Supporting Information

A high-throughput screen for the engineered production of β-lactam antibiotics.

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General Methods: Water used for media and buffers was deionized with Barnstead/Thermolyne HN Ulatrapure and HN Organic Rem. cartridges then distilled. LB^+ contained: 10.0 g/L tryptone, 10.0 g/L NaCl, 5.0 g/L yeast extract, 1.0 g/L NaAc, 10.0 g/l glycerol and 25 mg/L $Fe(SO)_4$, with a pH of 7.0. *E. coli* DH5α was used for routine DNA manipulations (Novagen) and *E. coli* SN0301[1] was used for *in vivo* and *in vitro* β-lactam detection assays. DNA purification was preformed with the GeneJET plasmid purification or GenJET PCR purification kits from Fermentas. T4 DNA ligase (New England Biolabs (NEB), Ipswitch MA), Phusion Polymerase (NEB), Antarctic Phosphatase (NEB), and all restriction enzymes (NEB) were purchased from the indicated suppliers. All fluorescence measurements were taken on a Typhoon 9140 phosphorimager (GE Healthcare, Pitscaway, NJ). DNA sequencing was performed by Genewiz (Germantown, MD). CarCopt was obtained from DNA 2.0 (Menlo Park, CA). Vectors pNU305,[2] pDB2-GFP,[3] pBR322,[4] pDIMC8-MalE,[5] and pET24a-CarAC[6] were all obtained from the sources cited. Oligonucleotides were obtained from either (Invitrogen, Carlsbad, CA) or Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed by Genewiz (Germantown, MD).

Construction of pRP5.199: The region coding for the *ampR* transcriptional regulator and the *ampC* promoter (*PampC*) were cloned out of pNU305 using the primers AmpCNheI and AmpRR2. AmpCNheI inserted an improved ribosome binding site (AGGAGG) followed by a NheI restriction site at the 3' end of the *ampC* promoter. AmpRR2 primed from the 3' end of *ampR* toward the divergent *PampC* segment, inserting an EcoRI restriction site at the 3' end of *ampR*. These primers afforded a PCR product containing the *ampR-ampC* promoter region flanked by EcoRI and NheI restriction sites at the 5' and 3' ends of the overall DNA segment. GFPMut2 (*gfp*) was cloned out of pDB2-GFP using GFPFNheI and GFPRPstI as the primers. The *ampR-PampC* fragment as well as the *gfp* PCR product were digested with NheI and ligated together using T4 ligase. This ligation put *gfp* under the control of *PampC.* The ligation reaction was run on a 0.8% agarose gel and the *ampR-PampC-gfp* fusion product was isolated and amplified using the outside primers (AmpRR2 and GFPRPstI). The *ampR-PampC-gfp* PCR product as well as pBR322 was digested with EcoRI and PstI. The portion of pBR322 containing the tetracycline resistance marker was ligated to the *ampR-gfp* PCR product using T4 ligase. The ligation was concentrated using Paint Pellet (Invitrogen, Carlsbad CA) co-precipitant and transformed into *E. coli* DH5α. Clones possessing tetracycline resistance were isolated and the

entire plasmid was sequenced to verify the proper plasmid containing *gfp* under control of the β-lactam regulated *ampC* promoter had been obtained.

Construction of pDIMC8K-MalE: The kanamycin resistance cassette was cloned out of pET28b (Novagen) using KanNheIF and KanHindR as the sense and anti-sense primers. The PCR product was purified and digested, along with pDIMC8-MalE, by NheI and HindIII. The appropriate DNA fragments were excised and purified from the gel. These two DNA fragments were ligated using T4 ligase. The DNA was concentrated using Paint Pellet (Invitrogen, Carlsbad CA) co-precipitant and transformed into *E. coli* DH5α. Colonies possessing kanamycin resistance were isolated. Plasmid DNA was sequenced to verify the plasmid obtained was that of pDIMC8K.

Construction of pDIMC8K-CarAC: The CarAC operon was cloned out of pET24a-CarAC using CarABamHIF as the sense and CarCSpeIR as anti-sense primers. pDIMC8K-MalE and the cloned CarAC operon were digested by BamHI and SpeI and was resolved on a 0.8% agarose gel. DNA corresponding to the appropriate sizes were excised and purified. These DNA fragments were ligated using T4 ligase and transformed into *E. coli* DH5α. Colonies containing kanamycin resistance were cultured so that plasmid DNA could be prepared for sequencing to verify that the plasmid was pDIMC8K-CarAC.

Construction of pDIMC8K-CarCopt: CarC was optimized by DNA 2.0 (Menlo Park, CA) and amplified using CarCoptEcF and CarCoptEcR as the sense and anti-sense primers respectively. pDIMC8K-MalE and cloned CarCopt were digested by BamHI and SpeI. DNA was resolved on a 0.8% agarose gel. DNA corresponding to the appropriate sizes was purified, ligated using T4 ligase and transformed into *E. coli* DH5α. Colonies containing kanamycin resistance were cultured so that plasmid DNA could be prepared for sequencing to verify that the plasmid was pDIMC8K-CarCopt.

