequency at 298K. Chemical shifts are quoted in parts per million (ppm,  $\delta$  scale) and are referenced to the carbon resonances of the NMR solvent ( $\text{CDCl}_3$  = 77.2 ppm). Coupling constants,  $J$ , are measured to the nearest 0.1 Hz and are presented as observed. Data is represented as: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, m = multiplet and/or multiple resonances), coupling constant ( $J$ ) in Hertz.

HPLC analysis was carried out using a Thermo Scientific Dionex UltiMate 3000 Series UHPLC instrument and a HyperSil Gold C18 column (150x3 mm x 3  $\mu\text{m}$ ). Analytes were detected at 206, 190, 260, and 270 nm.

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. All water used experimentally was purified with a Suez Select purification system (18M $\Omega$ .cm, 0.2  $\mu\text{M}$  filter). All NMR and HPLC solvents were purchased from commercial suppliers. For all quantitative measurements by  $^1\text{H}$  NMR spectroscopy, 1,3,5-trimethoxybenzene (TMB, 2.7 mM) was used as an internal standard. For all quantitative measurements by HPLC, caffeine (51  $\mu\text{M}$ ) was used as an internal standard.

All synthetic genes were codon-optimised for *E. coli* BL21(DE3) and then synthesised using GeneArt<sup>TM</sup> (Thermo Scientific). Oligonucleotide primers were synthesised by Integrated DNA Technologies. Recombinant plasmid DNA was purified with a Miniprep Kit (Qiagen). Chaperone plasmids were purchased from Takara Biosciences. Plasmid pET22b was obtained from Prof. Chris French (University of Edinburgh). Plasmid pETDuet-1 was purchased from Novagen. Plasmid pQLinkN was obtained from Dr Konrad Buessow (Helmholtz Centre for Infection Research in Braunschweig, Germany). All restriction enzymes were purchased from Thermo Fisher as FastDigest<sup>TM</sup> enzymes. Unless otherwise specified, all restriction enzyme digests were carried out at 37 °C using FastDigest<sup>TM</sup> Green buffer. All plasmids were sequenced by Sanger sequencing at Edinburgh Genomics (Edinburgh, UK). Taq

Polymerase (New England Biolabs) was used for all colony PCR reactions. Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used for all other PCR reactions. T4 DNA ligase (Thermo Scientific) was used for all ligation reactions. For pQLink cloning, T4 polymerase (New England Biolabs) was used in buffer 2.1 following the manufacturer's protocol. All standard Taq PCR reactions were performed using the following conditions: initial denaturation (95 °C, 30 s), 35 thermocycles (30s denaturation at 95 °C, annealing at 50-65 °C for 20 s, and extension at 68 °C for 60 s/kb), and final extension (68 °C for 10 min). All standard Phusion PCR reactions were performed using the following conditions: initial denaturation (98 °C, 30 s), 35 thermocycles (10 s denaturation at 98 °C, annealing at 50-72 °C for 20 s, and extension at 72 °C for 30 s/kb), and final extension (72 °C for 10 min).

For agarose gel electrophoresis, agarose (1% w/v) TAE gels containing a 1 kB Generuler ladder (Thermo Scientific) were run at 80 V for 45 min and visualised using SYBR Safe™. For SDS-PAGE, 12-well 12% acrylamide Bis-Tris NuPAGE gels (Thermo Scientific) containing an unstained Precision plus standard ladder (Biorad) were used to analyse samples. Gels were run in 1x MES buffer (Novagen) at 50 V for 30 min followed by 150 V for 2 h.

Optical densities of *E. coli* cultures were determined using a DeNovix DS-11 UV/Vis spectrophotometer by measuring absorbance at 600 nm. Unless stated otherwise, *E. coli* cells were cultured at 37 °C with shaking at 220 rpm.

### **Media, Strains and Culturing Conditions**

Luria Bertani Lennox (LB) Media was prepared according to the following recipe: bacto-tryptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L) in MQ H<sub>2</sub>O. LB was autoclaved at 121 °C for 20 min, cooled and stored at room temperature. LB agar was made using the same recipe but with the addition of agar (15 g/L). Terrific Broth (TB) Media was prepared according to the following recipe: yeast extract (24 g/L), tryptone (20 g/L), glycerol (4 mL/L), Potassium Phosphate Buffer (72 mM K<sub>2</sub>HPO<sub>4</sub>; 12.5 g/L), in 1 L MQ H<sub>2</sub>O. TB was autoclaved using the same protocol as LB. M9-glucose media was prepared according to the following procedure: A 5x stock solution was prepared dissolving Na<sub>2</sub>HPO<sub>4</sub> (85.5 g), KH<sub>2</sub>PO<sub>4</sub> (15 g) and NaCl (2.5 g) in 1 L of MQ H<sub>2</sub>O. The pH was adjusted to 7.4 using NaOH (5 M) and autoclaved at 121 °C for 20 min. Filter sterilised MgSO<sub>4</sub> (2 mL; 1 M), CaCl<sub>2</sub> (2 mL; 50 mM), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (3.8 mL; 2 M) and glucose (20 mL; 20% w/v) were added to a 5x buffer solution (200 mL) and the total volume adjusted to 1 L using autoclaved MQ H<sub>2</sub>O. All other reagents used in culturing were filter sterilised using a 0.22 µm filter (Millex).

All chemically competent cells were prepared via treatment with calcium chloride.<sup>1</sup> For use in protein expression and cloning, chemically competent *E. coli* BL21(DE3), *E. coli* Lemo21(DE3), *E. coli* BL21(DE3)\_pGro7, *E. coli* BL21(DE3)\_pKJE7, *E. coli* BL21(DE3)\_pTf16 and *E. coli* DH5α cells were prepared and transformed with an appropriate plasmid via heat-shock at 42 °C for 45 s. Cells were

recovered in 1 mL of SOC media for 1 h at 37 °C. Transformants were selected by plating on LB agar containing appropriate antibiotics and incubating at 37 °C overnight. A single colony was picked and grown overnight at 37 °C and 220 rpm in 10 mL of LB containing the appropriate antibiotics. The resulting overnight culture (0.5 mL) was added to 0.5 mL of 1:1 v/v water:glycerol solution, frozen in liquid nitrogen, stored at –80 °C and used as required. For all microbiological experiments, antibiotics were used at the following concentrations: 100 mg/mL ampicillin, 34 mg/mL chloramphenicol.

### **Adipic Acid Production Reactions**

A –80 °C LB:glycerol stock of *E. coli* BL21(DE3), *E. coli* Lemo21(DE3), or *E. coli* BL21(DE3) $\Delta$ *iscR*, harbouring the pAA or pAA', and pGro7, pKJE7 or pTf16 plasmids was inoculated into 10 mL of LB and grown at 37 °C (220 rpm) for 18 h. The saturated overnight culture (5 mL) was then inoculated into Terrific Broth (250 mL in a 500 mL baffled Erlenmeyer flask) containing appropriate antibiotic(s) and grown aerobically at 37 °C (220 rpm) until the cultures reached  $OD_{600} = 0.6-0.8$  (ca. 2.5-3 h). At this point, DMSO (2.5 mL of a 5M solution) was added. Cultures were then incubated at room temperature (21 °C, 200 rpm) for 30 min before being induced using IPTG (100  $\mu$ L of a 1M solution). As required, arabinose (12 mM), benzyl alcohol (10 mM), or rhamnose (10-100  $\mu$ M) were added. Following 18 h of incubation at room temperature (21 °C, 200 rpm), cultures were pelleted via centrifugation (2700 xg, 19 °C for 10 min). The cell pellet was resuspended in 50 mL of wash buffer (50 mM sodium phosphate, pH 7.2) and centrifuged at (2700 xg, 19 °C) for 10 min. The cell pellet was resuspended in 50 mL of wash buffer and 25  $\mu$ L of this was used to determine  $OD_{600}$  before being centrifuged (2600 xg 19 °C, 10 min) and resuspended in an appropriate volume of reaction buffer (50 mM sodium phosphate, pH 7.2, 3% w/v glucose, 0.3% w/v NaCl, 5 mM catechol) or M9-reaction buffer (M9 media containing 3% w/v glucose and 5 mM catechol) to give a final  $OD_{600} = 122$ . Resuspending the pellet in an appropriate volume of reaction buffer resulted in dilution of 5 mM catechol substrate due to the volume of the pellet. The resulting total volume of cell suspension was noted and used to calculate actual catechol concentration. This was used in subsequent yield calculations. For reactions at low cell density ( $OD_{600} = 12$ ), parent  $OD_{600} = 122$  cell suspensions in reaction buffer were diluted ten-fold with additional reaction buffer. For each reaction, 3 mL of cell suspension in reaction buffer were aliquoted into a 15 mL falcon tube, sealed and incubated at 37 °C (220 rpm) for the desired time period. Reactions were sampled following a 0-2 h incubation for the time-course analyses and after 24 h of incubation for all other experiments.

