Electronic supplementary information

KnowVolution of prodigiosin ligase PigC towards condensation of short-chain prodiginines

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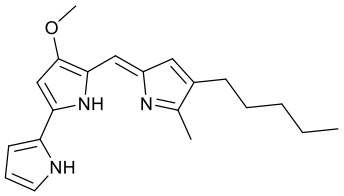
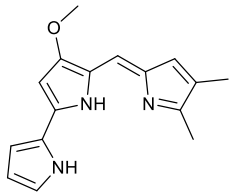
Table of contents

Overview of pyrrolic precursor and reference compounds

Pyrrolic compounds in Chart S1 were synthesised chemically as described before[1–4].



1a



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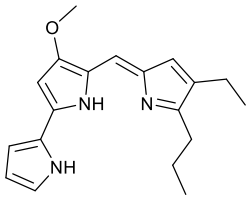

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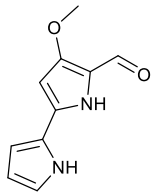

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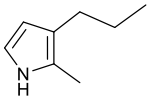
3b



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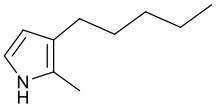

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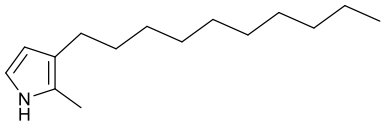

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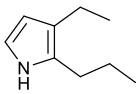

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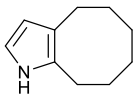

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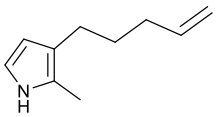

3g

Chart S1 Overview of pyrrolic compounds synthesised in this study. Prodigiosin (**1a**), prodiginine **1b** {4-methoxy-5-[(4,5-dimethyl-2*H*-pyrrol-2-yliden)methyl]-1*H*,1'*H*-2,2'-bipyrrole x HCl} and prodiginine **1c** {5-[(4-ethyl-5-propyl-2*H*-pyrrol-2-ylidene)methyl]-4-methoxy-1*H*,1'*H*-2,2'-bipyrrole}; MBC (**2;** 4-methoxy-2,2'bipyrrole-5-carbaldehyde; MAP (**3a;** 3-amyl-2-methyl-1*H*-pyrrole); diMe-pyrrole (**3b;** 2,3 dimethyl-1*H*-pyrrole); 2-methyl-3-propyl-1*H*-pyrrole (**3c**); 3-decyl-2-methyl-1*H*-pyrrole (**3d**); 3-ethyl-2-propyl-1*H*-pyrrole (**3e**); 4,5,6,7,8,9-hexahydro-1*H*-cycloocta[*b*]pyrrole (**3f**), and 2-methyl-3-pentenyl-1*H*-pyrrole (**3g**) were synthesised as published[1–4].

Site-saturation mutagenesis (SSM) primer sequences

For saturation of select amino acid positions in the PigC sequence, degenerate primers were used that contain an NNK codon (N = A/G/T/C; K = G/T) at the respective target site. Table S1 shows an overview of the targeted PigC positions and respective NNK-primers.

Table S1 Primers designed for site-saturation mutagenesis within PigC

Position		Target sequence	Primer sequence
D128	Fwd	TGCGTGGC**GAC**GACGAGGTGGTG	TGCGTGGC**NNK**GACGAGGTGGTG
	Rev	CACCACCTCGTC**GTC**GCCACGC	CACCACCTCGTC**MNN**GCCACGC
K134	Fwd	GAGGTGGTGCGC**AAG**GTGCAAAGCT GC	GAGGTGGTGCGC**NNK**GTGCAAAGCTG C
	Rev	GCAGCTTTGCAC**CTT**GCGCACCACCTC	GCAGCTTTGCAC**MNN**GCGCACCACCTC
N187	Fwd	ACCGGTGACGCC**AAC**CACATCGTGAT CG	ACCGGTGACGCC**NNK**CACATCGTGATC G
	Rev	ATCACGATGTG**GTT**GGCGTCACCGGT C	ATCACGATGTG**MNN**GGCGTCACCGGT C
I212	Fwd	CACCGACAGCTTC**ATC**CTGGACAAGG CCAG	CACCGACAGCTTC**NNK**CTGGACAAGGC CAG
	Rev	CTGGCCTTGTCCAG**GAT**GAAGCTGTC GGTG	CTGGCCTTGTCCAG**MNN**GAAGCTGTCG GTG
D235	Fwd	CAGCGC**GAT**CCGCAGGGTCGTGTG	CAGCGC**NNK**CCGCAGGGTCGTGTG
	Rev	CTGCGG**ATC**GCGCTGGCAGTAGTGC	CTGCGG**MNN**GCGCTGGCAGTAGTGC
Q259	Fwd	CCAGAA**CAG**CTGCAGCAACTGGCCCG TC	CCAGAA**NNK**CTGCAGCAACTGGCCCGT C
	Rev	GCTGCAG**CTG**TTCTGGGGTCAGCGAT G	GCTGCAG**MNN**TTCTGGGGTCAGCGAT G
A276	Fwd	GCATGATCTACGGT**GCC**GAGCTGGAC ATCG	GCATGATCTACGGT**NNK**GAGCTGGACA TCG
	Rev	CGATGTCCAGCTC**GGC**ACCGTAGATC ATGC	CGATGTCCAGCTC**MNN**ACCGTAGATCA TGC
T329	Fwd	GCATGGAT**ACC**GGTGAAATCGTGACC GGTC	GCATGGAT**NNK**GGTGAAATCGTGACCG GTC
	Rev	CGATTTCACC**GGT**ATCCATGCGGCTGA AG	CGATTTCACC**MNN**ATCCATGCGGCTGA AG
G330	Fwd	GGATACC**GGT**GAAATCGTGACCGGTC	GGATACC**NNK**GAAATCGTGACCGGTC
	Rev	CGATTTC**ACC**GGTATCCATGCGGCTG	CGATTTC**MNN**GGTATCCATGCGGCTG
V333	Fwd	GAAATC**GTG**ACCGGTCTGATGACC	GAAATC**NNK**ACCGGTCTGATGACC
	Rev	GACCGGT**CAC**GATTTCACCGGTATCC	GACCGGT**MNN**GATTTCACCGGTATCC
T334	Fwd	CGTG**ACC**GGTCTGATGACCCCAC	CGTG**NNK**GGTCTGATGACCCCAC
	Rev	CAGACC**GGT**CACGATTTCACC	CAGACC**MNN**CACGATTTCACC
I365	Fwd	CTGGCCGAC**ATC**GGCGACTGGCAGAT C	CTGGCCGAC**NNK**GGCGACTGGCAGATC