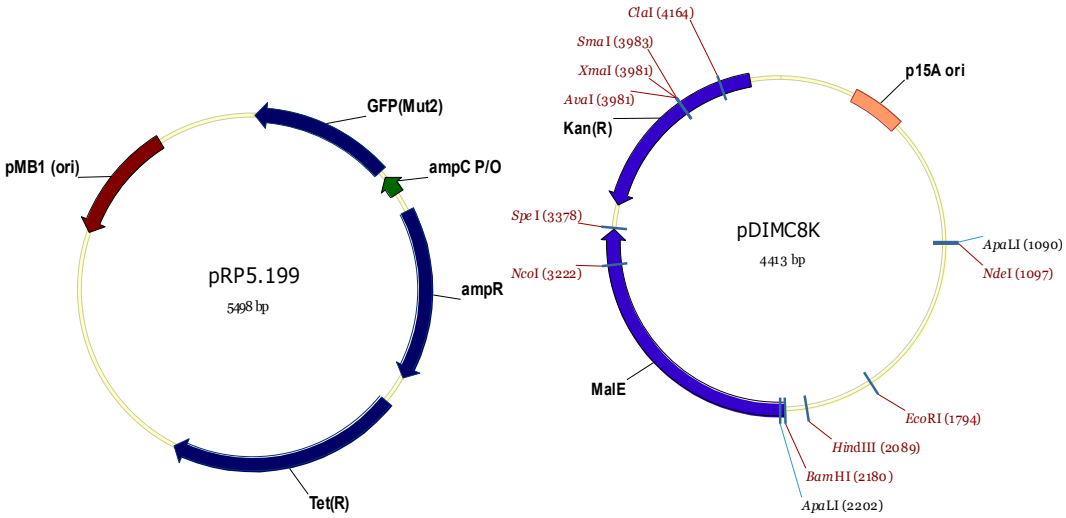

Figure S1. (left) pRP5.199, (right) pDIMC8K-MalE

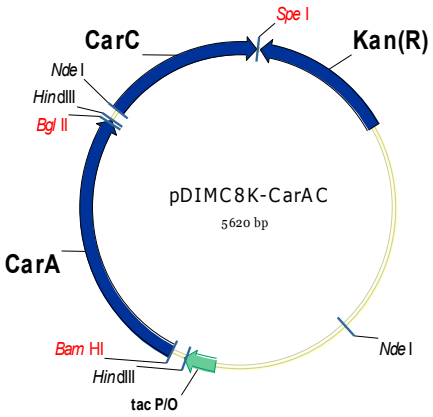

Figure S1. pDIMC8K-CarAC

Creation of CarC mutant libraries. CarC single residue mutants were prepared using overlap extension PCR (oePCR). Complementary mutagenetic primers (primers 14 and 15) were designed to incorporate the degenerate codons NNK/MNN which provided access to all amino acids, but cut the library size in half by limiting access to only 32 codons instead of the usual 64 codons when representing the mutant residue as NNN/NNN (**N** = A, C, G, T; **K** = G, T; **M** = A, C). pDIMC8K-CarAC, containing the polycistronic CarAC operon, served as the template with CarABamHIF2 and CarCSpeIR2 acting as the outside primers.

Production of the libraries began with amplification of CarC segments in two separate reactions. The CPS – 5' section of CarC was cloned using the forward outside primer and the reverse mutant primer. In a separate reaction the forward mutant primer was paired with the reverse outside to clone the 3' section of the CarC mutant gene. The thermocycling program went as follows: denaturation (98 °C, 2 min); (denaturation (98 °C, 15 sec.), annealing (50 °C, 20 sec.), extension (72 °C, 1 min)) x 5; (denaturation (98 °C, 15 sec.), annealing (55 °C, 20 sec.), extension (72 °C, 1 min)) x 25; extension (72 °C, 4 min). The resultant DNA was digested with DpnI to remove parental DNA, purified on a 0.8% agarose gel and extracted from the gel. The 5' and 3' CarAC operon mutant fragments were then used in a primerless oePCR reaction using the following thermocycler program: (98 °C, 2 min); (denaturation (98 °C, 15 sec.), annealing (50 °C, 20 sec.), extension (72 °C, 1 min)) x 30; extension (72 °C, 4 min). The oePCR products were amplified using the two outside primers and the following program: denaturation (98 °C, 2 min); (denaturation (98 °C, 15 sec.), annealing (50 °C, 20 sec.), extension (72 °C, 1 min)) x 5; (denaturation (98 °C, 15 sec.), annealing (55 °C, 20 sec.), extension (72 °C, 1 min)) x 25; extension (72 °C, 4 min).

