Electronic Supporting Information (ESI)

Colibactin biosynthesis and biological activity depends on the rare aminomalonyl polyketide precursor

A. O. Brachmann, C. Garcie, V. Wu, P. Martin, R. Ueoka, E. Oswald and J. Piel

1. Experimental methods

1.1. Fermentation

Escherichia coli DH5α, *E. coli* Arctic Express™ (DE3) and *E. coli* MG1655 were grown in Luria-Bertani (LB) broth or on LB Agar supplemented with kanamycin at 50 µg mL^{-1} or chloramphenicol at 37 µg mL^{-1} where appropriate. *E. coli* DH5α was routinely grown at 30 °C and 37 °C and *E. coli* MG1655 at 37 °C.

1.2. Verification of DNA integrity between *clbG* and *clbH*

The revision of ClbH A1 integrity was conducted by downstream and upstream sequencing of the *clbH* A1 domain. PCR products were generated by using DNA of pBAC*pks*$^+$ and oligonucleotides ColGap_Fw and ColGap_Rv for the upstream part of *clbH* A1 and oligonucleotides ColGap2_Fw and ColGap2_Rv for the downstream part of *clbH* A1. PCR products were cloned into sequencing vector pJET1.2 using Thermo CloneJet PCR cloning kit. Sequencing results were analyzed and interpreted with Geneious® 7 software.

1.3. Construction of expression plasmids for N-terminal His-tagged proteins

The DNA of pBAC*pks*$^+$ (Table S3) was used as template to PCR amplify *clbA*, *clbD*, *clbE*, *clbF*, *clbH-A1*, *clbH-A2* using Phusion DNA Polymerase (see Table S4 for primer sequences). The PCR products were ligated into the expression vector pCOLADuet™-1 (Novagen®) with the help of the Gibson Assembly® cloning kit (New England BioLabs®). For this purpose, pCOLADuet™-1 was linearized with *Bam*HI and both forward and reverse primers (Table S4) for the specific *clb* products were 5`-modified with an overlapping sequence complementary to the generated 5` and 3`-ends of the vector. ClbH-A2, ClbJ-A1 and ClbK-A2 were first cloned and expressed including the peptidyl carrier protein (PCP). Due to insufficient protein activity, ClbH-A2, ClbJ-A1 and ClbK-A2 were also expressed without PCP. For this, expression vectors ClbH-A2(PCP), ClbJ-A1(PCP) and ClbK-A2(PCP) were used as template to PCR amplify with primers Awo_PCP_Fw, A4wo_PCP_Rv and A6wo_PCP the expression plasmids excluding PCP domains. PCR products were ligated and transformed into *E. coli* DH5α by electroporation, and DNA integrity was verified by sequencing prior to transformation into the expression strain *E. coli* Arctic Express™.

1.4. Construction of colibactin mutants and complementation

Gene inactivations of *clbE* and *clbDEF* were engineered by using the lambda Red recombinase method.[1] Briefly, PCR products were generated that were homologous to regions adjacent to the gene to be inactivated and template plasmids carrying antibiotic resistance genes flanked by FLP recognition target sites. PCRs were conducted by using primers CG_clbE_P1/CG_clbE_P2 and CG_cblDEF_P1/CG_cblDEF_P2 (Table S4). Deletion mutants of the *clbE* gene or the *clbDEF* operon were isolated as antibiotic-resistant colonies after the introduction of synthetic PCR-generated DNA into bacteria carrying the lambda Red expression pKD46 plasmid. The resistance genes were then eliminated by using the pCP20 helper plasmid encoding the FLP recombinase. For complementation of the

Δ*clbE* mutant, the pCOLAclbE plasmid (described in 1.3, Table S3) was transformed into the MG1655 pBAC*pks*$^+$ Δ*clbE* strain. For complementation of the Δ*clbDEF* mutant, the DNA of pBACpks+ (Table S3) was used as template to PCR amplify the *clbDEF* operon using Phusion DNA Polymerase and primers CG_clbDEF_Fw/CG_clbDEF_Rv (Table S4). The PCR product was ligated into pCR-XL-TOPO vector using the TOPO® XL PCR Cloning Kit (Invitrogen). The generated pTOPOclbDEF plasmid (Table S3) was then transformed into the MG1655 pBAC*pks*$^+$ Δ*clbDEF* strain.

1.5. Overexpression and purification of His-tagged Clb enzymes

Large scale 1 L cultures of Luria Bertani broth supplemented with 50 µg ml^{-1} kanamycin were inoculated 1:400 with an overnight culture of the respective expression strain. Cultures were incubated at 30 °C at 200 rpm. As soon as the cultures reached an OD_{600} = 0.6-0.8 they were chilled on ice for 1 h and subsequently induced with 0.1 mM IPTG. Afterwards cultures were incubated at 16 °C for additional 20 h. The cells were harvested by centrifugation at 3000×g, 4 °C for 20 min and the cell pellet was quick-frozen with liquid nitrogen. The cell pellet was resuspendend in 4 mL lysis buffer (50 mM sodium phosphate buffer pH 8, 300 mM NaCl, 10 % glycerol, 20 mM imidazole) per gram cell pellet and lysozyme (1 mg mL^{-1}) was added. The suspension was allowed to stir at 4 °C for 1 h prior to sonification. The cell lysate was centrifuged at 18,000×g for 45 min. The supernatant was transferred into a fresh tube, mixed with Ni-NTA resin (approx.1 mg) and rocked at 4 °C for 1 h, followed by centrifugation at 800 g, 4 °C for 10 min. The supernatant was discarded and the resin was washed twice with lysis buffer and finally transferred to a column with frit. A stepwise imidazole gradient using thrice the bed volume for each step (40 mM, 60 mM, 80 mM and 250 mM) was deployed to elute the protein. The presence and purity of the proteins were monitored by SDS-PAGE analysis. Fractions with the respective proteins were dialyzed thrice against 1 L storage buffer (20 mM Tris-HCl pH 7.5, 10 % glycerol, 1 mM DTT).

