Supporting Information

Optimization of CoaD inhibitors against Gram-negative organisms through targeted metabolomics

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Table of Contents:9 pages

Supplementary Table 1. Effects of *tolC*-dependent efflux pumps on antibiotic susceptibility. Highlighted rows indicate a complementation with a significant (≥4-fold) effect on compound **4** potency.

Compound	NB27079-CDY0099[a]	+*acrB*	+*emrB*	+*entS*	+*acrF*	+*emrY*	+*mdtF*	+*acrD*	+*macB*	+*mdtBC*
Compound **4**	0.25	4	0.125	0.125	2	0.125	2	0.25	0.125	0.25
CHIR090	0.008	0.015	0.004	0.004	0.008	0.008	0.004	0.004	0.004	0.008
Triclosan	0.008	0.015	0.004	0.004	0.008	0.004	0.004	0.004	0.004	0.004
Gatifloxacin	0.004	0.008	0.004	0.002	0.004	0.002	0.002	0.002	0.002	0.002
Colistin	0.25	0.25	0.25	0.25	0.125	0.25	0.125	0.25	0.125	0.25
Trimethoprim	0.125	0.125	0.06	0.06	0.06	0.125	0.06	0.06	0.06	0.06
Kanamycin	1	0.5	0.5	0.5	1	1	4	0.5	0.5	0.5
Tetracycline	0.25	1	0.5	0.25	0.5	0.25	0.5	0.5	0.25	0.25
Rifampin	4	4	4	4	4	4	4	4	4	4
Erythromycin	1	16	1	0.5	8	0.5	8	0.5	1	1
Novobiocin	0.25	16	0.25	0.5	2	0.25	0.25	0.25	0.25	0.25
Cerulenin	8	32	8	8	8	8	8	8	8	8
Linezolid	8	128	8	8	16	8	8	8	8	8
Argyrin B	16	>64	8	8	>64	8	>64	8	16	16

[a]*E. coli*-Δ*acrB* Δ*acrD* Δ*acrF* Δ*emrB* Δ*emrY* Δ*entS* Δ*macB* Δ*mdtBC* Δ*mdtF*.

Supplemental Methods

Construction of *E. coli* strain JWK0002. JWK0002 expresses human c*oaSY* instead of *E. coli coaD.* Initial attempts to directly replace the chromosomal copy of *coaD* with human *coaSY* were not successful. Assuming the expression level may need to be higher when complemented with genes from different species, we thus constructed JWK0002 expressing the human *coaSY* plasmid. We first removed the existing kanamycin resistance cassette in *E. coli*-Δ*tolC* (JW5503-1)[1] using flippase by transforming pFLP2[2] plasmid into the strain and then cured the plasmid using plates with 5% sucrose. Colonies were confirmed by streaking on plates with or without kanamycin) and sequence confirmation of the *tolC* region. We then transformed the unmarked *E. coli*-Δ*tolC* cells with the pNOV016 plasmid, (Gm^R, IPTG inducible $coaSY_{hs}$ expression vector, Genbank number MF988354). Finally, we utilized a recombineering approach to replace the chromosomal *coaD* gene with a kanamycin resistance cassette.[3] The substrate used for recombineering was amplified using overlap extension PCR.[4] The primers

and plasmids used in this study are listed in **Supplementary Table 2** and **3**. The upstream sequence of *coaD* was amplified from the genome of *E. coli* BL21(DE3) with primer pair US coaD 245 and KTT274. The downstream sequence of *coaD* was amplified from *E. coli* BL21(DE3) with primer pair KTT275, and DS coaD 252. The *aph* (Km^R) marker was amplified using the primer pair KTT85 and KTT86. These amplified fragments have homologous sequences from the primers and were ligated together overlap extension PCR using primers US coaD 245 and DS coaD 252. The recombined mutants were isolated from kanamycin plates and confirmed by PCR sequencing. The confirmed clone was named JWK0002 and the growth is IPTG dependent. The viability of this strain indicates that single-copy expression of *coaSY* was not sufficient to complement loss of the bacterial *coaD.* In contrast, JWK0002 grows similarly to the parent *E. coli-ΔtolC* strain and has identical susceptibility to several classes of reference antibiotics[5].

