

1 Supplementary material for:

2

3 ***E. coli* EC93 deploys two plasmid-encoded class I CDI**
4 **systems for antagonistic bacterial interactions**

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24 **This supplementary file includes:**

25 Supplementary methods

26 Chromosomal and plasmid constructs

27 Supplementary tables S1-S6

28 Supplementary figures S1-S10

29

30 **Supplementary methods**

31 *Minimal inhibitory concentration (MIC) measurements*

32 MIC assays were carried out using E-test according to the instructions of the
33 manufacturer (AB bioMerieux, Solna, Sweden). E-tests were performed on LB agar
34 plates that were incubated for 16–18h at 37°C.

35

36 *Prophage identification*

37 The EC93 genome was analyzed for the presence of intact prophages and partial
38 prophage sequences using PHASTER (PHAge Search Tool Enhanced Release) [1, 2].

39

40 *Phage plaque assay*

41 Cultures of EC93 were grown overnight in LB liquid broth supplemented with 10mM
42 MgSO₄ and 5mM CaCl₂. Cultures were then diluted 1:10 in fresh LB supplemented
43 with 10mM MgSO₄ and 5mM CaCl₂ and 5mL were aliquoted in an empty petri dish
44 and subjected to varying degree of UV light (254 nm). After UV exposure cells were
45 left to recover for 2 h at 37°C to allow for prophage induction. Cultures were then
46 pelleted at 3000xg for 5min and the supernatant was filtered through a 0.2µm filter
47 before being spotted on a soft-agar plate (top-agar 0.5%, bottom agar 1.5%) made
48 with a lawn of *E. coli* MG1655.

49

50 *Transposon mutagenesis to identify EC93 CdiA-CT2 resistant mutants*

51 To generate CDI-resistant mutants, target cells were mutagenized, mixed with CDI⁺
52 inhibitor cells, and CDI-resistant mutants were selected as described previously [3].
53 The mariner transposon [4] was introduced into *E. coli* DL8705 by conjugation with
54 MFD_{pir} cells carrying plasmid pSC189. Donors were supplemented with 30µM
55 diaminopimelic acid in shaking liquid LB and grown to mid-log at 37°C. Donors and
56 recipients were mixed and plated on LB agar at 37°C for 5 h. Cell mixtures were
57 harvested with a sterile swab, collected in 1×M9 medium, and plated on kanamycin-
58 supplemented LB agar for selection of transposon integrants. Each mutant pool was
59 harvested into 0.5 mL 1×M9 media for selection. Each mutant pool was subjected to
60 selection by co-culture with EPI100 Δ*wzb* carrying plasmid pDAL879-*cdiA1*-
61 *CT2/cdiI2*. The surviving colonies were collected again into 1 mL 1×M9 media and
62 subjected to two more rounds of co-culture selection. Transposon insertion sites were
63 determined by rescue cloning. Chromosomal DNA from each resistant mutant was

64 digested overnight with NspI at 37°C followed by a 20 min inactivation step at 65°C.
65 Each reaction was supplemented with ATP and T4 Ligase and incubated overnight at
66 16°C. The reactions were electroporated into *E. coli* DH5 α *pir*⁺ cells. Plasmids from
67 the resulting kanamycin-resistant colonies were isolated and transposon insertion
68 junctions identified by sequencing using primer CH2260.

69

70 *CdiA* SDS-PAGE assay

71 MG1655 cells expressing CdiA from the chromosome or from a medium-copy
72 plasmid (table S4) were diluted 1/1000 from an overnight culture and grown to
73 logarithmic phase (OD₆₀₀ = 0.4). Bacterial cultures (1 mL) were pelleted at 21,000 x g
74 for 10 min and re-suspended in 100 μ l of a 1x membrane-protein sample buffer
75 [50mM Tris pH 6.8, 1% SDS (w/v)], 1% Triton X-100, 10% Glycerol, 0.2%
76 bromophenol blue) and boiled for 5 min at 95°C. DTT (150 mM) was added to each
77 sample before pelleting at 21,000 x g for 5 min, then supernatant were loaded and
78 analyzed by SDS-PAGE (NuPAGE Tris-Acetate 3-8% gel, Thermo Scientific, USA).
79 Total protein was detected *in situ* using SYPRO Ruby Protein Gel Stain (Thermo
80 Scientific, USA) and visualized by UV light (254 nm). Relative protein abundance
81 was calculated by densitometry using ImageJ.

82

83 **Chromosomal and plasmid constructs**

84

85 *Construction of EC93 cdi mutants*

86 The *cdi-1* and *cdi-2* loci were deleted by amplifying a *catR* selection cassette [5] using
87 primers 1185/1273, for *cdi-1*, and primers 767/1186 for *cdi-2*, and selecting for
88 recombinants using the phage λ Red system [6]. To prevent auto-inhibition of growth
89 during the KO of *cdi-1*, prior to recombineering, EC93 was first transformed with the
90 temperature-sensitive plasmid pSK2757 expressing cognate CdiI1 immunity, which
91 was subsequently cured from EC93 by growing cells at 42°C. Plasmid pSK2757 was
92 constructed by amplifying the *cdiA1* toxin-immunity region with primers 543/544,
93 then blunt-end ligating this fragment to a pSC101 vector backbone generated by
94 amplification using primers 477 and 921. To prevent auto-inhibition during the KO of
95 *cdi-2*, prior to recombineering, EC93 was transformed with plasmid pSK1244
96 expressing cognate CdiI2 immunity. Plasmid pSK1244 and pSIM27 [7] both share a

97 pSC101 origin of replication, but have distinct antibiotic selection markers (kanR and
98 tetR respectively). Plasmid pSK1244 was segregated out of the population by only
99 selection for pSIM27 after lambda red recombineering. Plasmid pSK1244 was
100 constructed by amplifying the *cdiA2* toxin-immunity region with primers 547/548,
101 then blunt-end ligating this fragment to a pSC101 vector backbone generated by
102 amplification using primers 477 and 921. The constructs were verified by PCR using
103 primers 1489 with 1502, 1491 with 630 and 564 with 1491.