## Cloning

### pAA plasmid construction

The *catA* (UniProt ID O33948) and *ER* (UniProt ID G2TQU6) were synthesised with desired restriction enzyme sites appended to the 5' and 3' ends of each coding sequence. The resulting pMAT-*ER* plasmid was digested with NcoI and NotI. Following gel purification, the *ER* coding sequence was then inserted into the MCS-1 of a linearized pETDuet-1 vector using NdeI and XhoI to afford *ER*-pETDuet-1. This was then repeated to clone the *catA* coding sequence into the MCS-2 of *ER*-pETDuet-1 using NdeI and XhoI to afford *ER-catA*-pETDuet-2 (pAA), which was confirmed by Sanger sequencing. Sequencing primers used were “*CatA Seq Primer 1*”, “*CatA Seq Primer 2*”, “*Enoate Reductase Seq Primer 1*”, “*Enoate Reductase Seq Primer 2*” and “*Enoate Reductase Seq Primer 3*” (Table S2).

### *ΔiscR* BL21(DE3) strain generation

*E. coli* BL21(DE3)*ΔiscR* was generated via modification of the reported protocol by Court *et. al.*<sup>2</sup> except using the following modifications: (i) the linear CamR cassette was purified via gel extraction and the coding DNA sequence was amplified a second time via PCR, (ii) cells were washed with glycerol (10% v/v aqueous solution) when preparing electrocompetent cells and were electroporated at 2.5 kV (200 Ω, 25 μF), (iii) cells were grown for 5 h following electroporation. The pSIM27 plasmid was used for recombineering and was obtained from the Court Lab (National Institute of Health, MD, USA). The chloramphenicol resistance cassette was constructed using “*CamR Forward*” and “*CamR Reverse*” primers (Table S2) using an annealing temperature of 65 °C under standard PCR conditions (see S1.1). Knockout colonies were confirmed via colony PCR using primers “*iscR KO A*”, “*iscR KO B*”, “*iscR KO C*”, and “*iscR KO D*” (Table S2) using an annealing temperature of 50 °C under standard PCR conditions (see S1.1). Genomic DNA of *E. coli* BL21(DE3)*ΔiscR* was purified using a GenElute™ Bacterial Genomic DNA Purification kit (Sigma Aldrich) following the manufacturer’s protocol. Figure S1 demonstrates construction of the *ΔiscR* knockout strain. Primers A and B anneal upstream and downstream of the *iscR* gene, respectively. Together they yield an approximately 600 bp PCR amplicon in unmodified *E. coli* BL21(DE3) cells. Upon insertion of the CamR cassette at the *iscR* locus, an approximately 1000 bp amplicon is produced. Primers C and D anneal to the CamR cassette, producing PCR products only when the CamR cassette has been inserted into the genome.

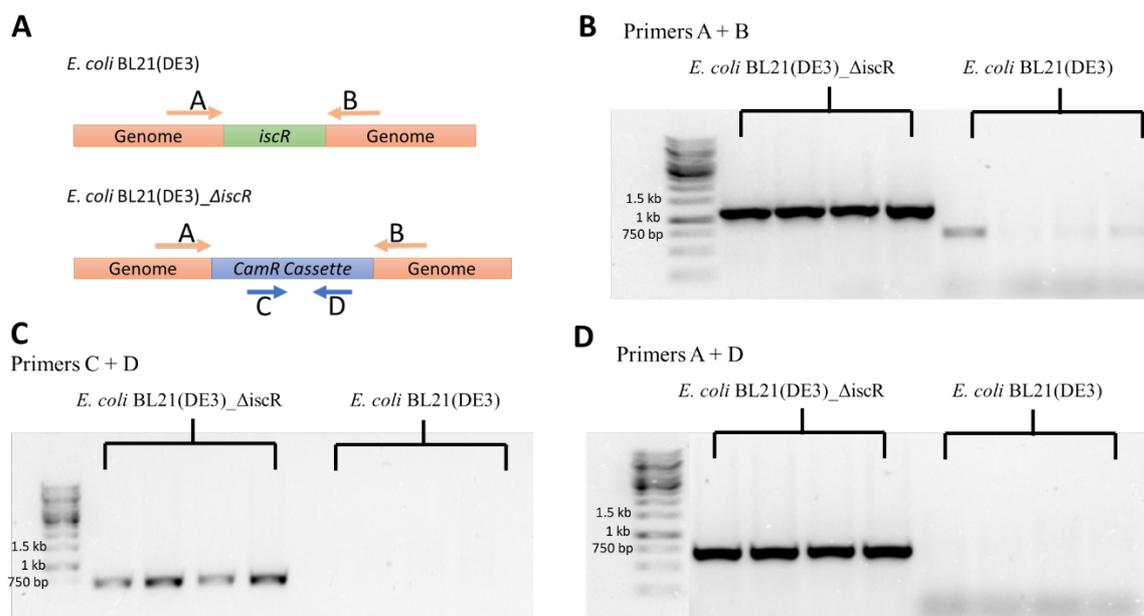


Figure S1. Confirming the  $\Delta$ iscR knock-out by colony PCR. A) Schematic showing primer design. B-D) Analysis of colony PCR reactions by agarose gel electrophoresis.

### pAA' plasmid construction

Coding sequence templates for pAA' construction are as follows: *gcoA* from *Amycolatopsis sp.* (UniProt ID P0DPQ7), *gcoB* from *Amycolatopsis sp.* (UniProt ID P0DPQ8), *gcoA* from *Rhodococcus rhodochrous* (GenBank ID MK007067.1), and *gcoB* from *Rhodococcus rhodochrous* (GenBank ID MK007068.1). Following PCR extension of the coding sequences (Table S1), the synthesized genes were sub-cloned into the pQLinkN vector using BamHI and NotI. The pAA' plasmid was assembled using the pQLink ligation independent cloning (LIC) method previously described by Büssow *et al.*<sup>3</sup> After Sanger sequencing, the pAA' plasmid was transformed into chemically competent *E. coli* BL21(DE3) cells.

Table S1:

Gene/Template	Forward Primer	Reverse Primer	Annealing Temp.
<i>catA</i> ( <i>P. putida</i> )	catA F pQLinkN	catA R pQLinkN	64 °C
<i>bcER</i> ( <i>B. coagulans</i> )	ER F pQLinkN	ER R pQLinkN	59 °C
<i>gcoA</i> ( <i>Amycolatopsis sp.</i> )	Aa-gcoA F pQLinkN	Aa-gcoA R pQLinkN	65 °C
<i>gcoB</i> ( <i>Amycolatopsis sp.</i> )	Aa-gcoB F pQLinkN	Aa-gcoB R pQLinkN	66 °C
<i>gcoA</i> ( <i>R. rhodochrous</i> )	Rr-gcoA F pQLinkN	Rr-gcoA R pQLinkN	65 °C
<i>gcoB</i> ( <i>R. rhodochrous</i> )	Rr-gcoB F pQLinkN	Rr-gcoB R pQLinkN	68 °C
Colony PCR	pQTEV3U	pQTEV3L	59 °C

## Oligonucleotide primers

Table S2:

Primer	Sequence
catA F petDuet1	CAGAGCTCACATATGACCGTGAAAATTAGCC
catA R petDuet1	CTGACGATCTCGAGTCATCATGAATTCCTTCTTGCAGTGCACG
catA F pQLinkN	CAGAGCTCAGGATCCATGACCGTGAAAATTAGCCATAACC
catA R pQLinkN	CTGACGATGCGGCCGCTCATCATGAATTCCTTCTTGCAGTGCA C
ER F pQLinkN	CAGAGCTCAGGATCCATGGGAAAATACAAGAACTGT
ER R pQLinkN	CTGACGATGCGGCCGCTCATCACGCGGCTGCTAAATTGGCTGCC ACTTC
<i>Aa-gcoA</i> F pQLinkN	CAGAGCTCAGGATCCATGACCACCACCGAACGTC
<i>Aa-gcoA</i> R pQLinkN	CTGACGATGCGGCCGCTCATCAAACCTCCCAGGTCACATGCAG
<i>Aa-gcoB</i> F pQLinkN	CAGAGCTCAGGATCCATGACCTTTGCAGTTAGCGTTGG
<i>Aa-gcoB</i> R pQLinkN	CTGACGATGCGGCCGCTCATCAGCTTGCCGGTGTGAATTTTTCA C
<i>Rr-gcoA</i> F pQLinkN	CAGAGCTCAGGATCCATGACCAGCACACTGAGCTG
<i>Rr-gcoA</i> R pQLinkN	CTGACGATGCGGCCGCTCATCAAATTTCCCAACGTGCATGCA
<i>Rr-gcoB</i> F pQLinkN	CAGAGCTCAGGATCCATGGGTGATCTGACCATGAGCTATACCCT GA
<i>Rr-gcoB</i> R pQLinkN	CTGACGATGCGGCCGCTCATCAACCTGCCGGTGAAAACGTTCA CGACGAATACGAC
T7-lac promoter F	CGACTCACTATAGGGGAATTGTG
T7-lac terminator R	CTAGTTATTGCTCAGCGGTGG
catA seq primer 1	GTACACCGCGTACCATTGAAGG
catA seq primer 2	CCAGCAGGTCCAGACATTCTTG
ER seq primer 1	CGTTATGCAATGGCACCGATG
ER seq primer 2	GCATTGTGCGCAAATTGGTG
ER seq primer 3	GAATATTCAAGCCTGGGTTGTGC
pQTEV3U	TATAAAAATA GGCGTATCAC GAGG
pQTEV3L	CCAGTGATTT TTTTCTCCAT TTT
CamR F	AAATACCCGACTAAATCAGTCAAGTAAATAGTTGACCAATTTAC TCGGGATGTGACGGAAGATCACTTCG
CamR R	TTAAGCGCGTAACTTAAACGTCGATCGCGTCTTGTGTGCGGGTGC GTGGCGACCAGCAATAGACATAAGCG
iscR KO A	ACGCCACGATAAAAAAATGGC
iscR KO B	GTGTTTACGGAGTATTTAGCAC
iscR KO C	GTATGGCAATGAAAGACGGT
iscR KO D	AAAACCTGGTGAAACTCACCC

## Microscopy

For each imaging experiment, reaction aliquots (1  $\mu\text{L}$ ) were added to MQ  $\text{H}_2\text{O}$  (5  $\mu\text{L}$ ) on a microscope slide coated with agarose (1% w/v in  $\text{H}_2\text{O}$ ). Images were acquired directly at a resolution of 0.1  $\mu\text{M}$  per pixel using a Zeiss Axiovert 200 fluorescence microscope equipped with a Photometrics Evolve<sup>TM</sup> 512 EMCCD camera.

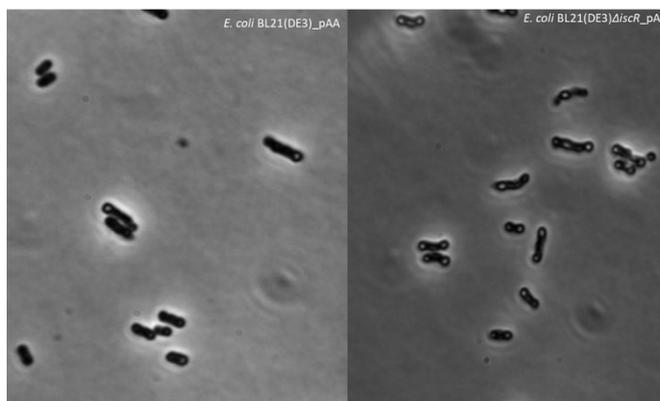


Figure S2. Microscopy images of *E. coli* BL21(DE3)\_pAA and *E. coli* BL21(DE3) $\Delta$ iscR\_pAA cells

## Quantification Methods

### <sup>1</sup>H NMR

For NMR analysis, reaction aliquots (900  $\mu\text{L}$ ) were pelleted via centrifugation (1666  $\times g$ , 2 min). The supernatant was transferred to a 1.5 mL Eppendorf tube, frozen at  $-80\text{ }^\circ\text{C}$  and lyophilised ( $-50\text{ }^\circ\text{C}$ , 16 h, 0.002 mbar). Dried samples were re-dissolved in  $d_6$ -DMSO (750  $\mu\text{L}$ ) containing TMB (2.7 mM) and filtered through a cotton wool plug into an NMR tube. Adipic acid was quantified using characteristic proton resonances at 2.20 ppm and 1.48 ppm. TMB peaks are at 6.08 ppm and 3.70 ppm.

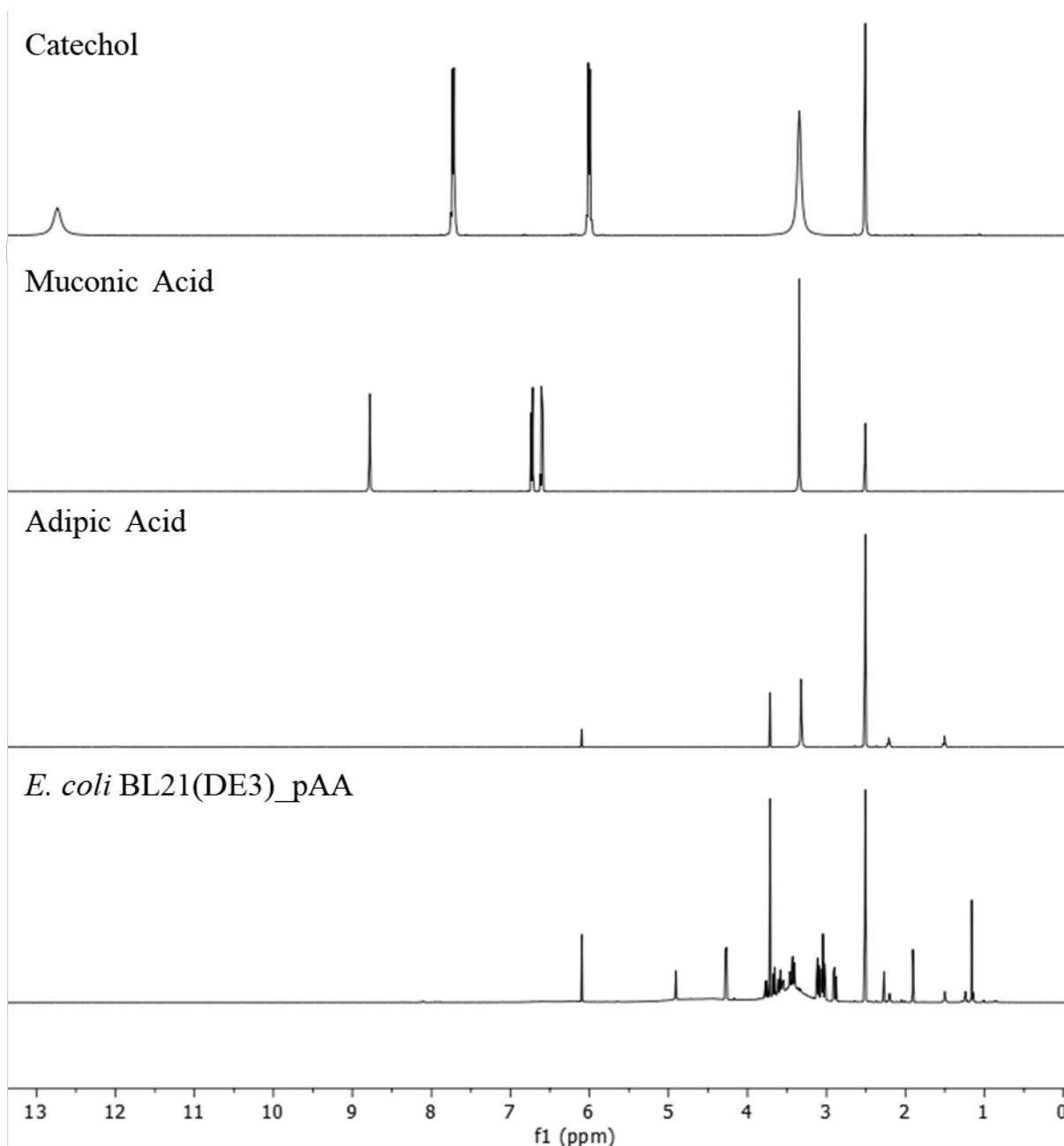


Figure S3.  $^1\text{H}$  NMR spectra of reaction intermediates and lyophilized culture extracts.

### Reverse-phase HPLC

Analytes were separated using a gradient from 5 to 10.5% MeCN (0.1% v/v TFA) in  $\text{H}_2\text{O}$  (0.1% v/v TFA). Peak areas were collected at 206 nm and integrated relative to an internal standard of caffeine. Elution times were as follows: 2-hexenedioic acid (7.6 min), adipic acid (8.8 min), catechol (10.6 min), muconic acid (12.2 min), caffeine (13.3 min) and guaiacol (17.9 min). A standard curve was constructed

over a defined range of analyte concentrations to ensure accurate detection via this method providing linear relationships of  $pA_{\text{adipic acid}}/pA_{\text{caffeine}} = 0.076c_{\text{adipic acid}} - 0.0031$ ,  $pA_{\text{muonic acid}}/pA_{\text{caffeine}} = 2.20c_{\text{muonic acid}} + 0.12$  and  $pA_{\text{catechol}}/pA_{\text{caffeine}} = 4.42c_{\text{catechol}} + 1.12$  (Figure S4,  $pA$  = peak area). Samples were diluted accordingly prior to injection. Calibration curves are provided below.