Construction of K. pneumoniae strains JWK0079 and JWK0080. JWK0079 is a *ΔacrB* derivative of ATCC 43816 and JWK0080 is an *E. coli-ΔtolC* derivative. These strains were constructed using overlap extension PCR recombineering as described above. For making *ΔacrB* derivative, the region upstream of *acrB* was amplified from the genome of *K. pneumoniae* ATCC 43816 using primer pair KTT737 and KTT741. The downstream sequence of *acrB* was amplified with primer pair KTT739 and KTT740. The *aph* (Km^R) marker was amplified using the primer pair KTT85 and KTT86. These amplified fragments have homologous sequence from the primers and were ligated together overlap extension PCR using primers KTT737 and KTT740. The recombined mutants were isolated from kanamycin plates and confirmed by PCR sequencing. The confirmed *ΔacrB* clone was named JWK0079. For making the *ΔtolC* derivative, the region upstream of *tolC* was amplified from the genome of *K. pneumoniae* ATCC 43816 using primer pair KTT742 and KTT743. The downstream sequence of *tolC* was amplified with primer pair KTT744 and KTT745. The *aph* (Km^R) marker was amplified using the primer pair KTT85 and

KTT86. These amplified fragments have homologous sequence from the primers and were ligated together overlap extension PCR using primers KTT742 and KTT745. The recombined mutants were isolated from kanamycin plates and confirmed by PCR sequencing. The confirmed *ΔtolC* clone was named JWK0080.

Supplementary Table 2. Primers used in this study

Primer	Sequence (5'-3')
US coaD 245	GAA GGA TGT TCA GCA CGT TTA TCT GC
KTT274	GCA ATT CCG GTT CGC TTG CTG TCA ACA ACC TCA ATG CGT TTT CGG TG
KTT275	GCC TTC TTG ACG AGT TCT TCT GAC GTT TAT GCC GGA TGG TAT GCC
DS coaD 252	AAG CGA AAA TCA AAT AAT TCT CGC TTT G
KTT85	GAC AGC AAG CGA ACC GGA ATT GC
KTT86	TCA GAA GAA CTC GTC AAG AAG GC
cPCR 245 US coaD	CAT CGT TAT CCT GAT TTA CCG ATT ACC
cPCR 252 DS coaD	GTACTGGTCACTTCTATACCACTACG
KTT737 - US acrAB F	TTT AAC GTA TTG AGC TGG CTC TGC
KTT741 - US acrAB KanR-R2	GCA ATT CCG GTT CGC TTG CTG TCATG TAA ACC TCG AGT GTC CAA TTT C
KTT739 - DS acrAB KanR-F	GCC TTC TTG ACG AGT TCT TCT GA TCT TCA CTC CTG AAC AAA GGG C
KTT740 - DS acrAB R	GCG GAT AAA TTT CCA GAC AGA AGT C
KTT742 - US tolC F	AGT GTA GCG GGT CGA TTC AAC TAT C
KTT743 - US tolC KanR-R	GCA ATT CCG GTT CGC TTG CTG TCTCC TTG TTG TGA AGC ATT TAG CGC
KTT744 - DS tolC KanR-F	GCC TTC TTG ACG AGT TCT TCT GAT TCT CAT ACT GTG ATG CGC ATC GC
KTT745 - DS tolC R	GAG GCA CAT CTG ATG TAG CTC AGC
KTT85 - KanR TOPO F	GAC AGC AAG CGA ACC GGA ATT GC
KTT86 - KanR TOPO R	TCA GAA GAA CTC GTC AAG AAG GC

Supplementary Table 3. Strains and plasmids used in this study

Strain code	Referred to as	Description	Source or Reference
P. aeruginosa			
PAO1	*P. aeruginosa* WT	K767; PAO1, prototroph	[6]
ATCC 35151	*P. aeruginosa-Z61*	Mutant 61 isolated by mutagenesis of ATCC 12055 and selection for antibiotic super susceptibility, prototroph	[7]
K1119	*P. aeruginosa-ΔmexAB*	K767 Δ*mexAB-oprM*	[8]