	Rev	GTCGCC**GAT**GTCGGCCAGGCCCATGG	GTCGCC**MNN**GTCGGCCAGGCCCATGG
		GAAACCCGTCGCTTC**CTG**GCGCTGGA	GAAACCCGTCGCTTC**NNK**GCGCTGGAC
L466	Fwd	CCTG	CTG
		CAGGTCCAGCGC**CAG**GAAGCGACGG	CAGGTCCAGCGC**MNN**GAAGCGACGGG
	Rev	GTTTC	TTTC
E563	Fwd	CGTGTTC**GAA**CGCCACAGCGCCCAAG	CGTGTTC**NNK**CGCCACAGCGCCCAAG
	Rev	GTGGCG**TTC**GAACACGGCCTTCAGC	GTGGCG**MNN**GAACACGGCCTTCAGC
		CGTCAAGAA**TTC**GAACTGAGCCTGCC	CGTCAAGAA**NNK**GAACTGAGCCTGCCA
F603	Fwd	ACG	CG
		CAGTTC**GAA**TTCTTGACGGCCACGGG	CAGTTC**MNN**TTCTTGACGGCCACGGGC
	Rev	CACC	ACC
M671	Fwd	AACTGTACGGCGTG**ATG**GCCGAGC	AACTGTACGGCGTG**NNK**GCCGAGC
	Rev	CTCGGC**CAT**CACGCCGTACAGTTTGG	CTCGGC**MNN**CACGCCGTACAGTTTGG
R674	Fwd	CGAG**CGT**CGCGAGGCGACCCGTCC	CGAG**NNK**CGCGAGGCGACCCGTCC
	Rev	CCTCGCG**ACG**CTCGGCCATCACG	CCTCGCG**MNN**CTCGGCCATCACG
		CGACCCGT**CCA**ACCTTCGTGACCGAAA	CGACCCGT**NNK**ACCTTCGTGACCGAAA
P680	Fwd	CC	CC
		CGAAGGT**TGG**ACGGGTCGCCTCGCGA	CGAAGGT**MNN**ACGGGTCGCCTCGCGA
	Rev	CG	CG
		GAGCTGCAGCCG**AAC**GAGATCCTGGT	GAGCTGCAGCCG**NNK**GAGATCCTGGT
N808	Fwd	G	G
	Rev	CACCAGGATCTC**GTT**CGGCTGCAGCTC	CACCAGGATCTC**MNN**CGGCTGCAGCTC
A852	Fwd	CATCCCA**GCC**GCCGTGAACCTGAAG	CATCCCA**NNK**GCCGTGAACCTGAAG
	Rev	CACGGC**GGC**TGGGATGCCGAACTC	CACGGC**MNN**TGGGATGCCGAACTC
		GAAGAACGCGACC**CAA**CTGATCAACT	GAAGAACGCGACC**NNK**CTGATCAACTC
Q861	Fwd	CG	G
		CGAGTTGATCAG**GTT**GGTCGCGTTCTT	CGAGTTGATCAG**MNN**GGTCGCGTTCTT
	Rev	C	C