104

105 *Construction of EC93 catR mutant*

106 An chloramphenicol resistant EC93 strain was created by inserting a *catR* selection
107 cassette [5] downstream of the *cdi-1* locus in EC93 by amplifying the *catR* cassette
108 using primers 1500/1501 and selecting for recombinants using the phage lambda Red
109 system [6]. The construct was verified by PCR using primers 1053 with 1502.

110

111 *Construction of EC93 cdiB-sYFP2 reporter constructs*

112 The *sYFP2* reporter cassette was amplified from plasmid pSK2991 with primers 876
113 and 877. The resulting PCR product was integrated into the *cdi-1* and *cdi-2* loci in
114 either a EC93 $\Delta cdi-1$ or $\Delta cdi-2$ strain by lambda red recombination [6]. The
115 constructs were verified by PCR using primers 1489 and 1490, as well as primers
116 1491 and 1492.

117

118 *Construction of acrB mutant*

119 An *acrB* mutant strain (CH14008) was generated by transducing the *acrB::kanR*
120 cassette from the Keio deletion strain into CH7175.

121

122 *Construction of acrB expression plasmid*

123 The *acrB* gene was amplified from *E. coli* MG1655 genomic DNA with primers
124 CH4498 and CH4355, digested with BamHI and XbaI and ligated into plasmid
125 pZS21.

126

127 *Construction of pCloDF1(PJ23109-cdiI2) expression plasmid*

128 The pCDF::*pJ23101-cdiI*^{EC93} expression plasmid [8] was modified to replace the
129 strong pJ23101 promoter with the weaker pJ23109 promoter as follows. The *cdiI2*

130 gene was amplified from EC93 genomic DNA using primers 1117 and 1118, then
131 ligated to the DNA fragment resulting from PCR-amplification of plasmid SK1757
132 with primers 1198 and 1200. This resulted in placement of the *cdiI2* gene under
133 control of the PJ23109 promoter. The resulting plasmid was verified by sequencing
134 using oligonucleotides 986 and 987.

135

136 *Construction of chimeric cdiA1-CT2 chromosomal insertion.*

137 The EC93 *cdiA*-CT/I1 region of the *cdiBAll* locus of EC93, previously inserted
138 down-stream of *lacA* in the chromosome of MG1655 [11], was replaced with the
139 EC93 *cdiA*-CT/I2 region of locus 2, at the conserved VENN motif. This was done by
140 PCR amplifying the EC93 *cdiA*-CT/I2 with primers 1862 and 1863 and a kanamycin
141 resistance cassette [5], using primers 1864 and 1865. Simultaneously inserting 40bp
142 homology to the *cdi-1* locus and to each PCR fragment. The PCR products were fused
143 by overlap-extension PCR (OE-PCR) using primers 1862 and 1865. The resulting
144 OE-PCR product was transformed into SK4790 by lambda red recombination [6]. The
145 construct was verified by PCR and sequenced using primers 1057 with 564.

146

147 *Construction of chimeric cdiA1-CT2 expression plasmid*

148 The EC93 *cdiA*-CT/I1 region from *cdi-1* was replaced with the EC93 *cdiA*-CT/I2
149 region of locus 2, at the conserved VENN motif. This was done using cosmid
150 recombineering with PheS* counter-selection using plasmid pCH10163, as previously
151 described [9]. EC93 *cdiA*-CT/I2 was amplified by PCR with primers CH3822 and
152 CH3823 and fused to upstream and downstream homology regions amplified from
153 EC93 *cdiA* locus 1 using primer pairs DL1527 and DL2470 or DL1663 and DL2368,
154 respectively. The PCR products were fused by overlap-extension PCR (OE-PCR)
155 using primers DL1527 and DL2368. The resulting OE-PCR product was transformed
156 along with pCH10163 into *E. coli* DY378 cells, as previously described [9]. The
157 resulting recombinants were selected on yeast extract glucose agar containing 33
158 µg/mL chloramphenicol and 10 mM D/L-p-chlorophenylalanine.