1.6. Enzyme Assays

For enzyme reactions the following stock solutions were prepared: 1 M Tris-HCl pH 8.5, 200 mM $MgCl_2$, 100 mM coenzymeA (Sigma-Aldrich GmbH), 1 M DTT, 200 mM L-serine, 200 mM L-serine-$^{13}C_3$ (Sigma-Aldrich GmbH), 100 mM ATP (Sigma-Aldrich GmbH), 20 mM NAD^+ (Sigma-Aldrich GmbH), 10 mM FAD (Sigma-Aldrich GmbH). The concentrations of the used enzyme stocks are metioned in the section before.

(1) The ACP/PPTase assay (100 µl) contained reaction buffer (100 mM Tris-HCl pH 8.5, 20 mM $MgCl_2$, 1 mM coenzyme A, 10 mM DTT), 10 µl ACP (ClbE), 2µl PPTase (ClbA) The volume was adjusted with H_2O to 100 µL and the assay was incubated at 30 °C for 1 h. A control experiment contained 2 µL water instead of PPTase (ClbA). All enzyme reactions were quenched by adding 100 µl (80 % acetonitrile + 0.1 % TFA) and analyzed by HESI-MS.

(2) The ACP loading assay contained in addition to assay (1) 3 mM ATP, 2 mM L-serine and 5 µl adenylation domain ClbA(A) and was incubated at 30 °C for 30, 60 and 120 min. Control reactions were conducted replacing the adenylation domain ClbA(A), L-serine and both with H_2O.

(3) The seryl-ACP assay contained in addition to assay (2) 2 mM NAD^+ in the presence of ClbD and 1 mM FAD in the presence of ClbF. The reactions were incubated at 30 °C for 2 h. Controls were conducted with boiled (98 °C for 5 min) ClbD and ClbF and H_2O instead of ClbD and ClbF.

(4) The characterization of adenylation domains was achieved by using the γ-[$^{18}O_4$]-ATP pyrophosphate exchange assay as described before.[2] Briefly, reaction mixtures each with a total volume of 6 µl consisted of 2 µM enzyme ClbH-A1, 1 mM amino acid, 0.3 mM DTT, 5 mM $MgCl_2$, 5 mM inorganic pyrophosphate, 1 mM γ-$^{18}O_4$ ATP and 20 mM Tris-HCl (pH 7.5). All canonical amino acids and three available non-canonical amino acids, ethylglycine (EtGly), 2,3-diaminopropionic acid (DAP), norvaline, were tested. ClbH-A1 and ClbH-A2 were also tested with 1-aminocyclopropanecarboxylic acid (ACC) (Sigma-Aldrich) and S-(5´-adenosyl)-L-methionine (SAM) (Sigma-Aldrich). The reaction was incubated at RT for 2 h, quenched with 6 µl MALDI matrix 9-aminoacridine in acetone (10 mg mL^{-1}) and analyzed using a MALDI LTQ Orbitrap instrument (Thermo Scientific).

1.7. MS analysis of ACP derivatives

ACP-assay samples were subjected to ultra-performance liquid chromatography-high resolution heated electrospray ionization mass spectrometry (UPLC HR HESI-MS) analysis. Data were collected on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Dionex Ultimate 3000 UPLC system. Obtained series of multiply charged ions were transformed into single molecular masses with the help of Thermo Protein Deconvolution 2.0 SP1 software. The following solvent gradient (A = H_20 + 0.1% formic acid, B = acetonitrile + 0.1% formic acid with B at 10% for 0-2.5 min, 10-100% for 2.5-8 min and 100% for 8-10 min at a flowrate of 0.5 ml min^{-1}) was used on a Phenomenex Aeris Widepore C4 3.6 µm C4 (50 X 2.1 mm) column at 50 °C. The MS was operated in positive ionization mode at a scan range of 333.4 -2000 *m*/*z* and a resolution of 140,000. The spray voltage was set to 3.5 kV, the S-lens to 100, the auxiliary gas heater temperature to 400 °C and the capillary temperature to 275 °C.

1.8. MALDI MS analysis of [$^{18}O_4$]-ATP pyrophosphate exchange assay products

The data were recorded on a Thermo Scientific™ MALDI LTQ Orbitrap™ XL equipped with a nitrogen laser at λ= 337 nm. The MS was operated in negative ionization and FTMS mode. The laser energy was tuned semiautomatically on 9-aminoacridine (10 mg ml^{-1} in acetone) matrix and set to 40 µJ. The following parameters were applied: automatic spectrum filtering (ASF) = off, automatic gain control (AGC) = on, number of laser shots = 1, microscans = 1, resolution 15000, scan range from 500-520 *m*/*z* and crystal positioning system (CPS). The average of 200 scans was used for each amino acid analysis. Data were normalized to the highest exchange rate for ClbJ-A1, ClbK-A2 and ClbH-A1.