DNA sequences of *pigC* and final variants S0-S3

The *pigC* sequence from *Serratia marcescens* strain W838 (DSM No. 12487) was codon optimised for *P. putida* expression.

```
>pigC wild type

ATGAACCCGACCCTGGTGGTGGAACTGAGCGGTGACAAGACCCTGGAACCGCACCGCCTGGGTGG
CAAGGCCCACAGCCTGAACCACCTGATCCAAGCCGGTCTGCCGGTGCCGCCAGCCTTCTGCATCA
CCGCGCAGGCCTACCGCCAGTTCATCGAGTTCGCCGTGCCAGGTGCGCTGCTGGACACCGGTGCG
CCAGGCAACGTGCGCGACATGATCCTGAGCGCGGCGATCCCAGCGCCACTGGATCTGGCCATCCG
CCACGCCTGCAAGCAGCTGGGTGATGGTGCCAGCCTGGCCGTGCGTAGCTCGGCCCTGGAAGAGG
ACGGTCTGACCCACAGCTTCGCCGGTCAGTACGACACCTACCTGCACGTGCGTGGCGACGACGAG
GTGGTGCGCAAGGTGCAAAGCTGCTGGGCCAGCCTGTGGGCCGAACGTGCCGCGCAGTATAGCCG
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GGTCTGGGCGAAGGCGTGGTGAGCGGTCAGGTGACCACCGACAGCTTCATCCTGGACAAGGCCAG
CGGTGAGATCCGCGAGCAGCAGATCCGCCACAAGCCGCACTACTGCCAGCGCGATCCGCAGGGTC
GTGTGACCCTGCTGCAAACCCCAGAAGCGCGTCGCGACGCCCCATCGCTGACCCCAGAACAGCTG
CAGCAACTGGCCCGTCTGGCGCGTCAGACCCGCATGATCTACGGTGCCGAGCTGGACATCGAGTG
GGCCGTGAAGGACGACCGCGTGTGGCTGCTGCAAGCCCGTCCGATCACCACCCAAGCGAAGCCGG
TGCAGATGCTGTACGCGAACCCGTGGGAGAGCGACCCAGCGATCAAAGAGCGTGCCTTCTTCAGC
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CTACCAGAAGCACATCCACGGTCCAGCCATCAAGACCATGGGCCTGGCCGACATCGGCGACTGGC
AGATCTACATGGGCTACCTGCAGGGCTACGTGTACCTGAACATCAGCGGCAGCGCCTACATGCTG
CGTCAGTGCCCACCGACCCGTGACGAGATGAAGTTCACCACCCGCTACGCCACCGCGGACATCGA
CTTCAGCGGCTACAAGAACCCGTATGGCCCAGGCGTGCAAGGCTGGGCCTACCTGAAAAGCGCCT
GGCACTGGCTGAAGCAGCAGCGCCACAACCTGCGTAGCGCCGGTGCCACCGTGGACGCCATGATC
GCCCTGCGCCAGCGTGAAACCCGTCGCTTCCTGGCGCTGGACCTGACCACCATGACCCACCAAGA
GCTGGAACGCGAGCTGAGCCGTATCGACGGCTACTTCCTGGACAGCTGCGCCGCCTATATGCCGT
TCTTCCTGCAGAGCTTCGCCCTGTACGACGCGCTGGCCCTGACCTGCGAGCGCTACCTGAAAGGC
CGTGGCAACGGTCTGCAGAACCGCATCAAGGCGAGCATGAACAACCTGCGCACCATCGAGGTGAC
CCTGGGCATCCTGAGCCTGGTGGAAACCGTGAACCGCCAGCCAGCGCTGAAGGCCGTGTTCGAAC
GCCACAGCGCCCAAGAACTGGTGACCGTGCTGCCGACCGATCCGGAAAGCCGTGCGTTCTGGCAG
AGCGACTTCTCGGCCTTCCTGTTCGAGTTCGGTGCCCGTGGCCGTCAAGAATTCGAACTGAGCCT
GCCACGCTGGAACGACGACCCGAGCTACCTGCTGCAGGTCATGAAGATGTACCTGCAACACCCGG
TGGACCTGCACACCAAGCTGCGCGAAACCGAGCGCCTGCGCCATGAGGATAGCGCGACCCTGCTG
AAGGCCATGCCGTGGTTCGGTCGCATGAAGCTGAAATTCATCACCAAACTGTACGGCGTGATGGC