159 **Table S1. Bacterial strains used in this study**

| Strain number | Genotype | Origin |
|---------------|--|------------|
| CH7175 | EPI100 Δwzb | This study |
| CH7176 | EPI100 $\Delta wzb::kanR$ | This study |
| CH14008 | EPI100 $\Delta wzb, \Delta acrB$ | This study |
| DL3717 | <i>E. coli</i> from Charles River K93 Rat colony (EC93 WT) | [10] |
| DL8705 | <i>E. coli</i> MG1655 $\Delta wzb, araBAD::spmR$ | This study |
| SK431 | <i>E. coli</i> K12 MG1655 /pDAL660 Δ 1-39 | [11] |
| SK1455 | <i>E. coli</i> MG1655 $\Delta wzb, ara::spec, galK::cat-RFP$ | This study |
| SK2540 | <i>E. coli</i> MG1655 <i>bamA</i> ^{LT2} | [11] |
| SK2661 | <i>E. coli</i> MG1655 <i>lacA-cdiBAlI-kanR</i> | [11] |
| SK2725 | EC93 /pSC101(ts):: <i>cdiA</i> -CT/I1 ^{EC93} (RepA A56V mutation) | This study |
| SK2773 | EC93 /pSC101(ts):: <i>cdiA</i> -CT/I1 ^{EC93} (RepA A56V mutation) /pSIM27 | This study |
| SK2837 | EC93 <i>cdiAII::catR</i> | This study |
| SK2968 | <i>E. coli</i> EPI100 Δwzb /pDAL660 Δ 1-39 (<i>cdiBAlI</i> ^{EC93}) | This study |
| SK2970 | <i>E. coli</i> EPI100 Δwzb /pDAL660 Δ 2-63:: <i>cat</i> | This study |
| SK2971 | <i>E. coli</i> EPI100 Δwzb /pSK2971 (<i>cdiBAlI</i> -CT/I ^{NC101}) | This study |
| SK2974 | <i>E. coli</i> EPI100 Δwzb /pSK2974 (<i>cdiBAlI</i> -CT/I2 ^{EC93}) | This study |
| SK3022 | <i>E. coli</i> MG1655 <i>lacA-cdiBAlI</i> (Δ CT+I) | [11] |
| SK3099 | EC93 DEL <i>cdiAII</i> | This study |
| SK3290 | <i>E. coli</i> MG1655 $\Delta wzb, ara::specR, galK::cat-RFP$ /pTrc99 (Empty) | This study |
| SK3291 | <i>E. coli</i> MG1655 $\Delta wzb, ara::specR, galK::cat-RFP$ /pTrc99:: <i>cdiI</i> ^{EC93} | This study |
| SK3292 | <i>E. coli</i> MG1655 $\Delta wzb, ara::specR, galK::cat-RFP$ /pTrc99:: <i>cdiI2</i> ^{EC93} | This study |
| SK3295 | <i>E. coli</i> MG1655 $\Delta wzb, ara::specR, galK::cat-RFP$ /pTrc99:: <i>cdiI</i> ^{<i>E. coli</i> NC101} | This study |
| SK3303 | <i>E. coli</i> MG1655 $\Delta wzb, ara::spe,c, galK::cat-RFP, \Delta acrB::kan$ | This study |
| SK3935 | <i>E. coli</i> MG1655 <i>rpsL</i> (K42R) | This study |
| SK3936 | <i>E. coli</i> MG1655 <i>bamA</i> ^{LT2} , <i>rpsL</i> (K42R) | This study |
| SK3954 | <i>E. coli</i> MG1655 <i>rpsL</i> (K42R) /pCDF1::pJ23101 | This study |
| SK3955 | <i>E. coli</i> MG1655 <i>rpsL</i> (K42R) /pCDF1::pJ23101- <i>cdiI</i> | This study |
| SK3956 | <i>E. coli</i> MG1655 <i>rpsL</i> (K42R) /pCDF1::pJ23109- <i>cdiI2</i> | This study |
| SK4062 | EC93 <i>cdiAII::catR, cdiB2-sYFP2-kanR</i> | This study |
| SK4096 | <i>E. coli</i> MG1655 /pDAL660 Δ 2-63:: <i>cat</i> | This study |
| SK4097 | <i>E. coli</i> MG1655 /pDAL660 Δ 1-39 (<i>cdiBAlI</i> ^{EC93}) | This study |
| SK4098 | <i>E. coli</i> MG1655 /pSK2974 (<i>cdiBAlI</i> -CT/I2 ^{EC93}) | This study |

| | | |
|--------|---|------------|
| SK4464 | EC93 /pSIM27 | This study |
| SK4465 | EC93 <i>DELcdiA11</i> /pSIM27 | This study |
| SK4466 | EC93 /pSIM27 /pSC101:: <i>cdiA</i> -CT/I2 ^{EC93} | This study |
| SK4467 | EC93 <i>DELcdiA11</i> /pSIM27 /pSC101:: <i>cdiA</i> -CT/I2 ^{EC93} | This study |
| SK4485 | EC93 <i>cdiBA11-orphan</i> CT/I- <i>catR</i> | This study |
| SK4487 | EC93 <i>DELcdiA11</i> , <i>cdiBAI2</i> :: <i>catR</i> | This study |
| SK4492 | EC93 <i>cdiBAI2</i> :: <i>catR</i> | This study |
| SK4493 | EC93 <i>cdiBAI2</i> :: <i>catR</i> , <i>cdiB1-sYFP2-kanR</i> | This study |
| SK4545 | EC93 <i>cdiBA11-orphan</i> CT/I- <i>catR</i> /pCDF1::pJ23101 | This study |
| SK4546 | EC93 <i>cdiBA11-orphan</i> CT/I- <i>catR</i> /pCDF1::pJ23101- <i>cdiI1</i> ^{EC93} | This study |
| SK4547 | EC93 <i>cdiBA11-orphan</i> CT/I- <i>catR</i> /pCDF::pJ23109- <i>cdiI2</i> ^{EC93} | This study |
| SK4548 | EC93 <i>cdiA11</i> :: <i>catR</i> /pCDF1::pJ23101 | This study |
| SK4549 | EC93 <i>cdiA11</i> :: <i>catR</i> /pCDF1::pJ23101- <i>cdiI1</i> ^{EC93} | This study |
| SK4550 | EC93 <i>cdiA11</i> :: <i>catR</i> /pCDF1::pJ23109- <i>cdiI2</i> ^{EC93} | This study |
| SK4551 | EC93 <i>cdiBAI2</i> :: <i>catR</i> /pCDF1::pJ2310 | This study |
| SK4552 | EC93 <i>cdiBAI2</i> :: <i>catR</i> /pCDF1::pJ23101- <i>cdiI1</i> ^{EC93} | This study |
| SK4553 | EC93 <i>cdiBAI2</i> :: <i>catR</i> /pCDF1::pJ23109- <i>cdiI2</i> ^{EC93} | This study |
| SK4554 | EC93 <i>DELcdiA11</i> , <i>cdiBAI2</i> :: <i>catR</i> /pCDF1::pJ2310 | This study |
| SK4555 | EC93 <i>DELcdiA11</i> , <i>cdiBAI2</i> :: <i>catR</i> /pCloDF1::pJ23101- <i>cdiI1</i> ^{EC93} | This study |
| SK4556 | EC93 <i>DELcdiA11</i> , <i>cdiBAI2</i> :: <i>catR</i> /pCloDF1::pJ23109- <i>cdiI2</i> ^{EC93} | This study |
| SK4790 | <i>E. coli</i> MG1655 <i>lacA-cdiBA1</i> (Δ CT+I) /pSIM27 | This study |
| SK4791 | <i>E. coli</i> MG1655 <i>lacA-cdiBA1</i> -CT/I2 ^{EC93} - <i>kanR</i> | This study |