For HPLC analysis, reaction aliquots (100  $\mu\text{L}$ ) were diluted into MQ  $\text{H}_2\text{O}$  (100  $\mu\text{L}$ ) before adding MeCN (400  $\mu\text{L}$  + 0.15% v/v TFA). Samples were vortexed for 10 s and left to stand at room temperature for 30 min. Samples were vortexed again for 10 s before being centrifuged (1666  $\times g$ , 10 min). The supernatant was transferred into 1.5 mL Eppendorf tubes and left to concentrate by evaporation in a fume hood for 48-72 h. Sample residues were then re-dissolved in water (300  $\mu\text{L}$ ) containing caffeine (0.01 mg/mL) and TFA (0.1% v/v). Samples were filtered through a spin column filter (Agilent) via centrifugation (1666  $\times g$ , 10 min). The filtrate (10  $\mu\text{L}$ ) was then analysed by HPLC. The average recovery of adipic acid from biological samples was determined to be  $88 \pm 3\%$ . Where appropriate, relative concentrations of guaiacol within batch samples are reported due to its volatility during sample preparation.

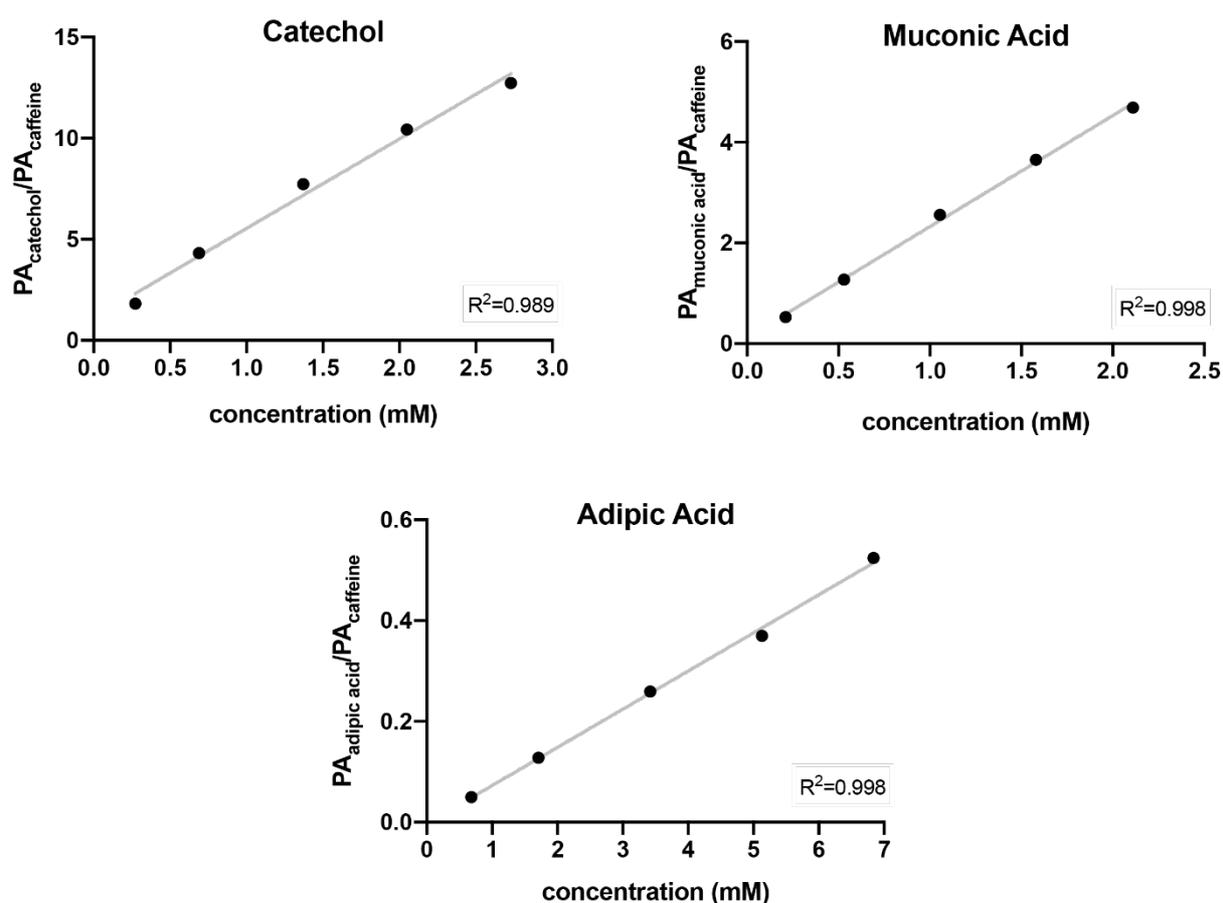


Figure S4. HPLC calibration curves

Table S3: Adipic acid production from catechol in Na-Pi<sup>[a]</sup>

entry	<i>E. coli</i> BL21(DE3)	Time (h)	OD <sub>600</sub> (a.u.)	Catechol (mM)	Muconic Acid (mM)	Adipic Acid (mM)	Yield (%)
1	_pET22b	24	122	5±0.04	0	0	0
2	_pAA	24	122	0	0	3.7±0.2	85±4.8
3	_pAA (dead)	24	122	0	2.6±0.22	0	0
4	_pAA + pTf16	24	122	0	0	4.0±0.3	88±6.9
5	_pAA + pGro7	24	122	0	0	3.7±1=0.2	84±4.0
6	_pAA + pKJE7	24	122	0	0	3.0±0.1	70±2.4
7	_pAA <i>ΔiscR</i>	24	122	0	0	4.1±0.1	89±1.2
8	Lemo <sup>[b]</sup>	24	122	0	0	3.6±0.03	86±2.0
9	Lemo <sup>[c]</sup>	24	122	0	0	2.3±0.3	56±4.6
10	Lemo <sup>[d]</sup>	24	122	0	0.45±0.1	1.4±0.8	32±0.7
11	_pAA <sup>[e]</sup>	24	122	0	0	4.2±0.2	98±4.6
12	_pAA <sup>[f]</sup>	24	122	0	0.9±1.6	1.7±1.2	38±28
13	_pAA	24	12	0	2.9±0.1	0	0
14	_pAA + pTf16	24	12	0	2.4±0.2	0	0
15	_pAA + pGro7	24	12	0	3.4±0.1	0	0
16	_pAA + pKJE7	24	12	0	3.0±0.2	0.6±0.2	13±3.0
17	_pAA <i>ΔiscR</i>	24	12	0	4.5±0.1	0	0
18	_pAA	6	122	0	0	4.0±0.1	91±2
19	_pAA + pGro7	6	122	0	0	3.9±0.01	89±0.01
20	_pAA + pTf16	6	122	0	0	3.8±0.04	87±0.9

[a] reactions were incubated for 24 h. [b] 10 μM L-rhamnose was added. [c] 40 μM L-rhamnose was added. [d] 100 μM L-rhamnose was added. [e] 10 mM benzyl alcohol was added. [f] 10% v/v D-glucose was added. “Lemo” refers to *E. coli* Lemo21(DE3)\_pAA

Table S4: Adipic acid production from catechol in M9<sup>[a]</sup>

entry	<i>E. coli</i> BL21(DE3)	OD <sub>600</sub> (a.u.)	Catechol (mM)	Muconic Acid (mM)	Adipic Acid (mM)	Yield (%)
1	_pAA	122	0	0	3.5±0.06	84±1.4
2	_pAA	12	0	0.32±0.46	2.6±0.77	59±21
3	_pAA + pTf16	12	0	0	2.5±0.14	77±1.3
4	_pAA + pGro7	12	0	0	4.0±0.04	89±1.0
5	_pAA + pKJE7	12	0	0	4.3±0.04	87±0.7
6	_pAA <sup>[b]</sup>	12	0	0.27±0.040	2.8±0.10	57±2.0

[a] reactions were incubated for 24 h. [b] 10 mM benzyl alcohol was added.

## Time-course analysis

Reactions were set-up as described previously. Each data point was generated from the analysis of separate culture tubes by HPLC.