CGAGCGTCGCGAGGCGACCCGTCCAACCTTCGTGACCGAAACCTGGTTCTACCGTCGCATCATGC
TGGAAGTGCTGCGTCGCCTGGAAGCCCAAGGCCTGGTGAAGCAGGCCGACCTGCCGTACGTGGAC
TTCGAGCGCTTCCGTGCCTTCATGGCCGGTGAGCAGTCGGCCCAAGAAGCCTTCGCCGCCGACCT
GATCGAGCGCAACCGCCACCAACATCTGCTGAACCTGCACGCCGAGGAACCGCCAATGGCCATCG
TCGGTGGCTACCAGCCACGCATGAAAGCCCCAACCGCCGAGAACGCCGCCGGTATGCTGAGCGGT
CTGGCCGCCTCGCCAGGTAAGGTGGTGGCCAAAGCGCGTGTGATCACCGACCTGCTGGCCCAAGC
GGGTGAGCTGCAGCCGAACGAGATCCTGGTGGCCCGTTTCACCGACGCCAGCTGGACCCCACTGT
TCGCCCTGGCCGCGGGTATCGTGACCGACATCGGTAGCGCCCTGAGCCACAGCTGCATCGTGGCC
CGTGAGTTCGGCATCCCAGCCGCCGTGAACCTGAAGAACGCGACCCAACTGATCAACTCGGGTGA
CACCCTGATCCTGGACGGCGACAGCGGCACCGTCATCATCCAACGTGGCGAGCGTGCCGACGGCT
GA

>PigC wild type

MNPTLVVELSGDKTLEPHRLGGKAHSLNHLIQAGLPVPPAFCITAQAYRQFIEFAVPGALLDTGA
PGNVRDMILSAAIPAPLDLAIRHACKQLGDGASLAVRSSALEEDGLTHSFAGQYDTYLHVRGDDE
VVRKVQSCWASLWAERAAQYSRTSAAQSDIAVVLQIMVDADAAGVMFTQDPLTGDANHIVIDSCW
GLGEGVVSGQVTTDSFILDKASGEIREQQIRHKPHYCQRDPQGRVTLLQTPEARRDAPSLTPEQL
QQLARLARQTRMIYGAELDIEWAVKDDRVWLLQARPITTQAKPVQMLYANPWESDPAIKERAFFS
RMDTGEIVTGLMTPLGLSFCQFYQKHIHGPAIKTMGLADIGDWQIYMGYLQGYVYLNISGSAYML
RQCPPTRDEMKFTTRYATADIDFSGYKNPYGPGVQGWAYLKSAWHWLKQQRHNLRSAGATVDAMI
ALRQRETRRFLALDLTTMTHQELERELSRIDGYFLDSCAAYMPFFLQSFALYDALALTCERYLKG
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LAASPGKVVAKARVITDLLAQAGELQPNEILVARFTDASWTPLFALAAGIVTDIGSALSHSCIVA
REFGIPAAVNLKNATQLINSGDTLILDGDSGTVIIQRGERADG

Table S2 Codon usage for substitutions in synthetic recombination genes A0-A3, S0-S4, P and R.

PigC	Position								
	134	**187**	**235**	**259**	**365**	**466**	**671**	**808**	**861**
WT	**K** AAG	**N** AAC	**D** GAT	**Q** CAG	**I** ATC	**L** CTG	**M** ATG	**N** AAC	**Q** CAA
A0	**S** AGC	**G** GGC	**S** AGC	**G** GGC	–	–	–	–	–
A1	**S** AGC	**G** GGC	–	–	–	–	–	–	–
A2	–	**G** GGC	–	**G** GGC	–	–	–	–	–
A3	–	**G** GGC	**S** AGC	–	–	–	–	–	–
S0	–	–	–	–	**G** GGG	**Q** CAG	**V** GTC	–	–
S1	–	–	–	–	**G** GGG		**V** GTC	–	–
S2	–	–	–	–	**G** GGG	**Q** CAG		–	–
S3	–	–	–	–	–	**Q** CAG	**V** GTC	–	–
P	–	–	–	–	–	–	–	**S** AGC	**G** GGC
R	**S** AGC	**G** GGC	**S** AGC	**G** GGC	**G** GGG	**Q** CAG	**V** GTC	**S** AGC	**G** GGC

Kinetic characterization of PigC variants