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161

162 **Table S2. Plasmids used in this study**

| Plasmid | Genotype | Origin |
|--|---|------------|
| pSK917 | pSC101 Empty | This study |
| pSK1244 | pSC101:: <i>cdiA</i> -CT/I2 ^{EC93} | This study |
| pSK1751 | pCDF::pJ23101 | [8] |
| pSK1752 | pCDF1::pJ23101- <i>cdiI</i> ^{EC93} | [8] |
| pSK2350 | pSC101(ts) Empty (RepA A56V mutation) | This study |
| pSK2351 | pSC101(ts):: <i>cdiA</i> -CT/I1 ^{EC93} (RepA A56V mutation) | This study |
| pSK2902 | pCDF::pJ23109- <i>cdiI</i> 2 ^{EC93} | This study |
| pCH10163 (<i>cdiBA</i> :: <i>kan-pheS</i>) | pDAL660Δ2-63::cat that carries a <i>kan-pheS</i> * cassette in place of the <i>E. coli</i> EC93 <i>cdiA</i> -CT/ <i>cdiI</i> coding sequence. | [9] |
| pDAL660Δ1-39 (<i>cdiB</i> AII ^{EC93}) | pWEB containing a 16,734bp region from EC93 including the <i>cdiB</i> AII locus | [10] |
| pDAL660Δ2-63::cat | pDAL660Δ1-39 with a <i>cdiB</i> AII deletion | [10] |
| pSK2974 (<i>cdiB</i> A1-CT/I2 ^{EC93}) | pDAL660Δ1-39 with <i>cdiA</i> 1-CT1 and <i>cdiI</i> replaced by <i>cdiA</i> -CT2 and <i>cdiI</i> 2 | This study |
| pSK2971 (<i>cdiB</i> A1-CT/I ^{NC101}) | pDAL660Δ1-39 with <i>cdiA</i> 1-CT1 and <i>cdiI</i> replaced by <i>cdiA</i> -CT and <i>cdiI</i> from <i>E. coli</i> NC101 | [3] |
| pSIM27 | Lambda red genes under thermal induction | [7] |
| pZS21- <i>acrB</i> | Derivative of pZS21 with constitutive expression of AcrB from <i>E. coli</i> MG1655 | This study |
| pSC189 | Mobilizable plasmid with R6Kγ replication origin; carries the mariner transposon containing kanamycin resistance cassette | [4] |

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165 **Table S3. Oligos used in this study**

| Oligo | Sequence (5' to 3') |
|-------|---|
| 274 | ATCGATAAACCTCACTTGTGGAAGTGCCATTAACGTCAACCCATATGAA TATCCTCCTTAGTTCC |
| 275 | CATATTCACTACCGCCAAGTAAATTATTCTCAACAACAAACCATATGAA TATCCTCCTTAGTTCC |
| 477 | GCTCGAGGTGAAGACG |
| 543 | TAGTTAGTTAGATTAGTTGAGAATAATGCAATGTCTGG |
| 544 | CATAGTTTTTCATCATTCGCGGCTTTTCTGTCTAAGATACTAAGGCC |
| 547 | TAGTTAGTTAGATTAGTTGAGAATAACTCACTCAGTGATG |
| 548 | CATAGTTTTTCATCATTCGCGGCACGATAAAAACGATTTAATATCAATAT G |
| 564 | GTGTTCCGCTTCCTTTAGCAG |
| 630 | ACCATACTATATCGACCAAG |
| 767 | GGCGCGGTTCTCCGTGGATATGCTATCTCTCCCTCTCCGTTGTAGGCTGG AGCTGCTTC |
| 876 | ATCTCACGGTTTACTGGCGCGTTGCCGTCGCGTTTTAAAGAGAAAGAGG AGAAATACTAG |
| 877 | GAAGCGAACGGGAGGCTGATGCATGGTAATAATCCCATATGAATATC CTCCTTAGTTCC |
| 921 | TTCAGAGAAAGAGGAGAAATACTAG |
| 986 | ATCACCACCACCATCACGTGG |
| 987 | GTGACCGTGTGCTTCTCAAATGC |
| 1053 | GCAAGATGTGGCGTGTTACG |
| 1057 | CCATTGCTTCAGGTATTGCAGG |
| 1117 | ATAAGGATCCCAACAAGTGAGGTTTATCGATATG |
| 1118 | TACAGTCGACAATTATTCTCAACAACAAATCAACG |
| 1185 | TTACTGGCGCGTTGCCGTCGCGTTTTAAGGGATTATTACCTGTAGGCTG GAGCTGCTTC |
| 1186 | CCATGCTGGCGTATCCACGCAGCATGAGCATATTCACTACCTTAGTTCC TATTCCGAAGTTCC |
| 1198 | TTTACAGCTAGCTCAGTCCTAGGGACTGTGCTAGCCTCGAGACTTGATA TCGCGTGG |
| 1200 | TGAGCGCAACGCAATTAATGTAAG |
| 1273 | GAGCAGCAACCGCACAGCCTCTTGCTATCAAGCTCAGCGACTTAGTTCC TATTCCGAAGTTCC |
| 1489 | GATAATCGCACTGACCAGGTAAC |
| 1490 | AGTGACTGGAATCATCGTTATTGC |
| 1491 | GGCAGAACAACATTCTGACG |
| 1492 | GGTCATCTCTGTCAGAGTGC |