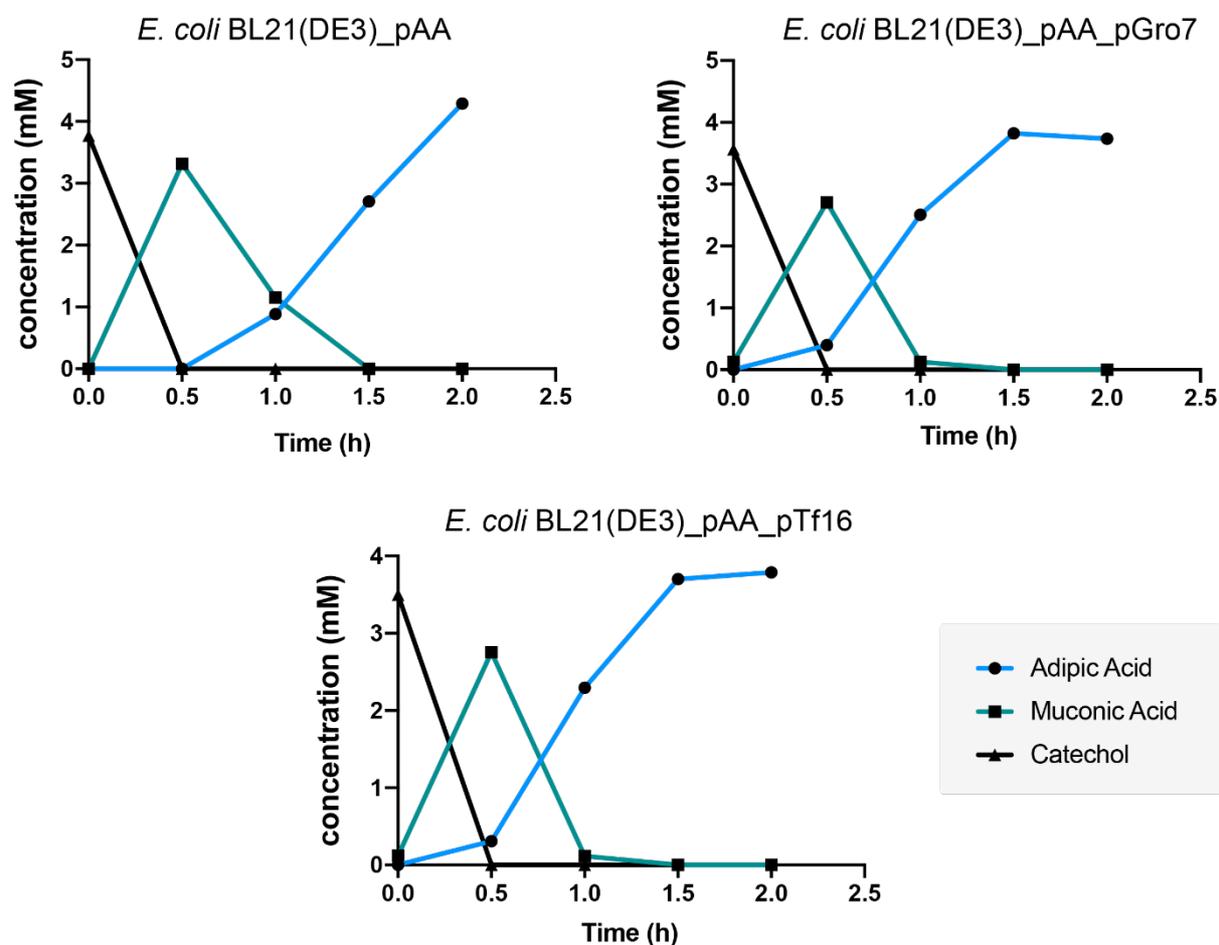


Figure S5. Concentration of intermediates during the reaction

## Protein solubility

Small-scale protein expression tests (10 mL cultures) were carried out as described previously (see General Materials and Methods). Aliquots (1 mL) were pelleted via centrifugation (1666 xg, 2 min), the supernatant was discarded and cells were resuspended in 650  $\mu$ L cell lysis buffer (40 mM sodium phosphate, pH 7.2, 100 mM NaCl). Chicken egg white lysozyme (Sigma Aldrich) was added to a concentration of 1 mg/mL and samples were incubated at room temperature for 10 min. Samples were then sonicated on ice (6 cycles of 10 s on/off at 5 amplitude microns). Aliquots of whole-cell extracts

(10  $\mu$ L) were extracted before centrifuging the samples (1666 xg, 4 °C, 20 min). Aliquots of supernatant containing soluble protein extract (10  $\mu$ L) were collected and the remaining supernatant was discarded. Cell pellets were rinsed with cell lysis buffer (3x 1 mL) before adding further cell lysis buffer (630  $\mu$ L) and sonicating the samples on ice (10 s sonication at 5 amplitude microns). Aliquots of the resulting solution (10  $\mu$ L) were used to analyse insoluble protein fractions.

For SDS-PAGE, the protein samples (10  $\mu$ L) were added to 5  $\mu$ L of 4x SDS PAGE loading dye (0.2 M Tris-HCl, pH 6.8, 8% v/v SDS, 20% v/v glycerol, 0.04% w/v bromophenol blue) and samples were heated at 95 °C for 2 min. Samples were centrifuged at (1666 xg, 2 min) and then 10  $\mu$ L of each sample was loaded onto a 12% NuPAGE Bis/Tris gel. The gel was run under standard conditions as outlined previously. Gels were stained using SimplyBlue Stain (Invitrogen) before imaging using a Gel Doc XR+™ (Biorad). Image Lab™ software (Biorad) was used to detect lanes and band intensities using the automatic detection function. Band intensities are reported as relative to the 50 kDa ladder band in the same gel.

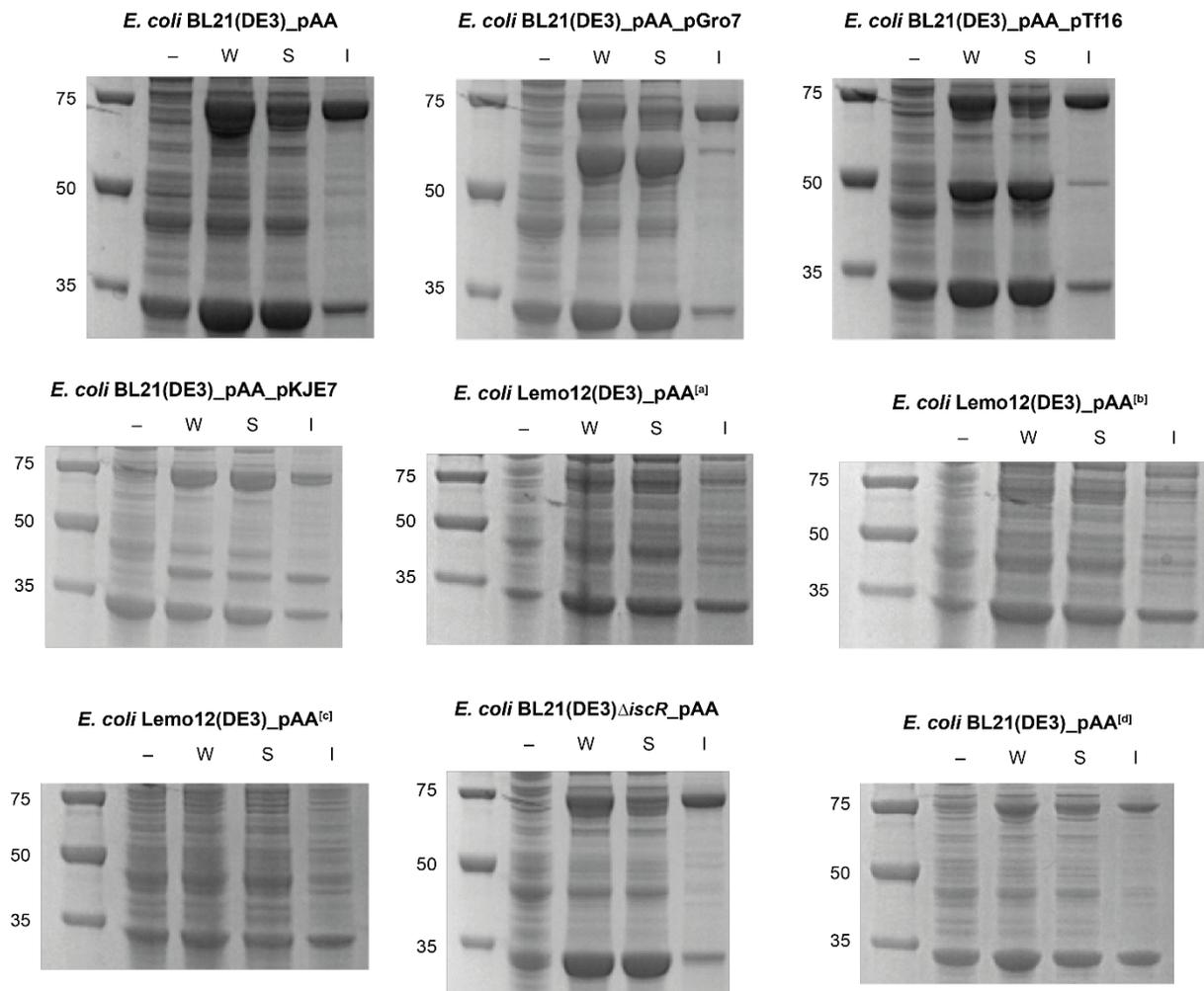


Figure S6. Analysis of protein solubility by SDS-PAGE. Whole cell, soluble and insoluble fractions are indicated by “W”, “S” and “I”, respectively. Uninduced controls are indicated by “-”. BcER is 75 kDa and CatA is 35 kDa. [a] 10  $\mu$ L L-rhamnose was added. [b] 40  $\mu$ M L-rhamnose was added. [c] 100  $\mu$ M L-rhamnose was added. [d] 10 mM benzyl alcohol was added.