The most promising recombinants S0-S3 were kinetically characterised in *E. coli* BL21(DE3) membrane fraction in triplicates [Gradients: 0-200 µM MBC (**2**) and 0-200 µM monopyrrole (**3b**/**3e**), respectively; see Material and Methods].

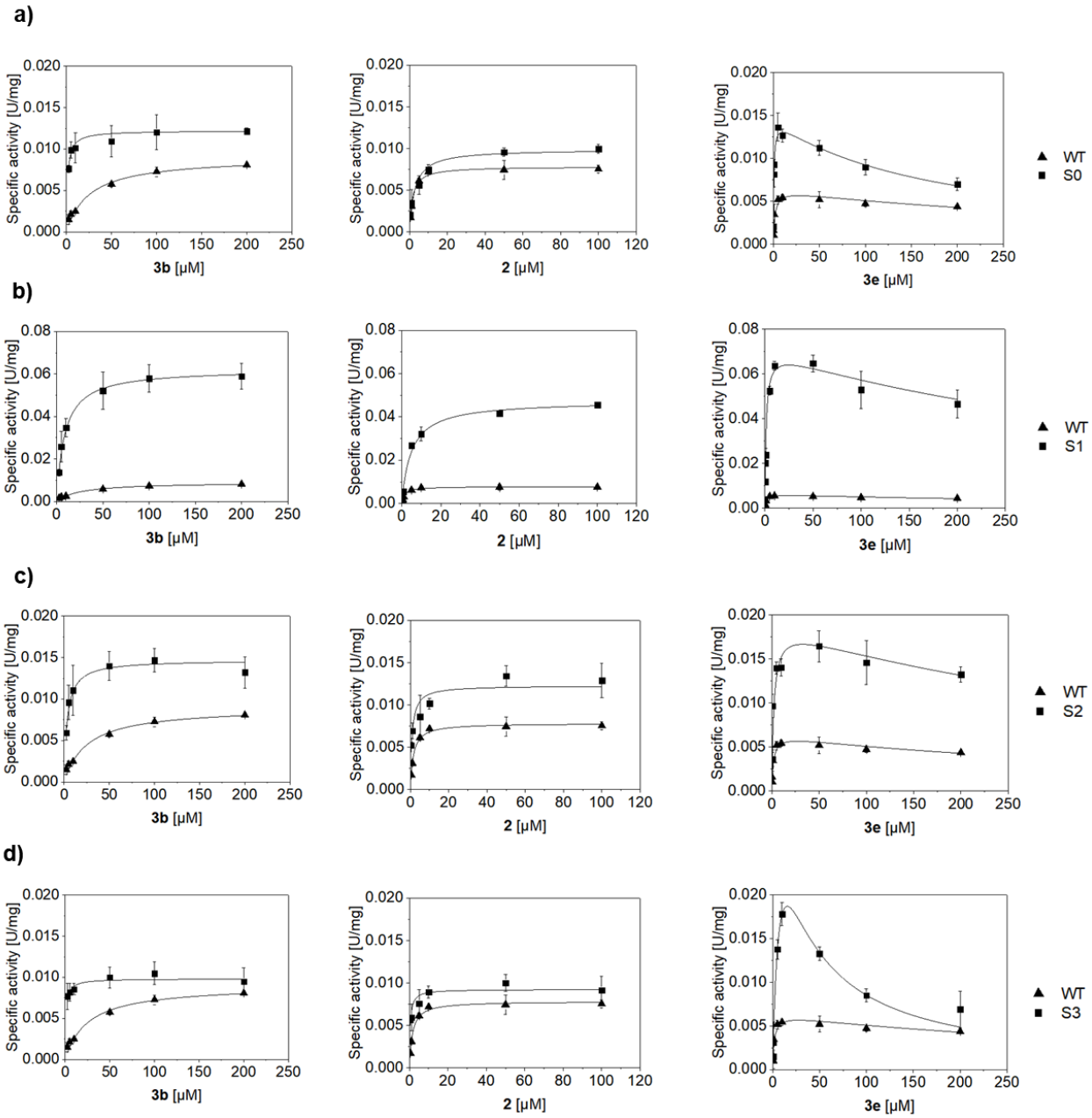


Fig. S1 *Michaelis-Menten* plots of variants S0-S3 with substrates **3b** (diMe pyrrole), **2** (MBC), and **3e** (3-ethyl-2-propyl-1*H*-pyrrole). The wild type (WT, ▲) kinetic curves with the substrates are shown in each diagram. The error bars mark the standard deviation of triplicate PigC reactions.

PigC quantification in isolated BL21 membrane fractions

PigC in isolated BL21(DE3) membrane fractions was quantified using an Experion Automated Electrophoresis System with Pro260 microchips (Bio-Rad Laboratories, Hercules, US-California). Samples were prepared in triplicates according to the Experion Pro260 Analysis Kit. PigC concentrations were calculated from the respective PigC fluorescence peak area in the virtual chromatogram by an internal standard in known concentration (250 ng/µL bovine serum albumin [BSA]). One sample of each membrane fraction was carried along as background control without BSA.

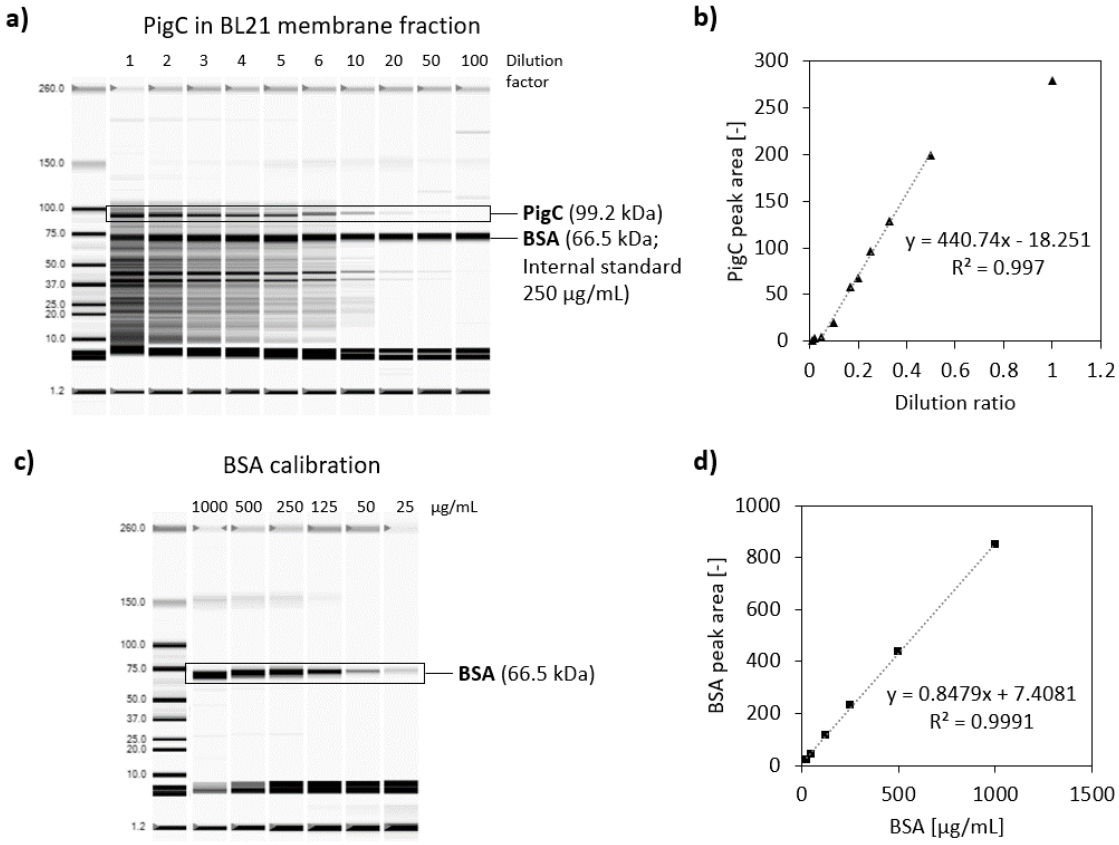


Fig S2 Experion automated gel electrophoresis calibration. **a)** Dilution row of the PigC membrane fraction from 1 (undiluted) to 0.01 (1:100 dilution with assay buffer). **b)** Linearity of fluorescence signal response from PigC dilution samples (1:1 – 1:100). **c)** Virtual electrophoresis gel of BSA in different concentrations (calibration and linearity control). **d)** BSA calibration curve. Different concentrations of BSA standard plotted against fluorescence peak area.

PigC half-life time at 30 °C

The PigC thermal resistance at 30 °C, (half-life at 30 °C as %-decrease in initial activity) of PigC wild type and variants S0-S3 has been determined in *E. coli* BL21(DE3) membrane fraction.

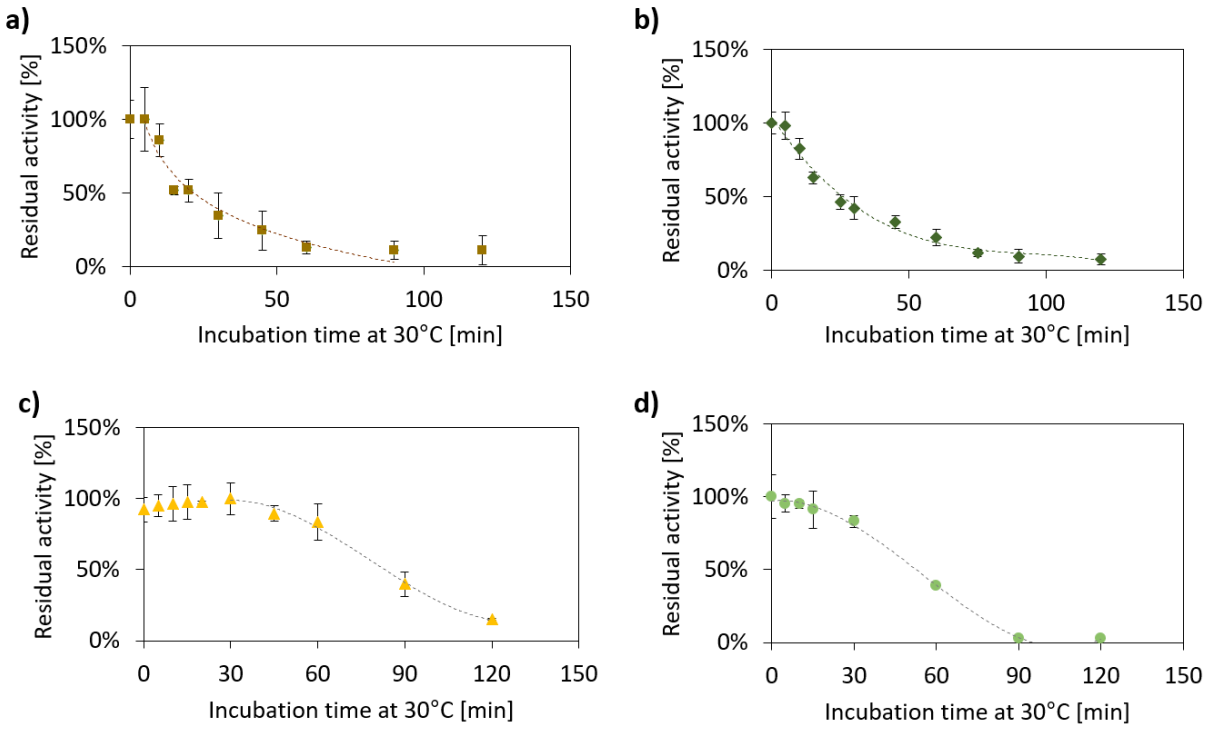


Fig S3 Half-life time of variants S0-S3 at 30 °C. **a)** Variant S0 (•); t½ = 21.1 ± 0.8 min. **b)** Variant S1 (•); t½ = 26.5 ± 0.7 min. **c)** Variant S2 (•); t½ = 83.0 ± 0.0 min. **d)** Variant S3 (•); t½ = 53.7 ± 0.2 min.