1.9. HPLC-MS analysis of crude extracts of *clbE*, *clbDEF*, and complemented deletion mutants

20 mL cultures of Luria Bertani broth containing XAD-16 resin (Sigma-Aldrich) were inoculated 1:100 with an overnight culture of the respective strain (*E. coli* MG 1655 pks^+ Δ*clbE*, *E. coli* MG 1655 pks^+ Δ*clbDEF*, *E. coli* MG 1655 pks^+ Δ*clbE* pTOPO*clbE*, *E. coli* MG 1655 pks^+ Δ*clbDEF* pTOPO*clbDEF*) and grown for 24 h at 37 °C. XAD-16 was harvested and washed with deionized water and extracted with 20 mL methanol. The extract was filtered, dried and redissolved in 1 mL methanol. 1:4 diluted samples were used for HPLC-MS analysis. Samples were analyzed by ultra-performance liquid chromatography-high resolution heated electrospray ionization mass

spectrometry (UPLC HR HESI-MS). Data were collected on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Dionex Ultimate 3000 UPLC system. The following solvent gradient (A = H_20 + 0.1% formic acid, B = acetonitrile + 0.1% formic acid with B at 5% for 0-2 min, 5%-98% for 2-25 min and 98% for 25-29.5 min at a flowrate of 0.5 ml min^{-1}) was used on a Phenomenex Kinetex XB-C18 2.6 µm 100 Å (150 X 4.6 mm) column at 30 °C. The MS was operated in positive ionization mode at a scan range of 250-2000 *m*/*z* and a resolution of 70,000. The spray voltage was set to 3.5 kV, the S-lens to 50, the auxiliary gas heater temperature to 430 °C and the capillary temperature to 270 °C.

1.10. Megalocytosis and genotoxicity assay

The megalocytosis effect induced by colibactin was determined as previously described.[3] Briefly, HeLa cells were dispensed in 96-well cell culture plate (5×10^3 cells/well) and incubated for 24 hours. For bacterial infections, overnight LB broth cultures of *E. coli* were diluted in interaction medium (DMEM, 5% FCS, 25mM HEPES) and cell cultures were infected with a multiplicity of infection (number of bacteria per HeLa cell at the onset of infection) of 6 to 200. Four hours post-inoculation, cells were washed 3 times with HBSS and incubated in cell culture medium with 200 µg/mL gentamicin until analysis. For megalocytosis quantification, cells were incubated for 72 h before protein staining with methylene blue (1% w/v in Tris-HCl 0.01M). The methylene blue was extracted with HCl 0.1 N. The quantification of staining was measured at OD_{660}.

1.11. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0c. The mean ± standard deviation (SD) is shown in figures, and p-values were calculated using a one-way ANOVA followed by a Bonferroni post-test unless otherwise stated. A p-value of less than 0.05 was considered statistically significant and is denoted by *. $p < 0.01$ is denoted by ** and $p < 0.001$ by ***. Non-significant result is denoted ns.