E. coli			
ATCC 25922	*E. coli* WT	ATCC 25922	
BW25113	*E. coli* WT	BW25113	[1]
NB27177	*E. coli*-Δ*tolC*	BW25113 *ΔtolC*, JW5503-1	[1]
NB27178	*E. coli*-Δ*acrB*	BW25113 *ΔacrB*, JW0451-2	[1]
NB27172	*E. coli-imp4213*	in frame deletion of 23 amino acids (D330 to D352) in *lptD* gene in *E. coli* MC4100	[9]
JWK0002	*E. coli*-Δ*tolC, coaSY*	*E. coli ΔtolC*::FRT Δ*coaD*:: *aph* (Km^R), + pNOV016 (pBRori *lacI* P_{tac}:: *coaSY*$_{hs}$ *aacC1* (Gm^R))	This study
NB27079-CDY0099	*E. coli*-9-pump KO	NB27079 Δ*acrB* Δ*acrD* Δ*acrF* Δ*emrB* Δ*emrY* Δ*entS* Δ*macB* Δ*mdtBC* Δ*mdtF*	[10]
K. pneumoniae			
ATCC 43816	*K. pneumoniae* WT	ATCC 43816	[11]
JWK0080	*K. pneumoniae*-Δ*tolC*	ATCC 43816 *ΔtolC*:: *aph* (Km^R)	This study
JWK0079	*K. pneumoniae*-Δ*acrB*	ATCC 43816 *ΔacrB*:: *aph* (Km^R)	This study
H. influenzae			
ATCC51907	*H. influenza* WT	ATCC51907	[12]
NB65044-CDS0020	*H. influenzae*-Δ*tolC*	ATCC51907 *ΔtolC*:: *aph* (Km^R)	[13]
NB65044-CDS0001	*H. influenzae*-Δ*acrB*	ATCC51907 *ΔacrB*:: *aph* (Km^R)	[13]
Plasmids			
pNOV016		IPTG inducible *coaSY*$_{hs}$ expression vector, (pBRori, *lacI,* P_{tac}:: *coaSY*$_{hs}$*, aacC1* (Gm^r))	This study
pAK1900		*E. coli – P. aeruginosa* shuttle vector, Ap^R	A. Kropinski, Queens University
pAK1900-acrB		pAK1900 harboring *acrB*, Ap^R	[10]
pAK1900-emrB		pAK1900 harboring *emrB,* Ap^R	[10]
pAK1900-entS		pAK1900 harboring *entS,* Ap^R	[10]
pAK1900-acrF		pAK1900 harboring *acrF,* Ap^R	[10]
pAK1900-emrY		pAK1900 harboring *emrY,* Ap^R	[10]
pAK1900-mdtF		pAK1900 harboring *mdtF,* Ap^R	[10]
pAK1900-acrD		pAK1900 harboring *acrD,* Ap^R	[10]
pAK1900-macB		pAK1900 harboring *macB,* Ap^R	[10]
pAK1900-mdtBC		pAK1900 harboring *mdtBC,* Ap^R	[10]

Ap^R, ampicillin- resistance marker, Tc^R, tetracycline resistance marker, Gm^R, gentamicin resistance marker, Km^R, kanamycin resistance marker

Mass Spectroscopy Assay Optimization

MS Tuning

Tuning was carried out by setting the RapidFire to continually inject solutions of pure standards (CoA-DP, CoA-SH, and CoA-Ac). Two ion pairs were optimized for CoA DP. A series of instrument parameters were optimized to provide the best sensitivity for each standard. For example, a parameter such as collision energy was tuned while the RapidFire continually injected standards. The parameter was adjusted such that a peak could be identified in the signal intensity as a function of the value changing. The peak where maximum signal intensity was observed was selected as the optimized parameter.

For the initial standard curve characterization, the instrument parameters in **Supplementary Table 4** were used. Mobile phase A consisted of 10 mM tributylamine and 15 mM acetic acid in water with 3% methanol, and mobile phase B consisted of methanol. A C4 column and detector voltage gain of -500 V were used.

Supplementary Table 4. Triple quadrupole mass spectrometer settings for standard curves

Analyte	Q1 (*m/z*)	Q1 (res.)	Q3 (*m/z*)	Q3 (res.)	Dwell (ms)	F (V)	CE (V)	CAV (V)	Polarity
CoA DP (1)	686.15	Unit	408	Unit	50	100	44	7.5	-
CoA DP (2)	686.15	Unit	39	Unit	50	65	44	7.5	-
CoA SH	766.12	Unit	408	Unit	50	100	43	7.5	-
CoA Ac	808.13	Unit	408	Unit	50	150	48	7.5	-
CoA IS	823.14	Unit	408	Unit	50	150	48	7.5	-

Subsequent cellular experiments used slightly different parameters (**Supplementary Table 5**). Mobile phase A consisted of 10 mM tributylamine and 15 mM acetic acid in water with 3% methanol, and mobile phase B consisted of acetonitrile:water (3:1). A phenyl column was used and detector voltage gain was set to -500 V.