Validation of the PigC homology model

SAVES v5.0 results

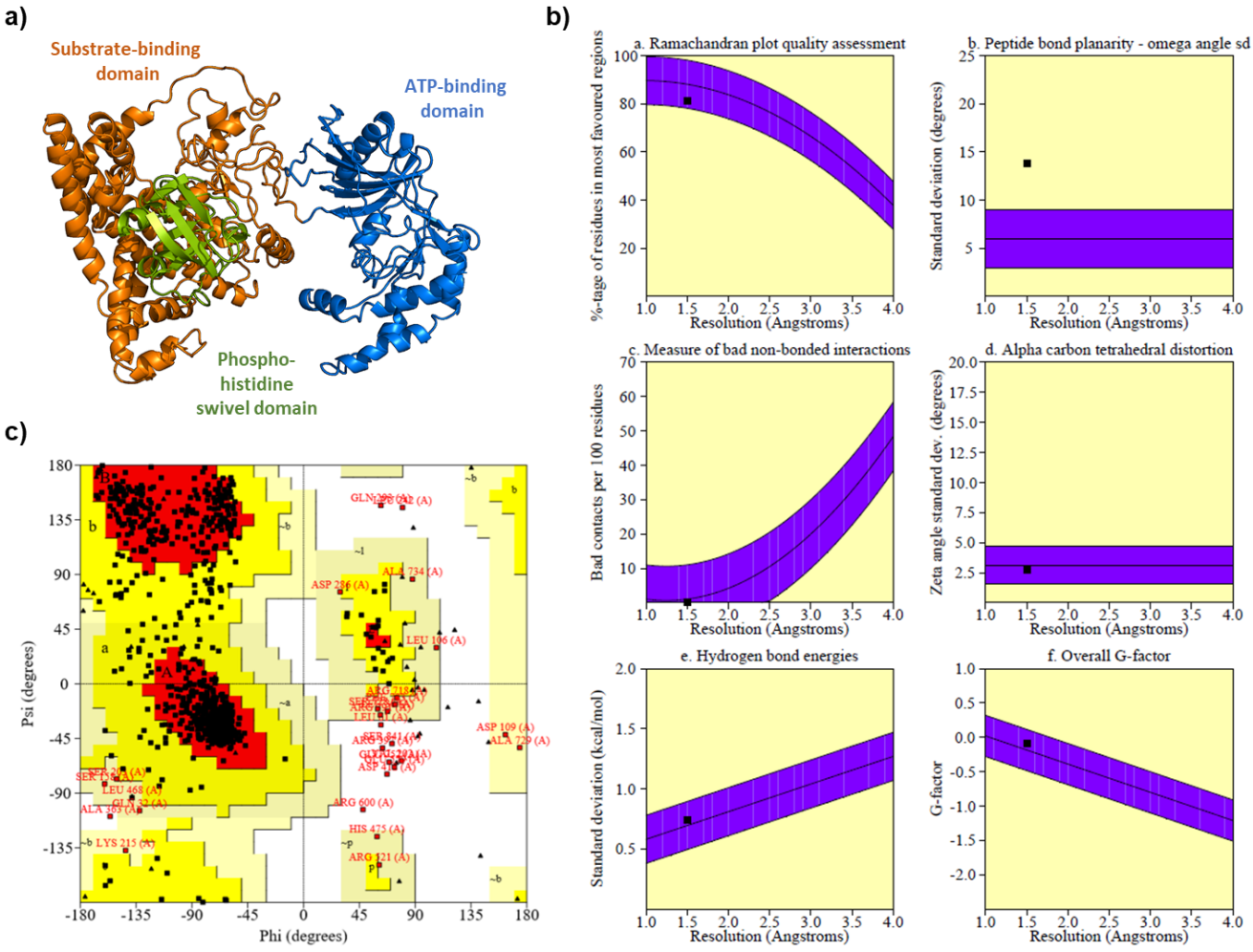


Fig S4 SAVES v5.0[5–11] validation of the PigC homology model. **a)** PigC homology model built on the I-TASSER web server as cartoon. **b)** PROCHECK main-chain parameters [graphs show: quality assessment of Ramachandran plot (81.4%, inside accepted purple region), peptide bond planarity (13.8 degrees, outside accepted region), measure of bad non-bonded interactions (0.0/100 residues), alpha carbon tetrahedral distortion (2.8 degrees, inside accepted region), hydrogen bond energies (0.7 kcal/mol, inside accepted region) and overall G-factor (-0.1, inside accepted region)]. **c)** Ramachandran plot of the PigC homology model. 81.4% of all residues are in favored regions, 17.3% in allowed regions, and 1.4% in disallowed regions.

ProSA web-tool results

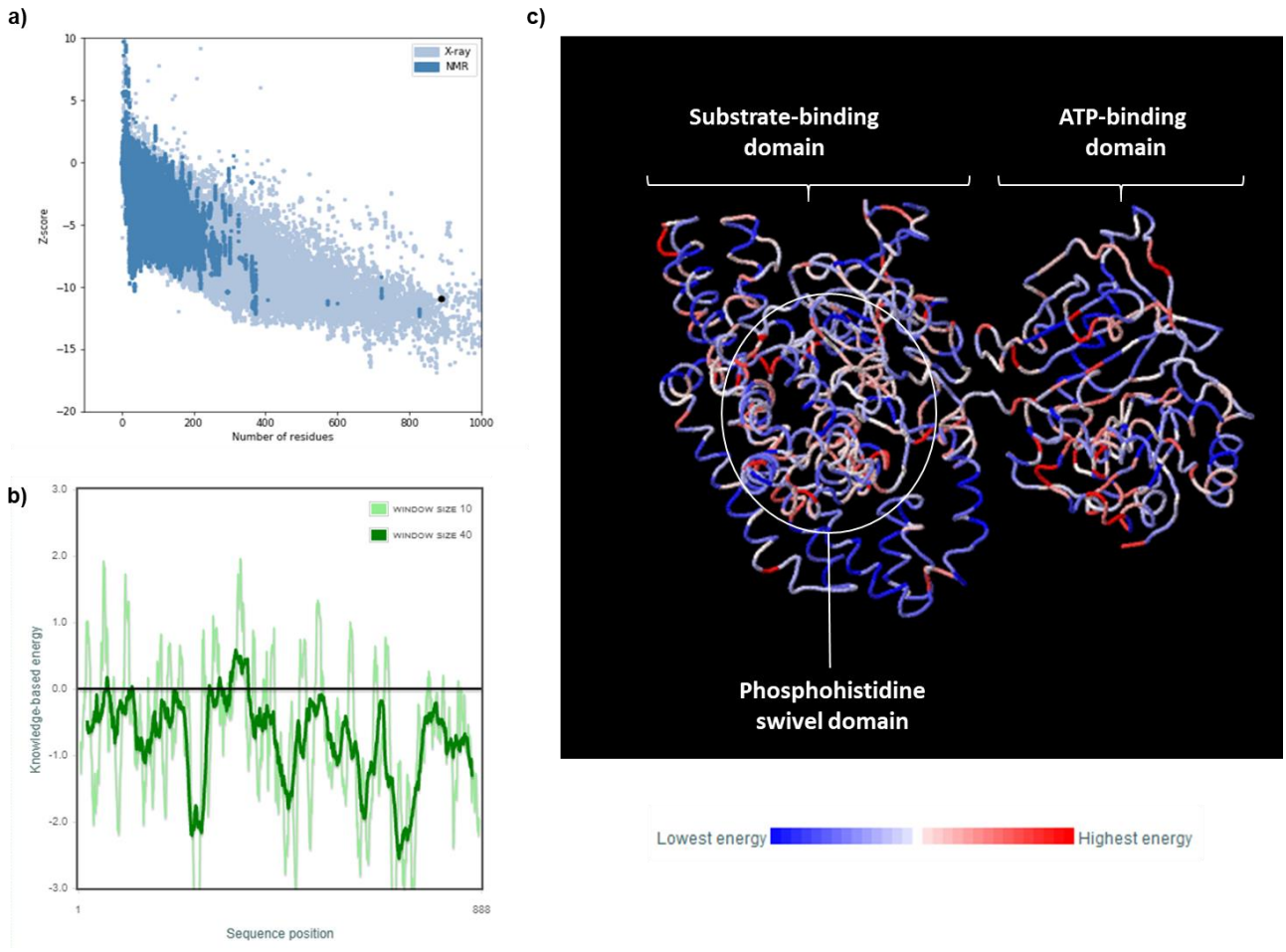


Fig S5 Validation of the PigC homology model by the PRoSA-web tool.[12] **a)** Overall model quality of PigC homology model (•; Z-Score = -10.92). The Z-score measures the overall model quality in comparison to experimentally determined protein chains in the PDB (blue dots). The PigC homology model Z-score (•) is within the range of proteins with similar size. **b)** Local model quality (energy profile). Positive values indicate possibly problematic regions of the model. The dark green graph shows the average energy over 40-residue fragments. The light green graph is calculated with a smaller window size of 10 residues. **c)** Visualization of residue energy in the PigC 3D model. Residues are coloured by their energy values from blue to red.

Relative free energy of folding ($\Delta\Delta G_{fold}$) analysis

Table S3 $\Delta\Delta G_{fold}$ prediction after *in silico* SSM (FoldX).[13] Colour code: green: stabilizing ($\Delta\Delta G_{fold}$ < 0.36 kcal/mol); yellow: unpredictable (0.36 kcal/mol < $\Delta\Delta G_{fold}$ < 7.52 kcal/mol); red: destabilizing ($\Delta\Delta G_{fold}$ > 7.52 kcal/mol). $\Delta\Delta G_{fold}$ was predicted via FoldX (YASARA[14] plugin 4.0) after energy minimization of the PigC homology model.