Table S5: BcER expression data

Strain	WCE	Soluble	Insoluble	Soluble:Insoluble
BL21(DE3)_pAA	1.6±0.40	0.75±0.05	1.6±0.33	0.49±0.12
BL21(DE3)_pAA_pGro7	1.0±0.13	0.57±0.02	1.2±0.12	0.49±0.06
BL21(DE3)_pAA_pKJE7	1.7±0.13	1.4±0.30	0.75±0.24	2.1±1.1
BL21(DE3)_pAA_pTf16	1.8±0.09	1.0±0.13	1.2±0.23	0.87±0.12
Lemo21(DE3)_pAA <sup>[a]</sup>	1.1±0.29	0.63±0.16	0.68±0.11	0.91±0.10
Lemo21(DE3)_pAA <sup>[b]*</sup>	0.43±0.10	0.25±0.11	0.21±0.05	1.2±0.33
Lemo21(DE3)_pAA <sup>[c]*</sup>	0.58±0.17	0.35±0.03	0.33±0.17	1.3±0.58
BL21(DE3) <i>ΔiscR</i> _pAA	2.0±0.10	0.65±0.06	1.6±0.09	0.40±0.04
BL21(DE3)_pAA <sup>[d]</sup>	0.87±0.12	0.60±0.12	0.56±0.03	1.1±0.2

\* indicates -IPTG control produces a 75 kDa band with an intensity within the standard deviation of WCE samples. [a] 10 μL L-rhamnose was added. [b] 40 μM L-rhamnose was added. [c] 100 μM L-rhamnose was added. [d] 10 mM benzyl alcohol was added.

Table S6: CatA expression data

Strain	WCE	Soluble	Insoluble	Soluble:Insoluble
BL21(DE3)_pAA	2.2±0.23	2.0±0.24	0.68±0.09	2.9±0.19
BL21(DE3)_pAA_pGro7	1.8±0.04	1.8±0.09	0.47±0.03	3.8±0.14
BL21(DE3)_pAA_pKJE7*	1.1±0.03	1.0±0.09	0.47±0.03	2.2±0.04
BL21(DE3)_pAA_pTf6	1.7±0.12	1.6±0.13	0.40±0.08	4.0±0.58
Lemo21(DE3)_pAA <sup>[a]</sup>	1.2±0.06	1.2±0.24	0.67±0.04	1.8±0.25
Lemo21(DE3)_pAA <sup>[b]</sup>	0.98±0.31	1.1±0.21	0.62±0.14	1.9±0.09
Lemo21(DE3)_pAA <sup>[c]</sup>	0.57±0.11	0.67±0.13	0.64±0.07	1.1±0.16
BL21(DE3) <i>ΔiscR</i> _pAA	2.1±0.12	1.9±0.13	0.61±0.14	3.2±0.89
BL21(DE3)_pAA <sup>[d]</sup>	1.1±0.12	0.76±0.10	0.65±0.11	1.2±0.21

\* indicates -IPTG control produces a 35 kDa band with an intensity within the standard deviation of WCE samples. [a] 10 μL L-rhamnose was added. [b] 40 μM L-rhamnose was added. [c] 100 μM L-rhamnose was added. [d] 10 mM benzyl alcohol was added.

## Production of adipic acid from guaiacol

To produce adipic acid from guaiacol, *E. coli* BL21(DE3)\_pAA' was cultured as described previously, except LB or M9-glucose media was used instead of TB and cells were induced using IPTG (0.4 mM) at OD<sub>600</sub> = 0.4-0.5. At this point, 5-aminolevulinic acid (0.76 mM) and ammonium iron(III) citrate (0.76 mM) were also added.<sup>4</sup> After 18 h of protein expression, whole-cell reactions were prepared as outlined previously. Reactions were carried out in M9 reaction buffer (M9 media, 3% w/v glucose, 5 mM guaiacol) and incubated at 37 °C (220 rpm) for 24 h prior to extraction and analysis by HPLC.

Table S7: Adipic acid production from guaiacol<sup>[a]</sup>

entry	<i>E. coli</i> BL21(DE3)	Protein Expression Media	OD <sub>600</sub> (a.u.)	Muconic Acid (mM)	Adipic Acid (mM)	Yield (%)
1	_pAA'	TB	122	0	0.61±0.02	14±0.47
2	_pAA'	LB	61	0	0	0
3	_pAA'	LB	30	0	2.6±0.28	53±5.6
4	_pAA'	LB	20	0	2.8±0.14	56±2.8
5	_pAA'	LB	12	0	2.3±0.50	47±10
6	_pAA'	LB	6	1.9±0.15	0.74±0.07	15±1.4
7	_pAA'	M9	61	0	0	0
8	_pAA'	M9	30	0	2.0±0.05	40±1
9	_pAA'	M9	20	0	3.0±0.67	61±14
10	_pAA'	M9	12	0.47±0.23	1.3±0.59	32±14
11	_pAA'	M9	6	3.3±0.06	0.16±0.15	3.2±3.1
12	_pAA'Rr	LB	61	0	0.26±0.05	5.6±1.1

[a] reactions were incubated for 24 h.

Table S8 Adipic acid production from catechol using *E. coli* BL21(DE3)\_pAA'

entry	<i>E. coli</i> BL21(DE3)	Formaldehyde added? (5 mM)	OD <sub>600</sub> (a.u.)	Muconic Acid (mM)	Adipic Acid (mM)	Yield (%)
1	_pAA'	yes	122	0	4.3±0.04	98±0.86
2	_pAA'	no	122	0	4.1±0.03	92±0.69

Table S9 Relative quantity of unreacted guaiacol

entry	<i>E. coli</i> BL21(DE3)	Protein Expression Media	OD <sub>600</sub> (a.u.)	Relative guaiacol concentration (%)
1	_pAA'	LB	61	95±5.4
2	_pAA'	LB	30	45±3.8
3	_pAA'	LB	20	36±3.9
4	_pAA'	LB	12	19±9.1
5	_pAA'	LB	6	13±4.1
6	_pAA'	M9	61	88±4.5
7	_pAA'	M9	30	63±5.4
8	_pAA'	M9	20	28±17
9	_pAA'	M9	12	7.9±2.5
10	_pAA'	M9	6	14±5

### Formaldehyde quantification

Quantification of formaldehyde was adapted from Yue et al., 2017.<sup>[5]</sup> A standard curve was generated by adding 25  $\mu$ L of aqueous formaldehyde solution (0.1-10 mM) to M9 media (975  $\mu$ L) containing glucose (3% w/v) and 4-amino-3-penten-2-one (Fluoral-P, 2 mM). Samples were mixed thoroughly and incubated at room temperature for 1 h before the absorbance at 420 nm was measured. This gave the linear relationship  $A_{420} = 0.0964c_{\text{formaldehyde}} - 0.038$  (Figure S7). To measure formaldehyde in biological samples, 200  $\mu$ L reaction aliquots were pelleted (1666 xg, 2 min) and then 25  $\mu$ L of supernatant was added to M9 media (975  $\mu$ L) containing glucose (3% w/v) and 4-amino-3-penten-2-one (2 mM). Samples were mixed thoroughly and incubated at room temperature for 1 h before the absorbance at 420 nm was measured. Dead cell solutions were prepared by heating cultures to 90 °C for 1 h before the addition of formaldehyde.