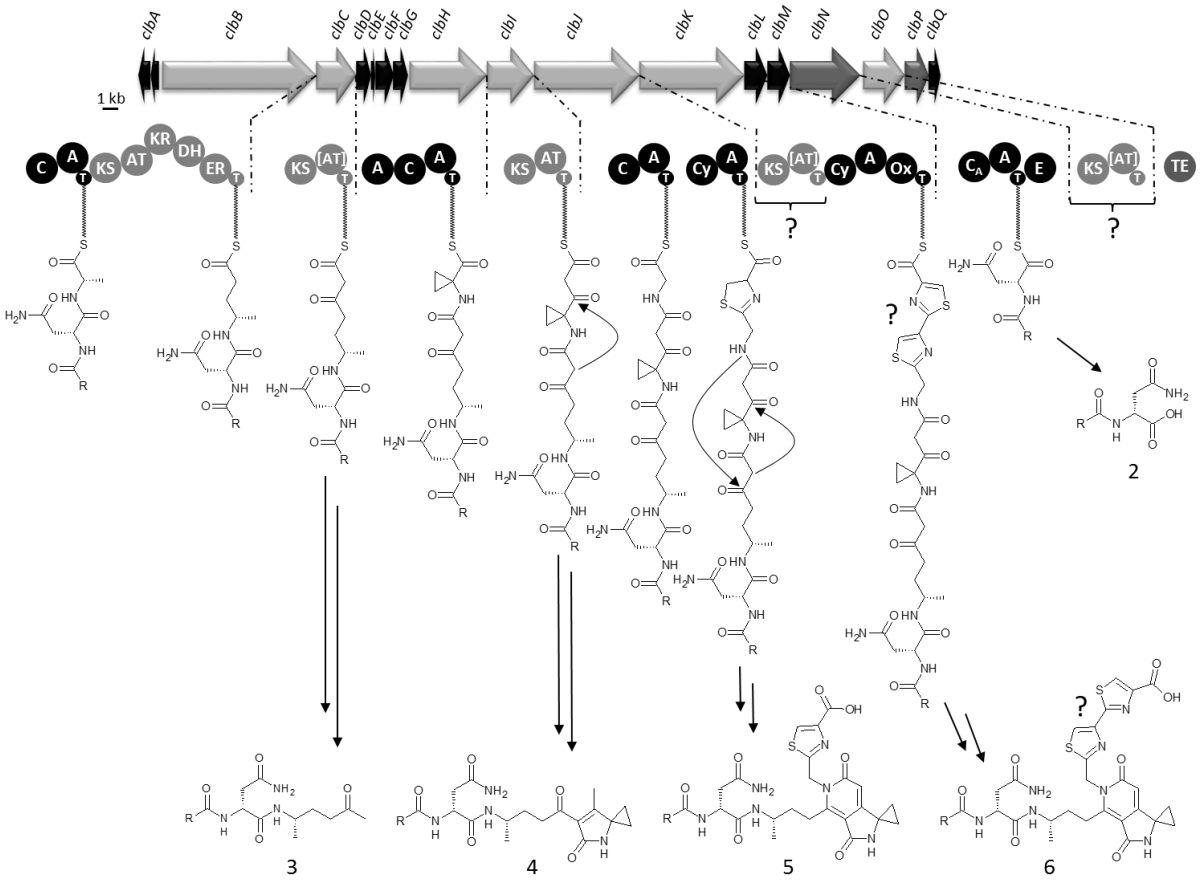

Figure S1. Architecture of the *clb* gene cluster from *E. coli* IHE2034 and model for colibactin biosynthesis based on previously available data. **6** is a hypothetical structure proposed on the basis of HRMS data.[4] NRPS modules are shown in black, PKS modules in grey. Numbered compounds were characterized in prior studies. Abbreviations: A, NRPS adenylation domain; AT, PKS acyltransferase; C, NRPS condensation domain; Cy, NRPS cyclization domain; DH, PKS dehydratase; E, NRPS epimerization domain; ER, PKS enoylreductase; KR, PKS ketoreductase; Ox, NRPS oxidation domain; T, PKS and NRPS thiolation domain (also referred to as ACP and PCP, respectively); TE, thioesterase domain. AT domains in parentheses are predicted to be inactive.