Position	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V	
128	-0.27	-1.70	-0.21	0.00	-1.29	-1.13	-1.30	0.77	-0.85	-2.15	-2.94	-1.06	-3.92	-0.37	-1.64	0.52	-0.17	0.09	0.49	-1.64	kcal/mol
134	0.75	0.63	0.04	1.68	-0.18	0.59	0.77	1.57	-0.27	-1.13	-1.41	0.00	-2.18	-1.33	3.22	0.90	0.95	-0.39	-1.68	0.58	kcal/mol
187	1.17	0.56	0.00	1.40	1.10	0.41	1.85	1.07	0.19	1.56	0.59	0.96	0.62	-0.27	3.14	0.70	0.55	-0.29	0.08	3.13	kcal/mol
212	2.07	-0.32	1.17	4.16	1.69	1.19	3.21	3.09	0.70	0.00	0.10	0.46	0.01	-0.78	3.45	2.75	1.84	0.11	-0.34	0.68	kcal/mol
235	1.00	1.07	0.70	0.00	1.22	0.86	0.89	2.61	1.90	4.14	-0.59	1.17	-0.08	-0.09	14.48	2.10	1.97	-0.09	-0.36	3.11	kcal/mol
259	1.12	1.62	0.18	1.83	0.95	0.00	1.47	2.49	2.81	-0.70	-1.56	1.17	-0.96	0.20	3.62	1.39	0.04	3.96	4.78	-0.19	kcal/mol
276	0.00	-1.94	-0.42	0.82	-0.50	-1.04	-0.12	0.39	0.03	-0.94	-1.11	-1.94	-1.93	-0.91	2.81	-0.31	-0.47	0.15	-0.27	-0.93	kcal/mol
365	-0.47	-0.65	-0.69	-0.69	-0.50	-0.65	-0.42	-0.68	-0.43	0.00	-0.63	-0.75	-0.55	-0.50	0.11	-0.18	-0.07	-0.59	-0.40	-0.27	kcal/mol
466	2.70	1.31	0.18	3.46	2.09	2.05	1.54	2.11	2.75	0.38	0.00	1.07	0.90	0.83	7.75	0.96	0.68	2.37	1.13	0.77	kcal/mol
542	-1.61	4.94	3.13	4.90	-0.02	1.12	3.00	-0.21	11.31	2.94	-0.63	1.85	0.20	15.99	4.50	-0.76	0.00	26.47	21.38	1.51	kcal/mol
563	-0.27	-0.73	-0.27	-0.34	-0.64	-1.12	0.00	0.00	1.39	-1.00	-0.26	-0.70	-0.94	0.66	1.57	-0.39	0.39	1.23	1.23	-0.59	kcal/mol
671	1.06	4.71	2.18	4.54	0.77	1.23	3.04	0.99	1.71	1.39	-0.51	0.97	0.00	3.70	6.04	0.09	0.63	14.81	9.98	0.50	kcal/mol
808	1.06	-0.75	0.00	0.39	1.49	-0.20	0.42	1.10	0.60	2.04	0.54	0.02	0.11	0.44	1.60	0.85	2.21	0.61	0.74	1.86	kcal/mol
852	0.00	3.04	1.86	4.68	-0.45	0.48	2.66	1.15	1.74	1.71	-1.42	0.90	-2.63	2.13	11.40	1.31	1.43	6.89	5.56	1.03	kcal/mol
861	0.58	-0.17	0.81	0.95	0.63	0.00	0.67	0.68	0.34	0.75	0.16	0.39	1.70	0.03	1.97	0.12	-0.32	0.07	0.39	0.72	kcal/mol

Locations of the final beneficial positions in the PigC structural model

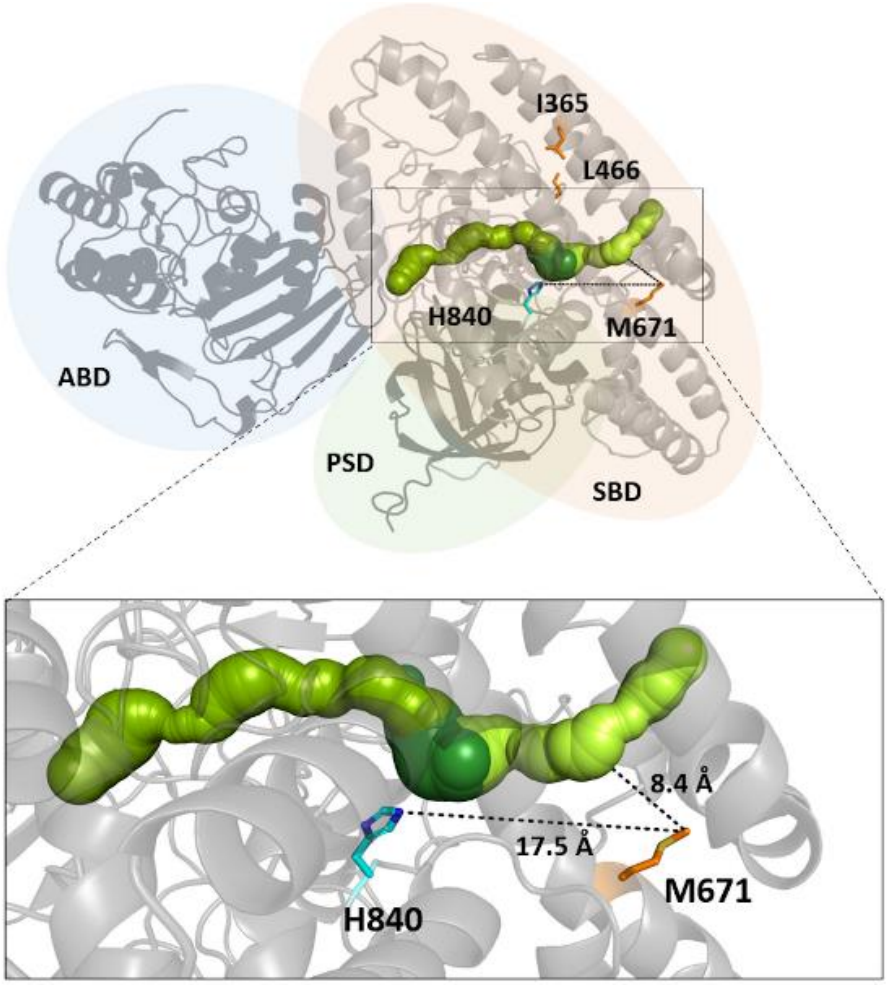
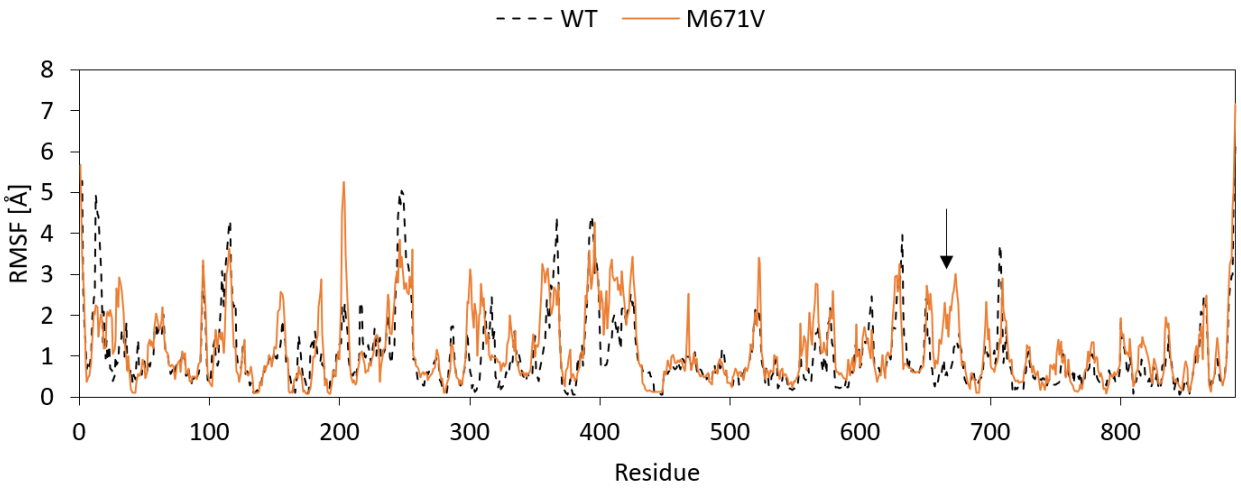