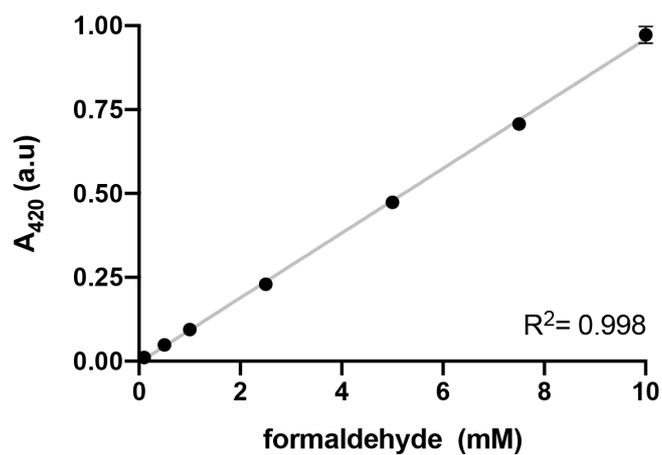


Figure S7. Calibration curve for formaldehyde

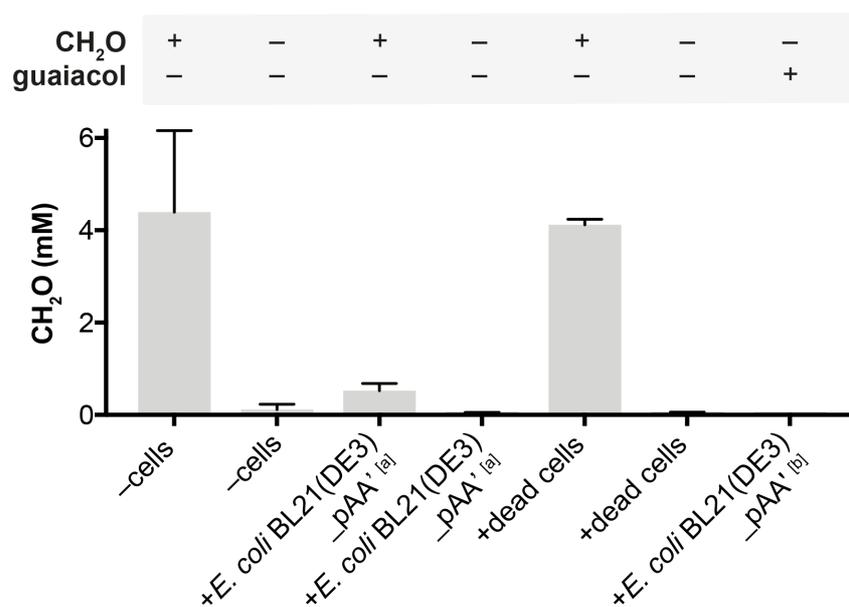


Figure S8. Analysing formaldehyde concentrations using a Fluoral-P colorimetric assay. [a] non-induced cells. [b] cells were induced using 0.4 mM IPTG.

### Cell viability

Reactions were set-up as described previously using Na-Pi buffer and M9 media and at OD<sub>600</sub>=122 and OD<sub>600</sub>=12. Following incubation of reactions for 24 h (37 °C, 220 rpm), 10 μL of each reaction was

inoculated into LB media (10 mL) and samples were incubated for a further 24 h (37 °C, 220 rpm). After this time the OD<sub>600</sub> of each sample was recorded.

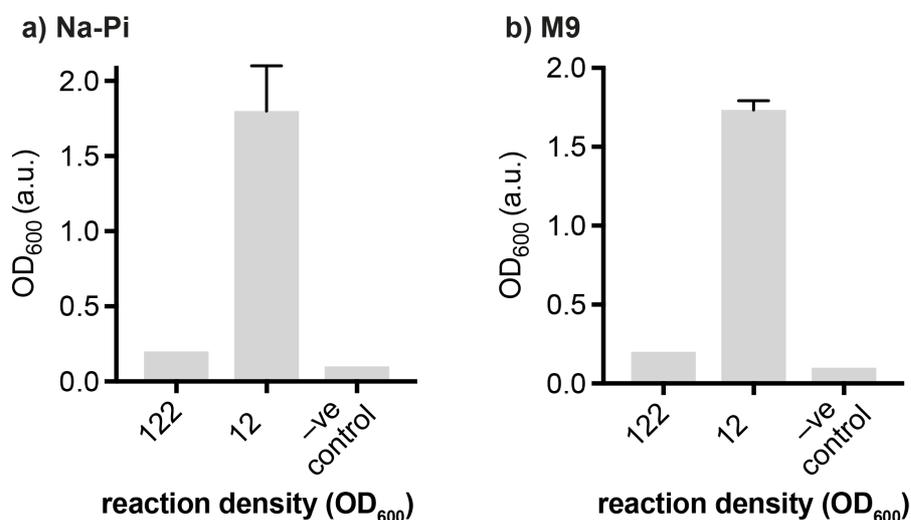
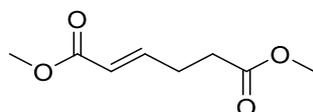


Figure S9. OD<sub>600</sub> of cell cultures inoculated from biocatalytic reactions. All values are average of 3 biological replicates +/- one standard deviation.

## Synthesis

### Synthesis of 2-hexenedioic acid

#### (E)-dimethyl hex-2-enedioate



Procedure adapted from Dannecker et al., 2018.<sup>[6]</sup>

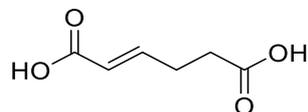
Methyl 4-pentenoate (0.55 ml, 4.4 mmol), methyl acrylate (4.13 ml, 44 mmol) and *p*-benzoquinone (0.049 g, 0.45 mmol) were degassed for 10 min. Hoveyda-Grubbs catalyst 2<sup>nd</sup> generation (25 mg, 0.04 mmol) was added and the mixture was heated to 50 °C for 16 h. The excess methyl acrylate was removed under reduced pressure. The product was purified by column chromatography (PET ether 5/1 diethyl ether, R<sub>f</sub> = 0.2) to yield (E)-dimethyl hex-2-enedioate, a clear oil (0.512 g, 3.0 mmol, 68%)

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) 6.96 (dt, *J* = 15.7, 6.4 Hz, 1H), 5.87 (dt, *J* = 15.7, 1.6 Hz, 1H), 3.74 (s, 3H), 3.70 (s, 3H), 2.59-2.51 (m, 2H), 2.51-2.46 (m, 2H)

$^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ) 172.67, 166.78, 146.85, 121.89, 51.78, 51.49, 32.25, 27.21

NMR data were in accordance with the published literature.<sup>7</sup>

### 2-hexenedioic acid



Procedure adapted from Sølvhøj et al., 2016.<sup>[7]</sup>

Lithium hydroxide (0.46 g, 20 mmol) was added to a stirred solution of (E)-dimethyl hex-2-enedioate in a mixture of THF:methanol:water (3:3:3, 18 ml). After 24 h the mixture was extracted with ethyl acetate (3 x 20 ml). The aqueous phase was adjusted to pH 3 with hydrochloric acid (2 M, 10 ml) and then extracted with ethyl acetate (3 x 20 ml). The combined organic layers were washed with water (20 ml) and brine (20 ml), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (8:2:0.05 ethyl acetate/methanol/acetic acid) to give  $\alpha$ -hydrinuconic acid as a white crystalline solid. (0.180 g, 1.25 mmol, 42 %)

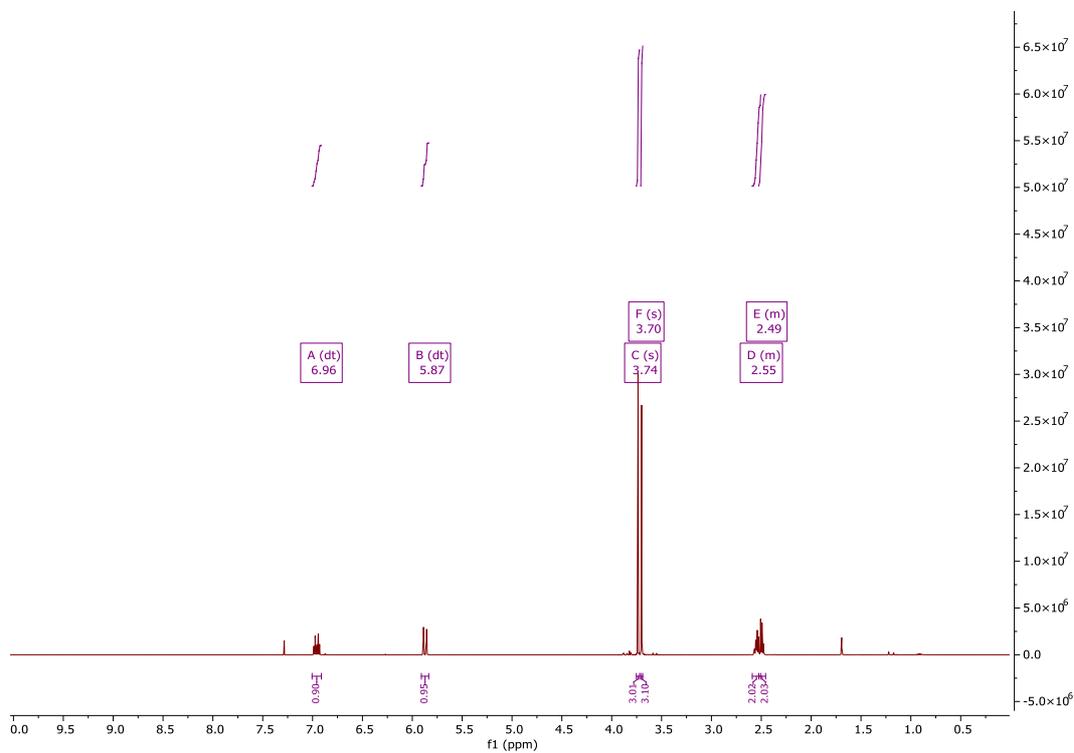
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ) 7.03-6.93 (m, 1H), 5.86 (dt,  $J = 15.7, 1.5$  Hz, 1H), 2.58-2.43 (m, 4H)