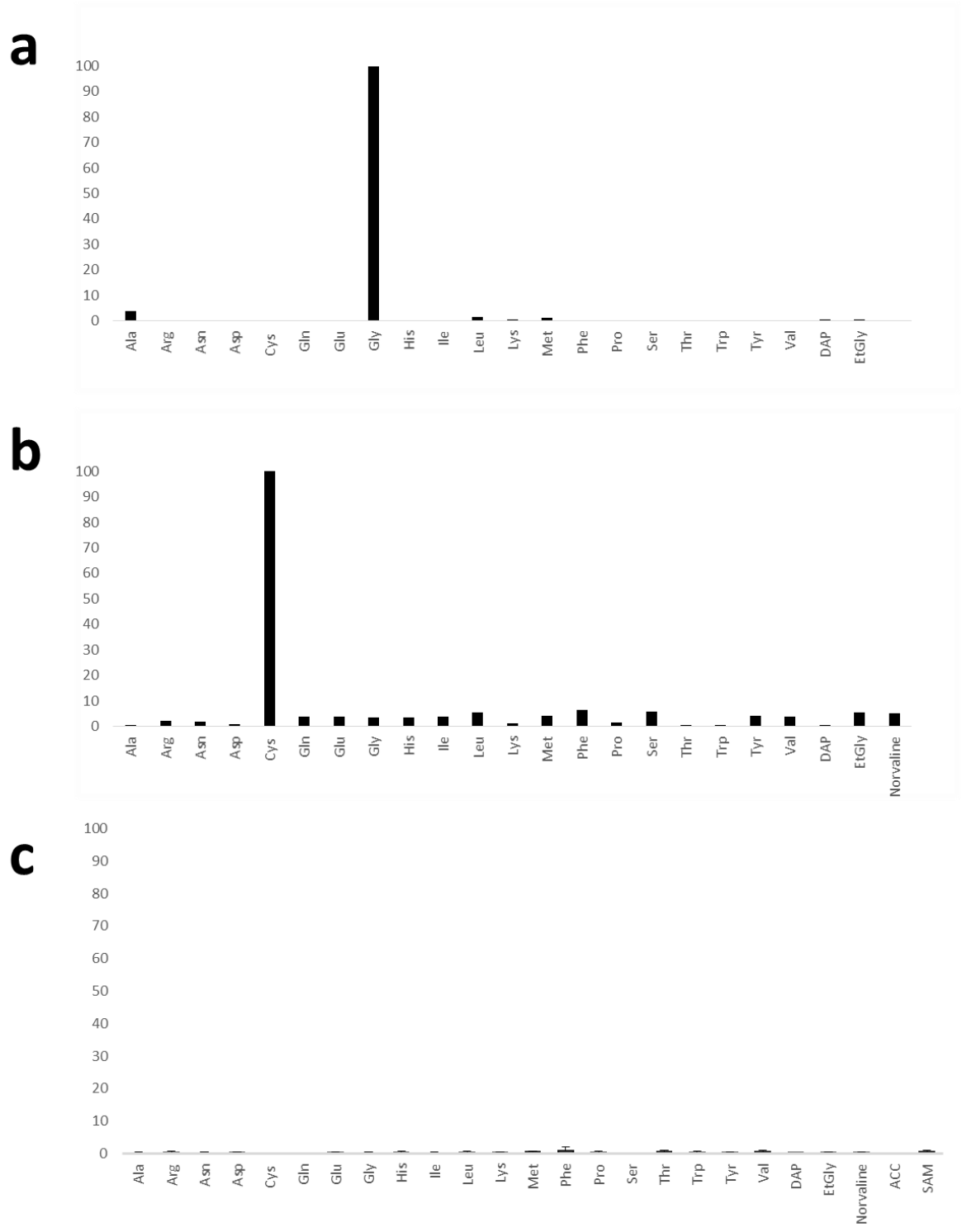

Figure S2. Exchange rates for the A domains ClbJ-A1 (a), ClbK-A2 (b) and ClbH-A2 (c) for various amino acid test substrates. EtGly, ethylglycine DAP, 2,3-diaminopropionic acid; ACC, aminocyclopropanecarboxylic acid; SAM, S-(5´-adenosyl)-L-methionine.

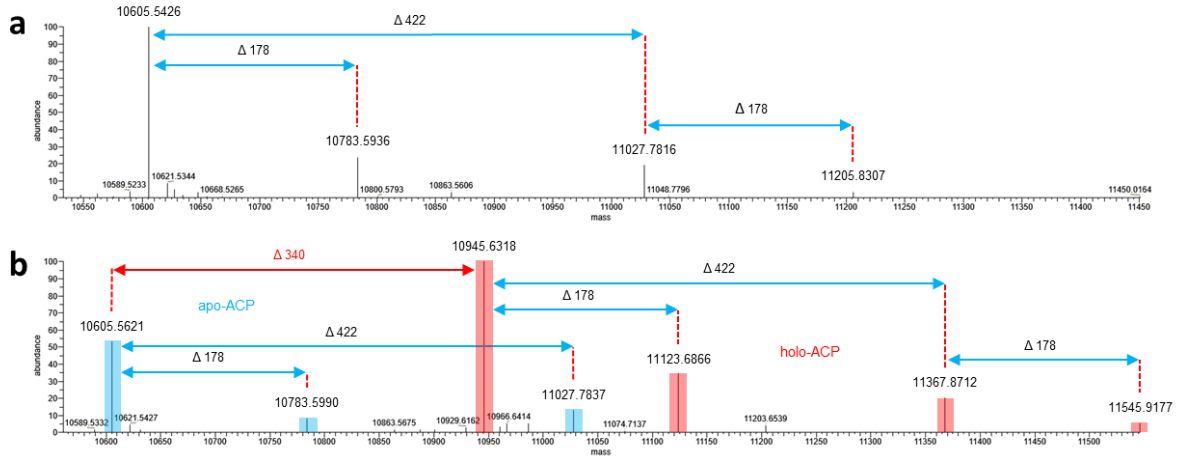

Figure S3. Activation of the ACP ClbE by the PPTase ClbA. (a) The deconvoluted spectrum shows ions for incubations containing the *apo*-ACP (calculated 10605.5524 Da) without PPTase. The mass shift of 178 Da corresponds to a modification of His-tagged proteins by gluconoylation, the 422 Da shift is as-yet unknown.[5] (b) Representative MS spectrum for assays containing the *apo*-ACP, PPTase ClbA, and coenzyme A. Ions of *apo*-ACP species are highlighted in blue, those of *holo*-ACP variants in red. The calculated mass of the *holo*-ACP is 10945.6382 Da. The covalent attachment of the phosphopantetheine moiety is indicated by a mass shift of 340 Da.