| | |
|--------|---|
| 1500 | TATGGATATAGCAAAAATAGATAAGCTATCTAATGCCTAATGTAGGCTG GAGCTGCTTC |
| 1501 | GCGAAGCAAAAAATCCCGACTTTGAGACCAGAATATCTATCCATATGA ATATCCTCCTTAGTTCC |
| 1502 | GGTGAGAAGCAGGCTATC |
| 1862 | AAGTGCTGCCGGTACGGGGGCACAGGCAGGTAGGAACTCGGTTGAGAA TAACTCACTCAGTG |
| 1863 | TTGCGCCCTGAGTGCTTGCGGCAGCCAACAAATCAACGATAAAAACG |
| 1864 | AATCGTTTTTATCGTTGATTTGTTGGCTGCCGCAAGCACTCAGGG |
| 1865 | GTCGTTAATCCATGCCCGATTATTCAATGTTCTGTGTCCAGAAGAACTC CAGCATGAGATCC |
| CH2260 | CAAGCTTGTCATCGTCATCC |
| CH2131 | TGCAGATCTTGTGGCAACAATTTCTAC |
| CH2132 | GCGACCCGGGTGTATCAAACAAGACG |
| CH3822 | CAGGTAGGAACTCGGTTGAGAATAATTCCTCAGTGATGGCTGGAAC |
| CH3823 | GGTCTGGTGTCTAACCTTTGGGTTAACGATAAAAACGATTTAATATCAA TATGATGA |
| DL1527 | GAACATCCTGGCATGAGCG |
| DL1663 | CCCAAAGGTTAGACACCAGACC |
| DL2368 | GTTGGTAGTGGTGGTGCTG |
| DL2470 | ATTATTCTCAACCGAGTTCCTACCTG |
| CH4355 | TTTGAGCTCTAGAATCAATGATGATCGACAGTATGGC |
| CH4498 | TTCGGATCCGTTAAGACATGCC |

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168 **Table S4. Predicted virulence factors and competition systems in the genome of**
 169 **EC93**

| Predicted fitness factors on the chromosome | Description |
|--|----------------------------------|
| <i>gad</i> | Glutamate decarboxylase |
| <i>iss</i> | Increased serum survival |
| <i>ipfA</i> | Long polar fimbriae |
| <i>fimH38</i> | Type 1 fimbriae |
| T6SS locus | Type VI secretion system |
| 3 <i>vgrG</i> genes | T6SS component |
| 8 <i>rhs</i> genes | T6SS toxic effector protein |
| <i>mdfA</i> | Multidrug efflux pump |
| Predicted fitness factors on the pCP127 plasmid | Description |
| <i>pic</i> | Serine protease auto-transporter |
| K88 fimbriae locus | Aggregation protein |
| RTX family exoprotein A | Putative virulence protein |
| 2 <i>cdiBAI</i> loci | T5SS, subclass b |

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172 **Table S5. MIC ($\mu\text{g}/\text{mL}$) of EC93 and MG1655 towards antibiotics used in this**
173 **study**

| | EC93 | <i>E. coli</i> MG1655 |
|-----------------|-------------|------------------------------|
| Streptomycin | 8 | 6 |
| Chloramphenicol | 8 | 3 |
| Kanamycin | 8 | 8 |

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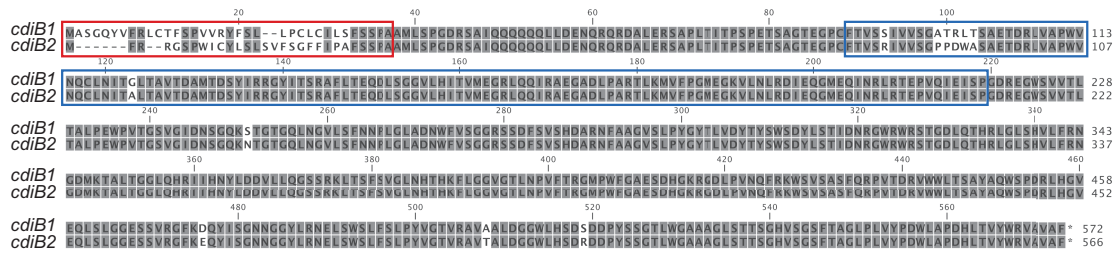
176 **Table S6. EC93 phage release after exposure to UV light (254 nm). Plaque assay**
177 **was performed on *E. coli* MG1655 (n=3).**

| UV light (mJ/cm ²) | Plaques |
|--------------------------------|---------|
| 0 | 0 |
| 25 | 0 |
| 50 | 0 |
| 100 | 0 |
| 200 | 0 |
| 400 | 0 |
| 600 | 0 |
| 800 | 0 |
| 1000 | 0 |

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179

180 **Table S7. Chromosomally located intact *E. coli*-like *cdi* systems.**
181 List of all 177 *E. coli cdi* systems identified to be present in 133 unique bacterial
182 genomes by blastn search, using the *cdiA1* gene from EC93 as query.
183
184 (See supplementary excel file, available with the online version of this paper.)
185