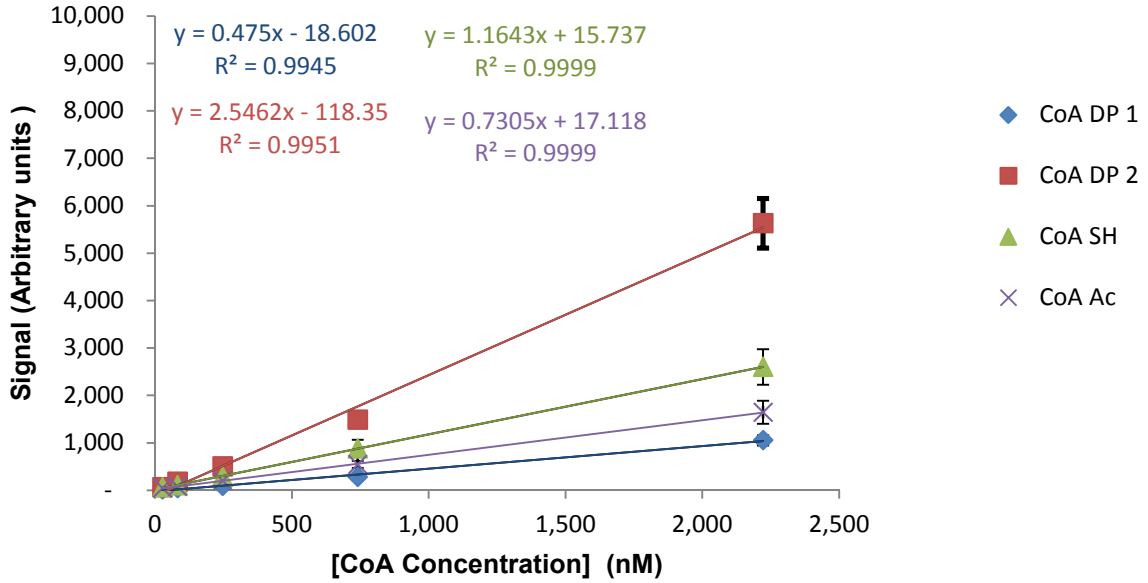
Supplementary Table 5. Triple quadrupole mass spectrometer settings for cellular experiments

Analyte	Q1 (*m/z*)	Q1 (res.)	Q3 (*m/z*)	Q3 (res.)	Dwell (ms)	F (V)	CE (V)	CAV (V)	Polarity
CoA SH	766.12	Wide	408.00	Wide	75	220	38	7.5	-
CoA Ac	808.13	Wide	408.00	Wide	75	220	35	7.5	-
CoA IS	823.13	Wide	408.00	Wide	75	220	35	7.5	