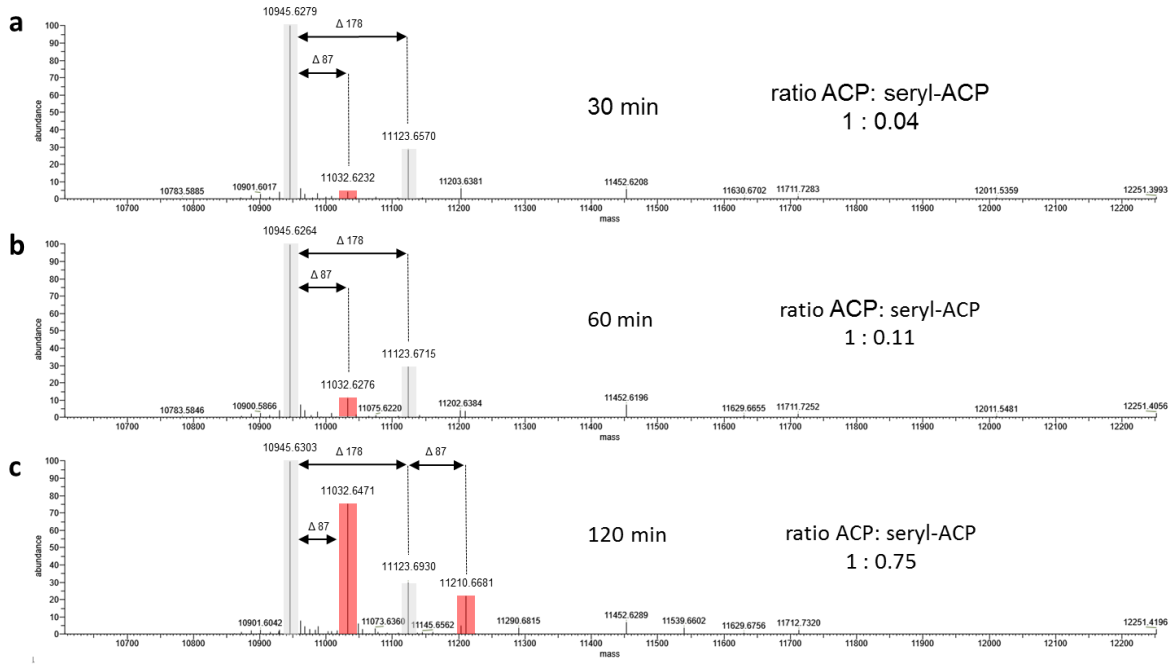

Figure S4. Deconvoluted spectra of ACP species formed in incubations of the *holo*-ACP ClbE with the A domain ClbH-A1 and serine. Formation of seryl-ACP over time was monitored. Ions corresponding to the *holo*-ACP (calculated 10945.6382 Da) are indicated by grey bars and those attributed to seryl-ACP (calculated 11032.6702 Da) are marked in red. The mass shift of +87 Da is consistent with loading of L-serine onto the *holo*-ACP. The mass shift of 178 Da corresponds to a glycosylated species.[5]

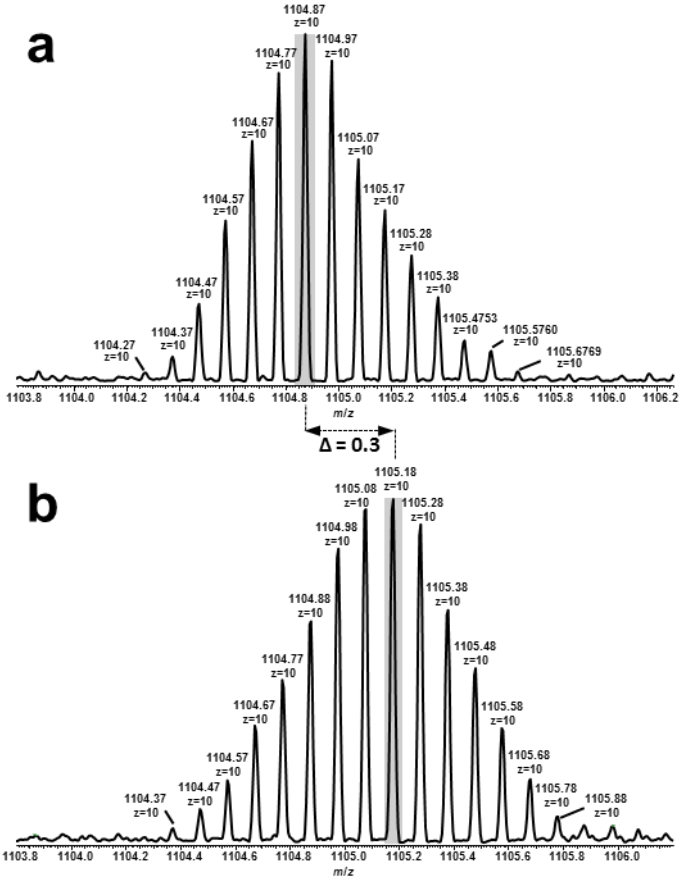

Figure S5. Isotopically resolved spectra of seryl-ACP $[M+10H]^{10+}$ generated by incubation of *holo*-ClbE with ClbH-A1 and serine species. (a) Seryl-ACP using unlabelled L-serine as substrate. (b) Seryl-ACP using [$^{13}C_3$]-L-serine. The mass shift between the dominant ions corresponds to the incorporation of three ^{13}C labels.