The resultant CarAC(mut) DNA sequences were digested using manufacturer's specifications, along with pDIMC8K-MalE, using BamHI and SpeI. The desired fragments, pDIMC8K (~3000 b.p.) and CarA-MutantCarC (~2750 b.p.), were purified from undesired DNA fragments on a 0.8% agarose gel after treatment with the appropriate restriction enzymes. Extraction from the gel with the GeneJET PCR purification kit afforded two fragments that could be ligated together to form pDIMC8K-CarAC(mut). Ligation reactions were done in a 1:5 ratio of pDIMC8K vector backbone to CarAC(mut) insert. Ligation reactions were performed with T4 ligase (2,000,000 U/μL) at 16 °C for 16 h and contained 50 ng/μL of the vector backbone per reaction. Purification of the ligation mixture was performed using the GeneJET PCR purification kit and the purified DNA was concentrated *in vacuo*. 1 μL of concentrated plasmid was transformed into DH5αE electrocompetent cells and let grow overnight. Libraries were recovered from the medium and prepared using the GeneJET plasmid purification kit. Purified CarC mutants libraries contained in the pDIMC8K-CarAC(mut) plasmid were stored at -80°C for later transformation into electrocompetent *E. coli* SN0301/pRP5.199 for investigation of catalytic activity.

Oligonucleotides

Number	Name	Sequence (5' → 3')
1	AmpCNheI	CGATTTTTTCATCATGCTAGCCCTCCTTTAATT
2	AmpRR2	GGAATTCTGACCATCTAAGTACATGCGTTAATT
3	GFPFNheI	ACGGAACTGCTAGCATGAGTAAAGGAG
4	GFPRPstI	CGATTAAGTTGGCTGCAGCCAGG
5	KanNheIF	GCTAGCGCTGGTAGCTCTTGATCCGGCAAA
6	KanHindR	AAGCTTGGTGGCACTTTTCGGGAAAT
7	CarABamHIF	GGATCCATGAGCAATAGTTTTTGCGTTGT
8	CarCSpeIR	ACTAGTTTAGATATCGGCGGTTTGTCC
9	CarABamHIF2	GGAGGAAGGATCCATGAGCAATAGTTTTTGCG
10	CarCSpeIR2	GAATTAATACTAGTTCATGAGCGGATACATATTTG
11	CarCSpeIR3	CAGTCTACTAGTTTAGATATCGGCGGT
12	CarCoptEcF	GGAAGGATCCATGAGCGAGATCGTCAAATTCAACCC
13	CarCoptEcF	GCCACCACTAGTCAGATATCAGCGGTTTGACCACG
14	CarCY67NF	GCTTACGGTACGATCGTGGAG**NNK**GCGGATGAAAAGATCGGC
15	CarCY67MR	GCCGATCTTTTCATCCGC**MNN**CTCCACGATCGTACCGTAAGC

Table S1. Fluorescence data for the *in vivo* carbapenem synthesis trials with CarCopt and MalE with the half-reaction.

	Avg.(RFU)	St. Dev.	C.V.
CarCopt	45370	2858	6.3
MalE	1000	112	11.2

Table S2. Fluorescence data for the *in vivo* carbapenem synthesis trials with *E. coli* RP1, RP2, RP3.

	RP1	RP2	RP3
Avg.(RFU)	58734.9	49220.3	1502.1
St. Dev.	2755.2	2856.3	178.6
C.V.	4.7	5.8	11.8

Table S3. Fluorescence data for the *in vivo* screen of CarC(Y67X) with the (3*S*,5*S*)-carbapenam.

Y67X (3S,5S)	
Y67	
Avg.(RFU	29599.2
St. Dev.	3396.3
C.V.	11.4

Table S4. Fluorescence data for the *in vivo* screen of CarC(Y67X) with the (3*S*,5*R*)-carbapenam.

Y67X (3*S*,5*R*)			
	Y67	Y67W	Y67F
Avg	73692.2	23105.0	25024.0*
St. Dev.	9955.0	2203.3	
C.V.	3.5	9.5	

* only one clone

Y67W/Y67	0.313
Y67F/Y67	0.339

References:

1. Lindquist, S.; Galleni, M.; Lindberg, F.; Normark, S., *Mol. Microbiology*, **1989**, *3*, 1091 – 1102.

2. Lindberg, F.; Lindquist, S.; Normark, S., *J. Bact.* **1987**, *169*, 1923 – 1928.

3. Sohka, T.; Heins, R. A.; Phelan, R. M.; Greisler, J. M.; Townsend, C. A.; Ostermeier, M., *Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 10135 – 10140.

4. Watson, N., *Gene*, **1998**, 70, 399-403.

5. Ostermeier, M., Shim, J.H., Benkovic, S.J., *Nat. Biotech.*, **1999**, *7*, 1205 - 09.

6. Bodner, M. J.; Li, R.; Phelan, R. M.; Freeman, M. F.; Moshos, K. M.; Lloyd, E. P.; Townsend, C. A., *ChemBioChem*, **2011**, *12*, 2159 - 2165.