186

187 **Figure S1. Alignment of CdiB1 and CdiB2 proteins from *E. coli* EC93.**

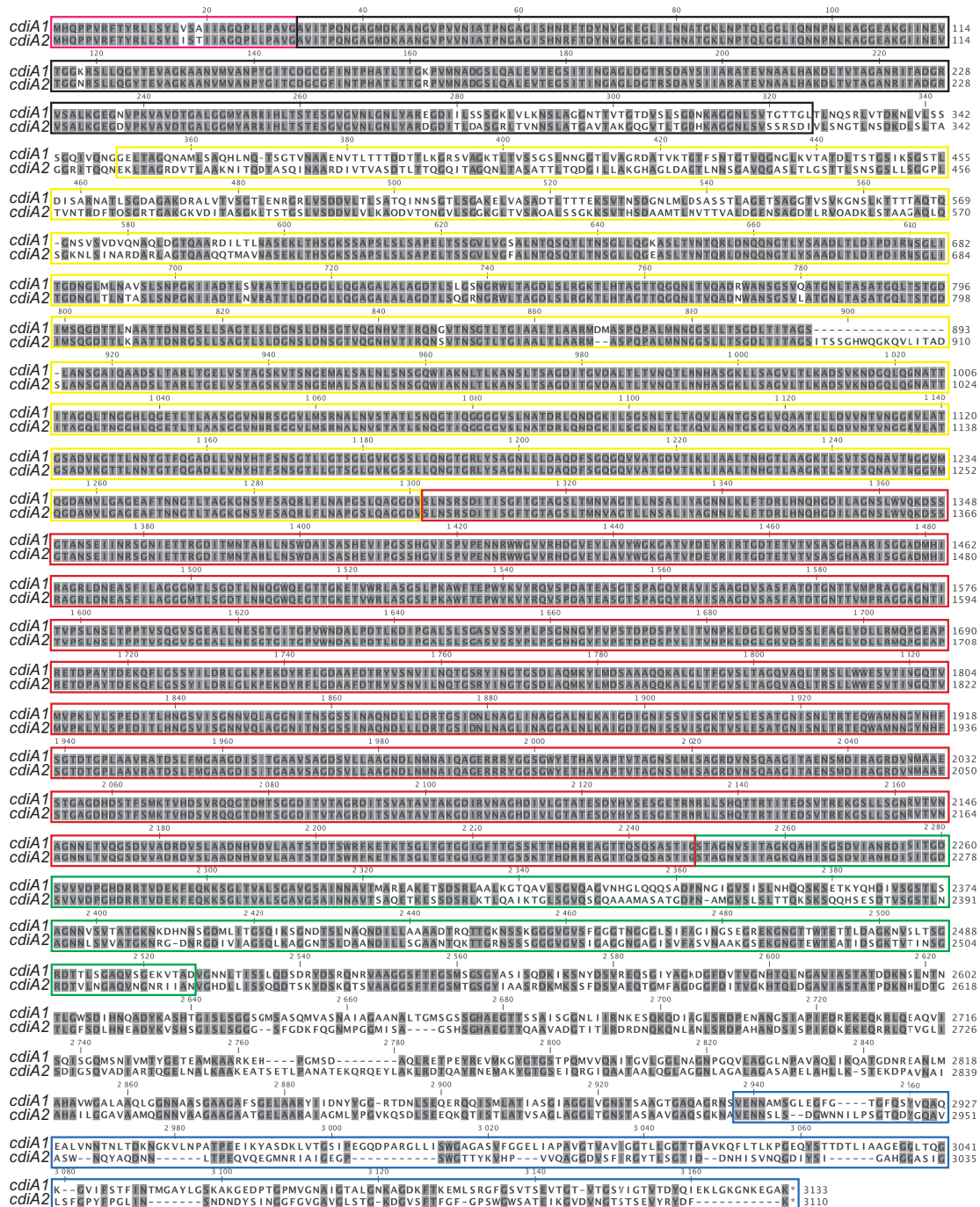
188 Identical residues are shown in grey and non-identical residues are white. Sequences

189 boxed in red encompass the putative SEC-signal peptide and sequences boxed in blue

190 encompass the putative POTRA domains.

191

192



193

194 **Figure S2. Alignment of CdiA1 and CdiA2 proteins from EC93.**

195 Identical residues are shown in grey and non-identical residues are white. Sequences
 196 boxed in pink encompass the putative SEC-signal peptide, sequences boxed in black
 197 encompass the Two-Partner Secretion domain (TPS), sequences boxed in yellow
 198 encompass the FHA-1 domain, sequences boxed in red encompass the extended
 199 Receptor-Binding Domain (RBD) region (class I), sequences boxed in green
 200 encompass the FHA-2 domain and sequences boxed in blue encompass the C-terminal
 201 domain containing toxin.


```

                20          40          60          80          100
cdi11  KKK-----L-----LKK-----P-----MLTVLGLGIP-----LILVNLRTGSDFN---D 47
cdi12  I LNNFYSLFLMLALSIFSFVFAFKDLGGVKFARDFLFDYSSDMRANASALSVIVFIFGAFDHRNPLVKNYLVDIVWFLVVLFIHCRFS--NIEYGT 114

                120          140
cdi11  EDV---LTTGGIPIGI--GLGWGLSD---K 79
cdi12  KIFRELFNKKLRLILWISRLFIILNFFYR* 153

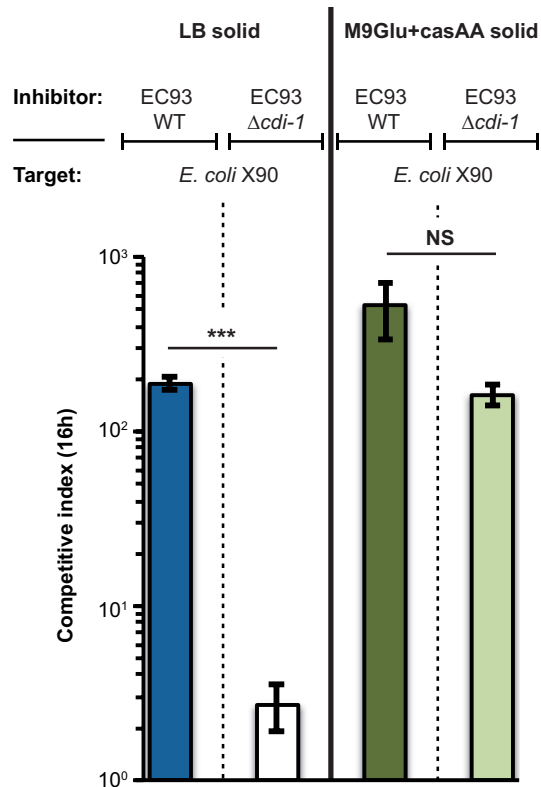
```