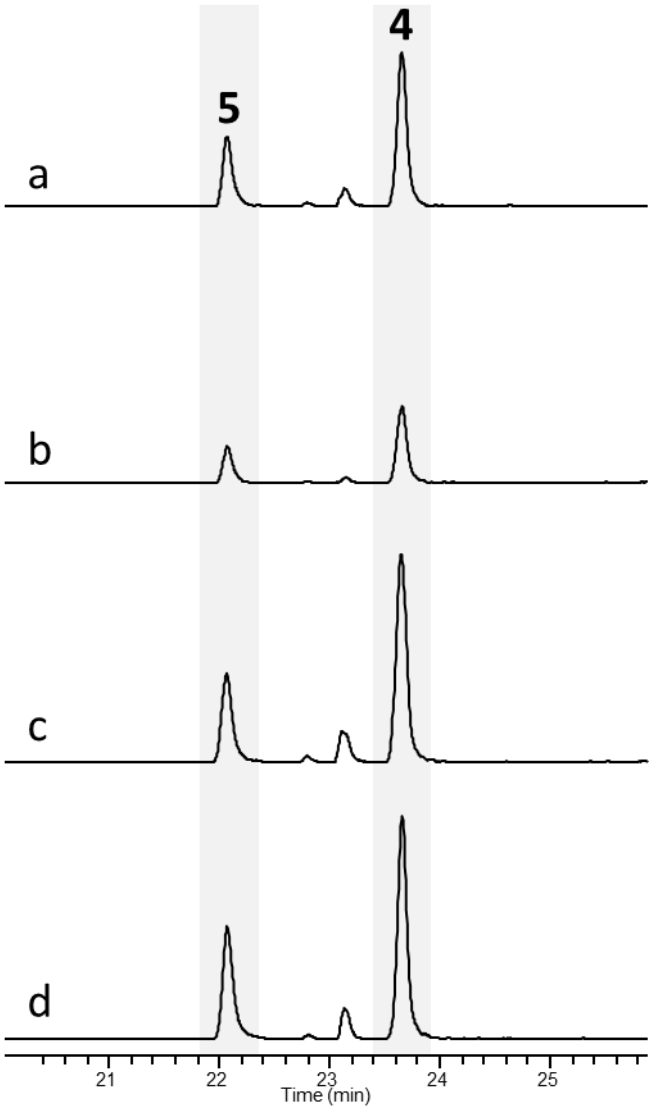

Figure S6. Extracted ion chromatograms of ACC-containing colibactin products **4** and **5**. The other two peaks belong to unknown compounds with ions of *m/z* = 547.3 and 713.3. (a) *E. coli* MG1655 pBAC*pks*$^{+}$ Δ*clbE* (b) *E. coli* MG1655 pBAC*pks*$^{+}$ Δ*clbDEF* (c) *E. coli* MG1655 pBAC*pks*$^{+}$ Δ*clbE* pTOPO*clbE* (d) *E. coli* MG1655 pBAC*pks*$^{+}$ Δ*clbE* pTOPO*clbDEF*.

Figure S7. ClustalW analysis of AT domains of ClbB, ClbC, ClbI, ClbK and ClbO (Fig. S1). The highly conserved GHSxG motif of ATs is highlighted in blue. The HAFH motif correlated to malonyl-CoA-specific ATs is highlighted in green.

Table S1. Comparison of accessory proteins in the colibactin and zwittermicin cluster.

***E. coli* IHE 3034**					***Bacillus cereus* UW85[a]**		
Protein	**Accession No.**	**Size (aa)**	**Deduced function**	**Identity/ similarity**	**Protein**	**Size (aa)**	**Accession No.**
ClbA	CAJ76300.1	244	phosphopantetheinyl transferase	--[b]	--	--	--
ClbD	CAJ76296.1	289	3-hydroxyacyl-CoA dehydrogenase	44 / 66	ZmaG	282	AAR87756.1
ClbE	CAJ76295.1	82	acyl-carrier protein	31 / 60	ZmaH	81	AAR87757.1
ClbF	CAJ76294.1	376	acyl-CoA dehydrogenase	34 / 56	ZmaE	382	AAD40109.1
				36 / 58	ZmaI	397	AAR87758.1
ClbH (A1)	CAJ76292.1	(1598)	NRPS A1-domain	43 / 59	ZmaJ	525	AAR87759.1
ClbG	CAJ76293.1	422	malonyl-CoA transacylase	34 / 54	ZmaF	405	AAD40110.1

[a]*Bacillus cereus* strain UW85 zwittermicin A biosynthetic gene cluster, GenBank accession number: FJ430564.1

[b]There is no homolog for ClbA in the zwittermicin cluster

Table S2. Identified MS ions corresponding to ACP species in coincubations of ClbA, ClbE, ClbH-A1, ClbD, ClbF, coenzyme A, serine, NAD^+ and FAD (Fig. 2e).

ACP species	molecular formula	calculated mass	detected mass[a]	mass error (ppm)	relative abundance
apo-ClbE	$C_{470}H_{752}N_{136}O_{142}S$	10605.5524	10605.5356	1.58	25.59
holo-ClbE	$C_{481}H_{773}N_{138}O_{148}S_2P$	10945.6382	10945.6281	0.92	100
seryl-ClbE	$C_{484}H_{778}N_{139}O_{150}S_2P$	11032.6702	11032.6423	2.53	20.96
glycyl-ClbE	$C_{483}H_{776}N_{139}O_{149}S_2P$	11002.6675	11002.6307	3.34	64.48

[a]spectra deconvoluted with Thermo Protein Deconvolution Software 2.0

Table S3. Plasmids used in this study.