$^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ) 174.75, 168.51, 147.61, 121.87, 31.84, 26.90

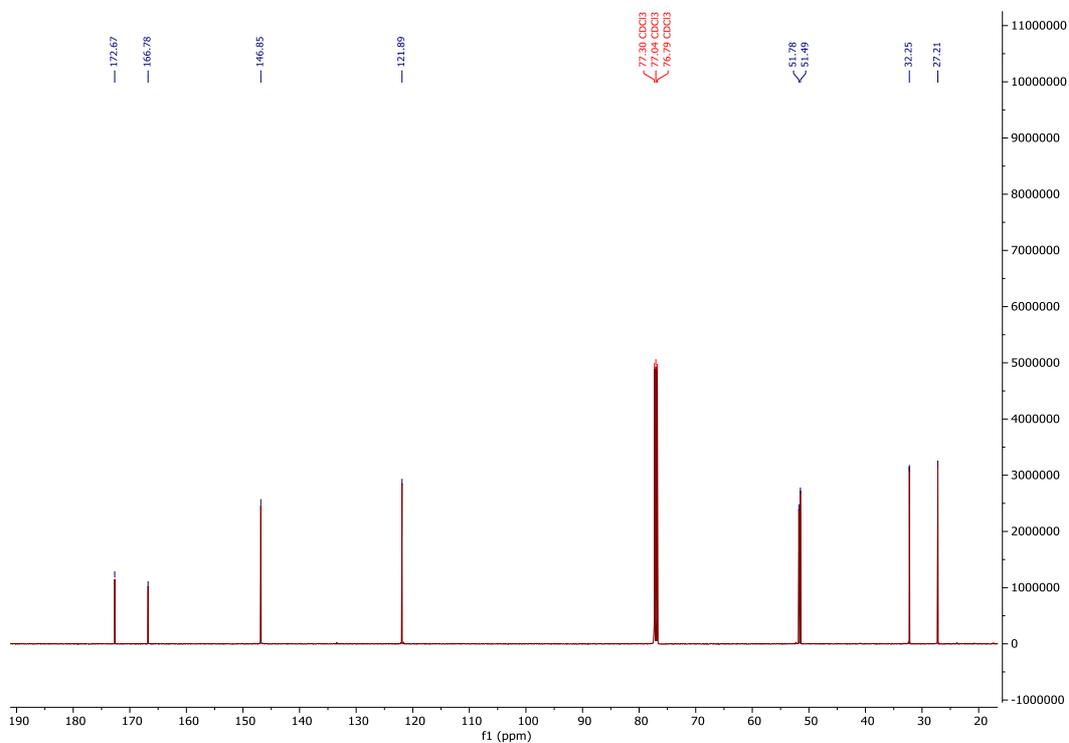
NMR data were in accordance with the published literature<sup>[8]</sup>.

# (E)-dimethyl hex-2-enedioate

$^1\text{H}$  NMR

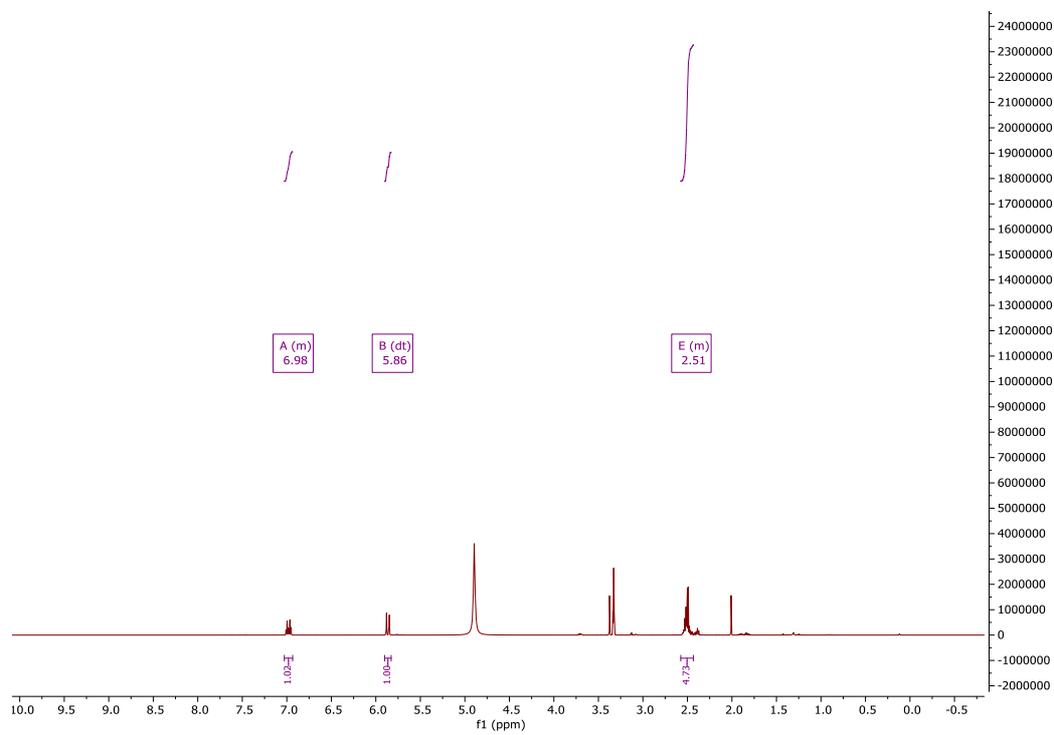


$^{13}\text{C}$  NMR

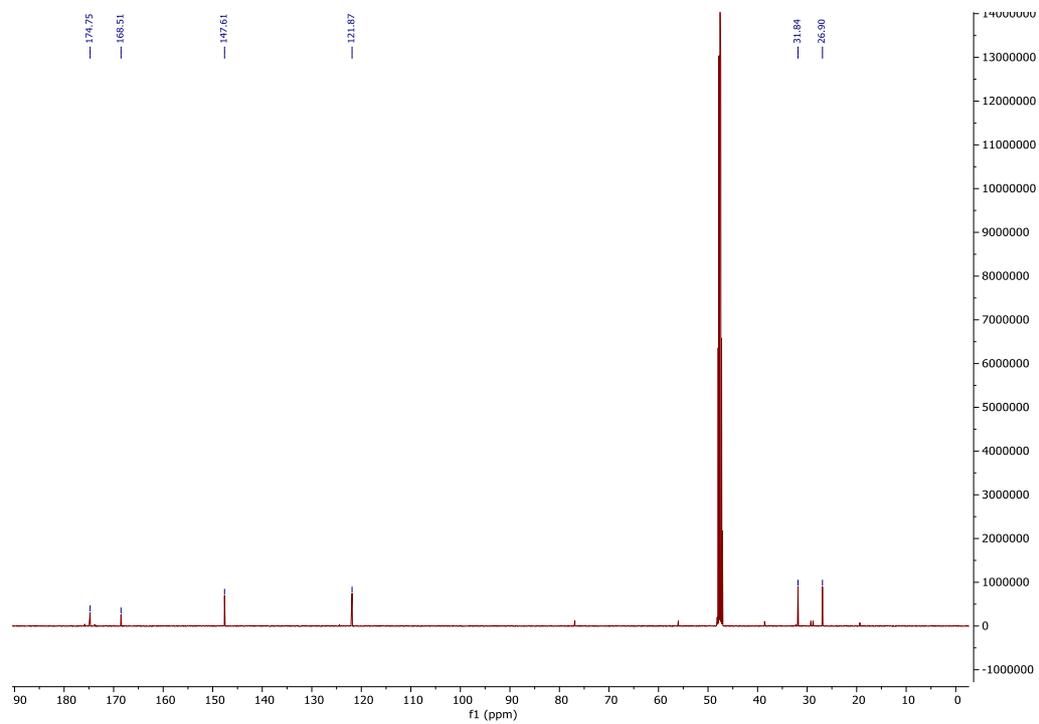


## 2-hexenedioic acid

$^1\text{H}$  NMR



$^{13}\text{C}$  NMR



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