202

203 **Figure S3. Alignment of CdiI1 and CdiI2 proteins from EC93.**

204 Identical residues are shown in grey and non-identical residues are white.

205



206

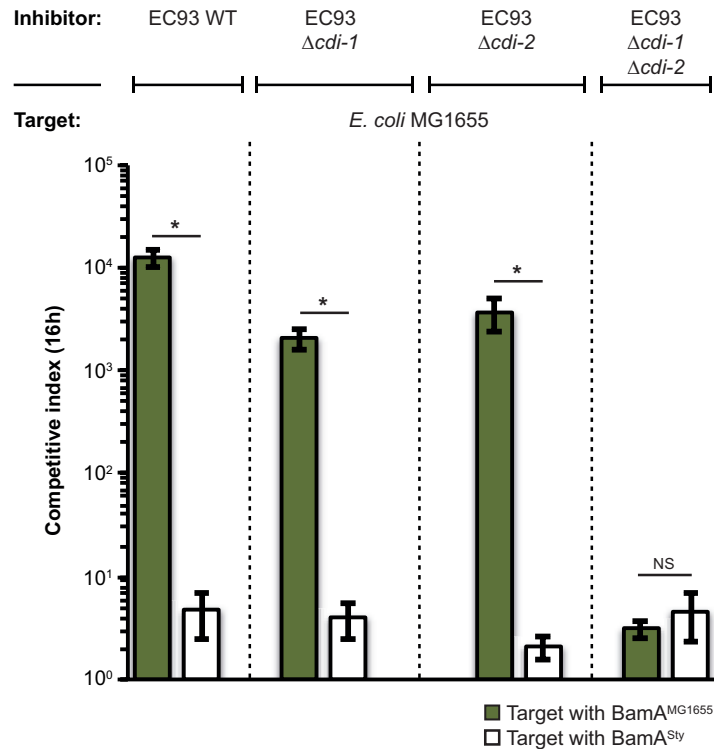
207 **Figure S4. The growth inhibitory activity of the *cdiBAI2* locus of EC93 was not**
 208 **identified because the system is not active on LB media.**

209 Average competitive index (CI) of wild-type EC93 and EC93 $\Delta cdi-1$ cells co-cultured
 210 with *E. coli* X90 for 16h on LB solid medium or M9Glu+casAA solid medium (n=3
 211 biological replicates \pm SEM). Competitions were performed as previously described [12].

212 Statistical significance was determined by two-tailed, un-paired t-test (p-value:

213 ***= <0.0005).

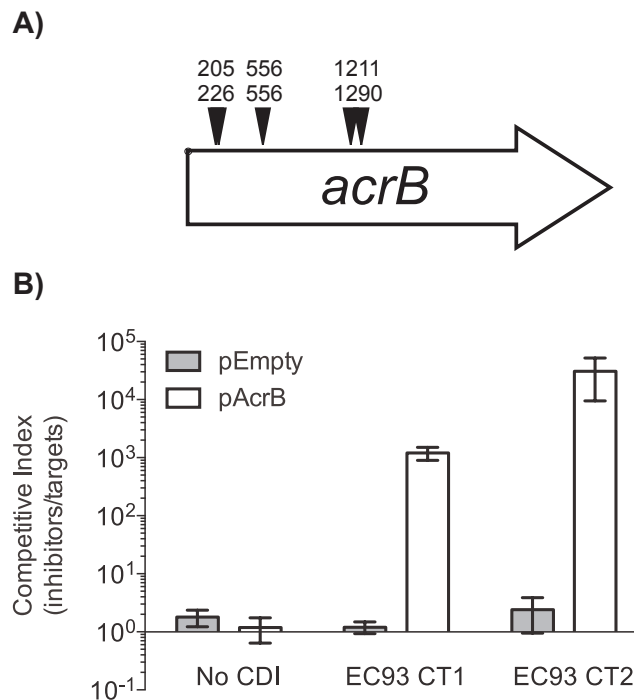
214



215

216 **Figure S5. EC93 CdiA1 and CdiA2 use BamA as a receptor.**

217 EC93, EC93 $\Delta cdi-1$, EC93 $\Delta cdi-2$ or EC93 $\Delta cdi-1 \Delta cdi-2$ cells were co-cultured with
 218 MG1655 cells expressing *E. coli* BamA (BamA^{MG1655}) or MG1655 expressing
 219 *Salmonella* BamA (BamA^{Sty}). Growth competitions were carried out for 16 h on
 220 M9Glu+casAA solid media (n=3 biological replicates \pm SEM). Competitive indices
 221 are shown on the y-axis. Statistical significance was determined by two-tailed, un-
 222 paired t-test (p-value: *<0.05).

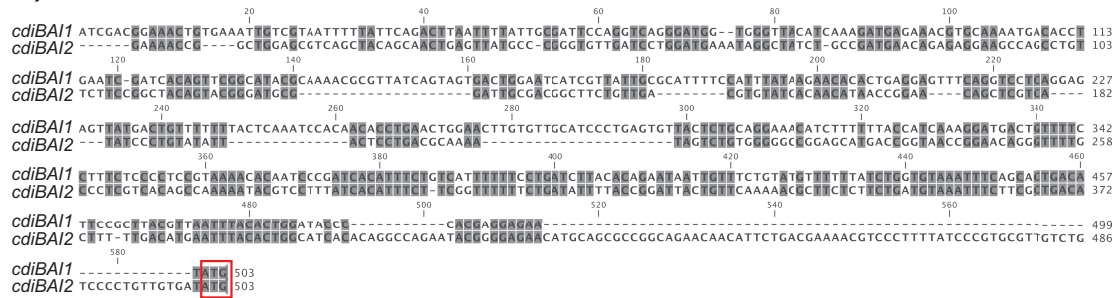


223

224 **Figure S6. EC93 CdiA1 and CdiA2 both require AcrB for inhibition of target**
 225 **cells.**

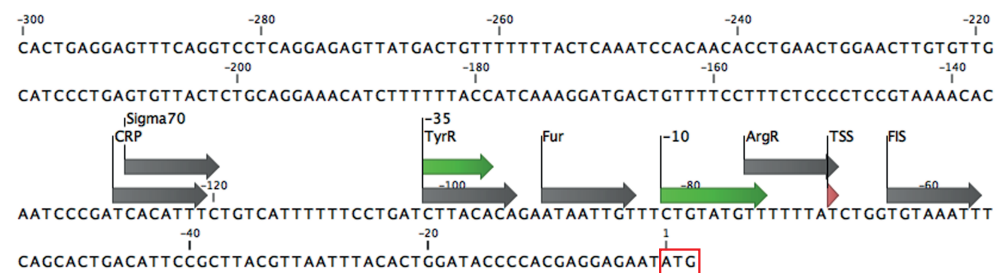
226 A) Schematic representation of mariner transposon insertions in *acrB* that conferred
 227 resistance to the Cdi-2 system (see Materials and Methods). B) Dependence of the
 228 *cdi-2* system on AcrB for growth inhibitory activity. EPI100 inhibitor cells expressing
 229 plasmid-encoded Cdi-1 delivering either the CdiA-CT1 toxin or the CdiA-CT2 toxin
 230 were co-cultured with EPI100 Δ *acrB* target cells transformed with an empty pZS21
 231 vector control or a pZS21-AcrB expression vector. Competitions were carried out for
 232 24h on LB solid media (n=3 biological replicates \pm SEM). Competitive indices are
 233 shown on the y-axis.