Plasmid	Characteristics	Source / Reference
pCOLADuet™-1	Expression vector, IPTG inducible T7 promoter, Km^R	Novagen®
pCR-XL-TOPO®	Expression vector, IPTG inducible T7 promoter, Km^R	Invitrogen™
pBAC*pks*$^+$	colibactin cluster from *E. coli* IHE3034, Cm^R	Ref. 5
pCOLAclbA	pCOLADuet™-1, *clbA* with N-terminal His-tag in MCS1	This work
pCOLAclbD	pCOLADuet™-1, *clbD* with N-terminal His-tag in MCS1	This work
pCOLAclbE	pCOLADuet™-1, *clbE* with N-terminal His-tag in MCS1	This work
pCOLAclbF	pCOLADuet™-1, *clbF* with N-terminal His-tag in MCS1	This work
pCOLAclbH(A1)	pCOLADuet™-1, *clbH*(A1) with N-terminal His-tag in MCS1	This work
pCOLAclbH(A2)	pCOLADuet™-1, *clbH*(A2) with N-terminal His-tag in MCS1	This work
pCOLAclbJ-A1(PCP)	pCOLADuet™-1, *clbJ*(A1) PCP with N-terminal His-tag in MCS1	This work
pCOLAclbK-A2(PCP)	pCOLADuet™-1, *clbK*(A2) PCP with N-terminal His-tag in MCS1	This work
pCOLAclbJ-A1	pCOLADuet™-1, *clbJ*(A1) with N-terminal His-tag in MCS1	This work
pCOLAclbK-A2	pCOLADuet™-1, *clbK*(A2) with N-terminal His-tag in MCS1	This work
pTOPOclbDEF	pCR-XL-TOPO®, *clbDEF* operon	This work
pTOPOclbE	pCR-XL-TOPO®, *clbE*	This work

Table S4. Oligonucleotides used in this study.

Oligonucleotide	5´to 3´Sequence
GB_clbD_Rv	gcgccgagctcgaattcggttaacccagaatcactgctcg
GB_clbD_Fw	catcatcaccacagccagaacgtggcggtaataggtgca
GB_clbE_Rv	gcgccgagctcgaattcgtttactccaggcctgtttgtc
GB_clbE_Fw	catcatcaccacagccagaaaaagcaagatatgaaagcc
GB_clbF_Rv	gcgccgagctcgaattcggacatccttcgtcatgcttc
GB_clbF_Fw	catcatcaccacagccagtgcactgaaaattatgagctg
GB_clbG_Rv	catatgttagaaaataatattacacagatttc
GB_clbG_Fw	gagctccatttgatttacaatactgtgatgttc
GB_clbA_Rv	catatgaaaaatgcagtgcaaattgtgaatga
GB_clbA_Fw	ctgcagaccaagttatcttcttctttactgaatctt
GIB_A2_Ser_Rv	catatgaaagatagcatggctaaaaaggaaat
GIB_A2_Ser_Fw	gagctcagtaagtcaggattaaaactctcttccgc
GIB_A2_unknown_Fw	catcaccacagccagatcgatgggcgcatcacc
GIB_A2_unknown_Rv	ccgagctcgaattcgttacagacgctgttccagttcg
GIB_A4_Gly_Fw	catcatcaccacagccaggacatgacagtcgaggtgttcc
GIB_A4_Gly_Rv	gcgccgagctcgaattcgttatgctgtgcaatacgcgcactc
GIB_A6_Cys2_Fw	catcatcaccacagccagtacagtatttcacagacaccgc
GIB_A6_Cys2_Rv	gcgccgagctcgaattcgattgtgcagttgctcatcaagg
Awo_PCP_Fw	taacgaattcgagctcggcgc
A6wo_PCP_Rv	atagctgcggcgtttgatg
A4wo_PCP_Rv	ttccgtgtcgctcagcgc
ColGap_Fw	catgatcgtgcgcaactagc
ColGap_Rv	aggcaccaccagcttttaca
ColGap2_Fw	gtatctcgctggtatcgggc
ColGap2_Rv	atcatcactcaccgagggga
CG-clbE-P1	actgcctcttgctgagccagctcataattttcagtgcacatagtttactcgtgtaggctggagctgcttc
CG-clbE-P2	agacgtacgccgagcagtgattctgggttaaccagatagggatatgaatacatatgaatatcctccttag
CG-clbDEF-P1	attgcgaaccggagccagggaacatcagtgcgacatccttcgtcatgcttgtgtaggctggagctgcttc

CG-clbDEF-P2	actgggggcgaagacaacctcgaaatagcctgtcagtaaaggagctcaacatatgaatatcctccttag
CG_clbDEF_Fw	gaaccggagccagggaac
CG_clbDEF_Rv	aatccgcgcaatatctacgc

1. K. A. Datsenko and B. L. Wanner, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 6640-6645.
2. V. V. Phelan, Y. Du, J. A. McLean and B. O. Bachmann, *Chem. Biol.*, 2009, **16**, 473-478.
3. P. Martin, I. Marcq, G. Magistro, M. Penary, C. Garcie, D. Payros, M. Boury, M. Olier, J. P. Nougayrede, M. Audebert, C. Chalut, S. Schubert and E. Oswald, *PLoS Path.*, 2013, **9**, e1003437.
4. Z. R. Li, Y. Li, J. Y. Lai, J. Tang, B. Wang, L. Lu, G. Zhu, X. Wu, Y. Xu and P. Y. Qian, *ChemBioChem*, 2015, online early version, doi: 10.1002/cbic.201500239.
5. K. F. Geoghegan, H. B. Dixon, P. J. Rosner, L. R. Hoth, A. J. Lanzetti, K. A. Borzilleri, E. S. Marr, L. H. Pezzullo, L. B. Martin, P. K. LeMotte, A. S. McColl, A. V. Kamath and J. G. Stroh, *Anal. Biochem.*, 1999, **267**, 169-184.