Fig S6 Visualization of beneficial amino acid positions I365, L466, and M671 in the PigC homology model. PigC tunnels are depicted in light green and the active pocket in dark green. Locations of phosphohistidine H840 (cyan) and beneficial positions I365, L466 and M671 (orange) are highlighted in the model. Dotted lines and labels mark distance measurements in [Å]. **ABD**: ATP-binding domain, **SBD**: Substrate-binding domain, **PSD**: Phosphohistidine swivel domain of PigC.

Flexibility analysis PigC wild type and M671V

The residue mean-square-fluctuation (RMSF) profiles of PigC wild type and M671V have been calculated on the CABS-flex server[15] in order to compare their structural flexibility. Energy minimised structures of the PigC wild type homology model and M671V were used as input PDB files with standard settings. Prior to analysis, protein residues of the PigC homology model were treated with the AMBER14[16] force field in YASARA[14] (first *in vacuo*, and secondly in a simulation cell filled with water), and subjected to FoldX[13] mutagenesis (accessed as YASARA plugin FoldX 4.0) in case of M671V, before the model of M671V was repaired with the FoldX[13] repair function.



	WT	**M671V**
Total average RMSF	1.02 Å	1.17 Å
Average RMSF (SBD only)	0.93 Å	1.17 Å
RMSF at position 671	0.90 Å	2.52 Å

Fig S7 Residue mean-square fluctuation (**RMSF**) of PigC wild type (**WT**) and variant M671V. The arrow indicates position 671. **SBD** substrate-binding domain of PigC (residues 299-779).

PigC flask expression

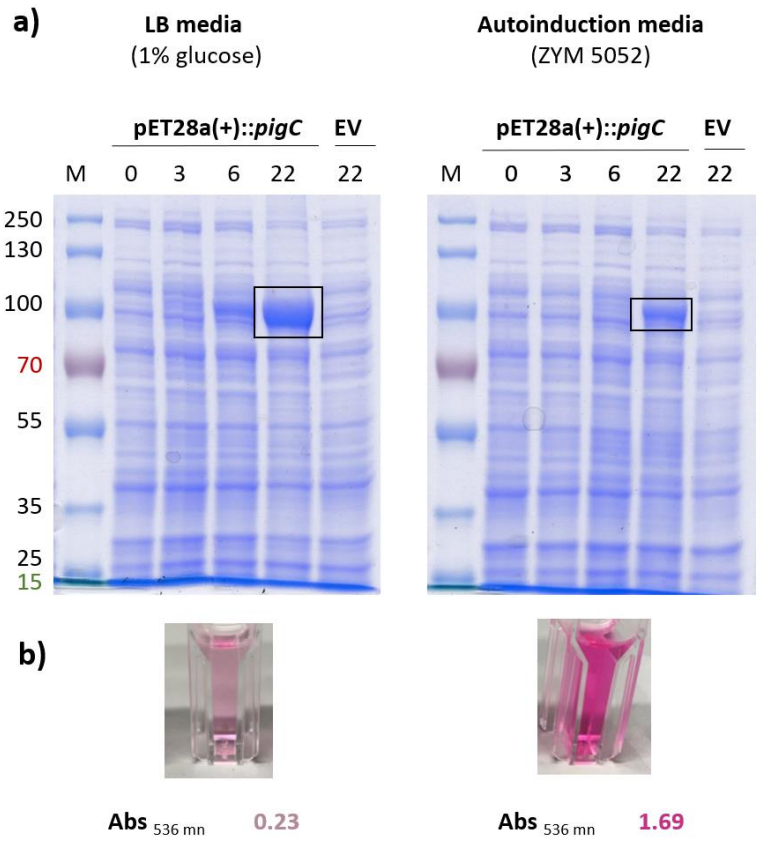


Fig S8 a) PigC flask expression in *E. coli* BL21 (DE3) in different media (LB 1% glucose *versus* ZYM 5052) 0-22 h after inoculation (2-3 h at 37 °C and 250 rpm, then 16-18 h at 18 °C and 250 rpm). **EV** – empty vector control pET28a(+). In LB media, *pigC* expression was induced by addition of 0.1 mM isopropyl-β-*D*-1-thiogalactopyranoside at OD_{600} = 0.6-0.8 (approx. 2-3 h after inoculation). **b)** PigC volumetric activity in 1 mL samples of the expression cultures (adjusted OD_{600} = 15) after 30 min incubation with 100 µM **3a** and **2** at 25 °C. Prodiginine formation was indicated by the pink color of the ethanolic extracts of the samples and their absorbances at 536 nm.

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