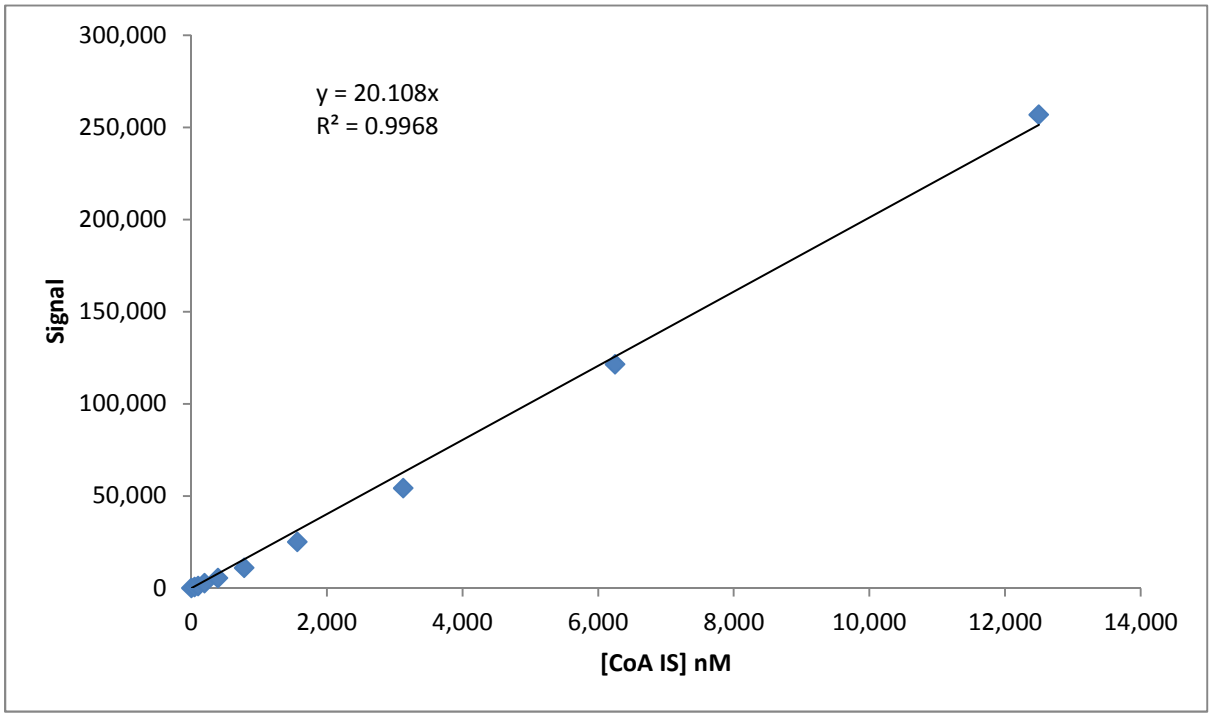
Standard curves of CoA metabolites

Initial chromatography conditions were based on methods described in the literature.[14] Using the optimized tuning parameters, standard curves were then generated. Three-fold dilutions of CoA DP, CoA SH, and CoA Ac were made in 75% acetonitrile, resulting in an 11-point dilution series from 20 µM to 0.11 nM final concentrations. Control wells without compound were also included. Signal could be observed for CoA DP, CoA SH, and CoA Ac from 27 nM – 7 µM (**Supplementary Figure 1**). The signal was linear from approximately 82 nM - 2 µM. R^2 values ranged from 0.994-1.000 with % standard deviations (n=4) of 1-40% in an inverse relation to compound concentration. Carry over was low for CoA DP at 1%, but moderate for CoA SH and CoA Ac at 28% and 27%.

Supplementary Figure 1. Standard curves for CoA metabolites



Supplementary Figure 2 Standard curves for CoA IS



Supplementary References

1. Baba, T. *et al.* Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2,** 2006.0008 (2006).
2. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. *Gene* **212,** 77–86 (1998).
3. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97,** 6640–6645 (2000).
4. Thornton, J. A. Splicing by Overlap Extension PCR to Obtain Hybrid DNA Products. in *Methods in molecular biology (Clifton, N.J.)* **1373,** 43–49 (2014).
5. Appleton, B. *et al.* Discovery and Optimization of Phosphopantetheine Adenylyltransferase Inhibitors with Gram-Negative Antibacterial Activity. *J. Med. Chem.* **under revi,** (2017).
6. Masuda, N. & Ohya, S. Cross-resistance to meropenem, cephems, and quinolones in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **36,** 1847–51 (1992).
7. Zimmermann, W. Penetration through the gram-negative cell wall: a co-determinant of the efficacy of beta-lactam antibiotics. *Int. J. Clin. Pharmacol. Biopharm.* **17,** 131–4 (1979).
8. Li, X. Z., Zhang, L., Srikumar, R. & Poole, K. Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **42,** 399–403 (1998).
9. Ruiz, N., Wu, T., Kahne, D. & Silhavy, T. J. Probing the Barrier Function of the Outer Membrane with Chemical Conditionality. *ACS Chem. Biol.* **1,** 385–395 (2006).
10. Jones, A. K. *et al.* Determinants of Antibacterial Spectrum and Resistance Potential of the Elongation Factor G Inhibitor Argyrin B in Key Gram-Negative Pathogens. *Antimicrob. Agents Chemother.* **61,** e02400-16 (2017).
11. Broberg, C. A., Wu, W., Cavalcoli, J. D., Miller, V. L. & Bachman, M. A. Complete Genome Sequence of Klebsiella pneumoniae Strain ATCC 43816 KPPR1, a Rifampin-Resistant Mutant Commonly Used in Animal, Genetic, and Molecular Biology Studies. *Genome Announc.* **2,** e00924-14-e00924-14 (2014).
12. Fleischmann, R. *et al.* Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science (80-.).* **269,** (1995).
13. Dean, C. R. *et al.* Role of the AcrAB-TolC Efflux Pump in Determining Susceptibility of Haemophilus influenzae to the Novel Peptide Deformylase Inhibitor LBM415. *Antimicrob. Agents Chemother.* **49,** 3129–3135 (2005).
14. Lu, W.; Bennett, B. D.; Rabinowitz, J. D. Analytical strategies for LC–MS-based targeted metabolomics. *J. Chromatogr. B* **871,** 236–242 (2008).