A)



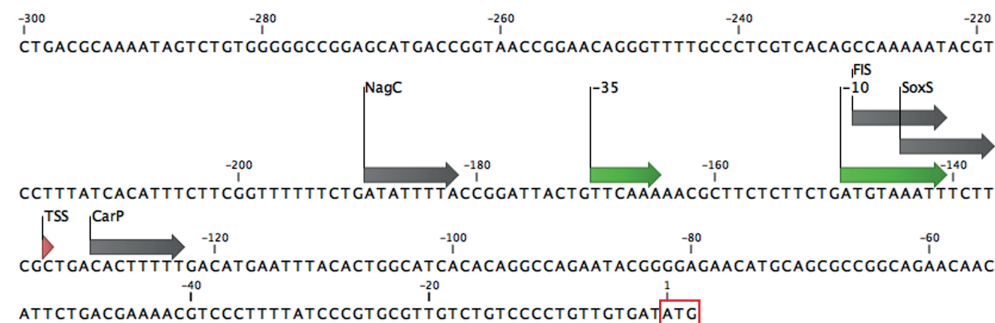
B)

cdiBAI1 promoter prediction



C)

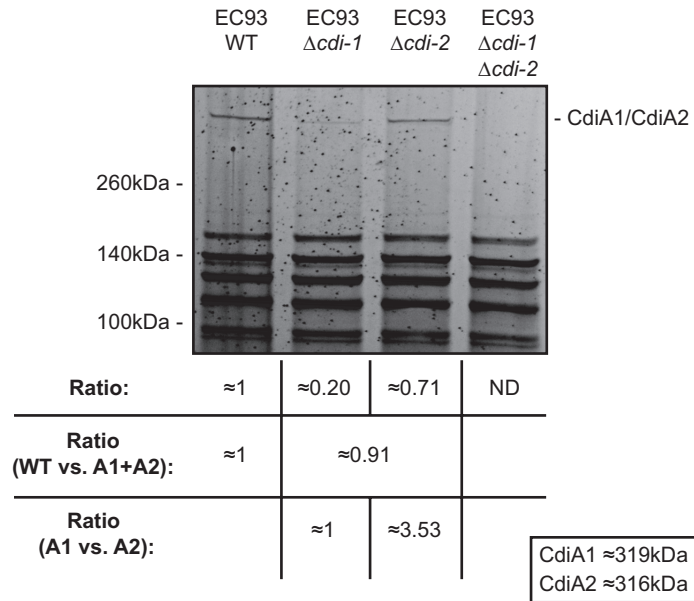
cdiBAI2 promoter prediction



234

235 **Figure S7. The *cdiBAI1* and *cdiBAI2* promoter regions.**

236 A) Alignment of the *cdiB1* and *cdiB2* upstream regions (500bp). Identical residues are
 237 shown in grey and non-identical residues are white. The DNA sequences encoding
 238 *cdiB* AUG start codons are indicated by a red-box. B, C) Putative promoter
 239 predications of the two *cdiBAI* loci promoters of EC93. Red-arrows indicate the
 240 transcriptional start-site (TSS). Grey-arrows indicate putative binding-sites for
 241 transcriptional regulators. Green-arrows indicate the location of the putative -10 and -
 242 35 motifs and the AUG start codons of *cdiB1* and *cdiB2* are indicated by a red-box.



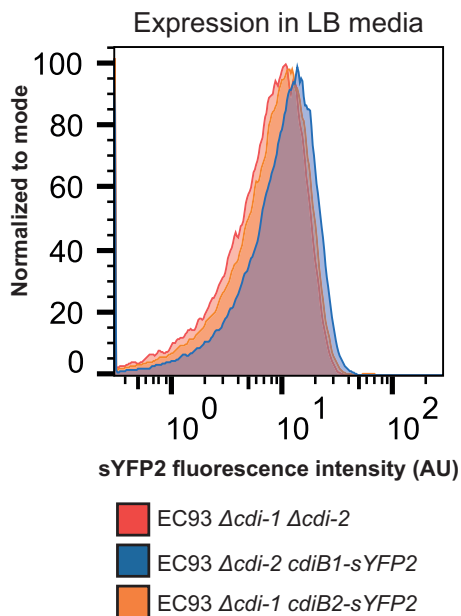
243

244 **Figure S8. EC93 CdiA1 and CdiA2 are differentially expressed.**

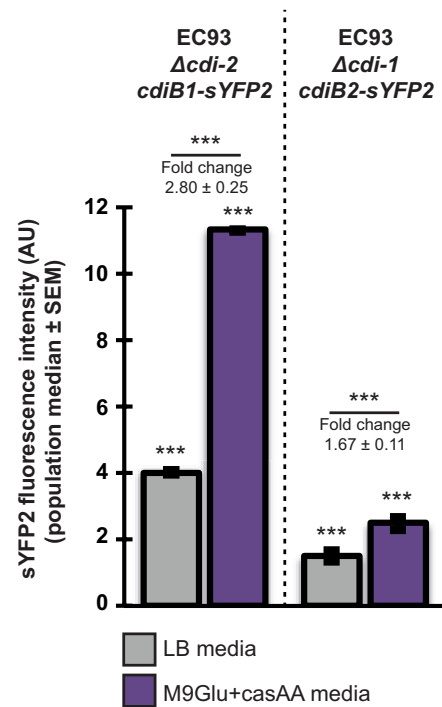
245 The relative expression of the *cdi-1* or *cdi-2* loci in EC93 was analyzed by SDS-
 246 PAGE after cells were grown in M9-Glu+casAA media. Total protein was visualized
 247 by SYPRO Ruby staining. Relative protein abundance was calculated by densitometry
 248 using ImageJ.

249

A)



B)

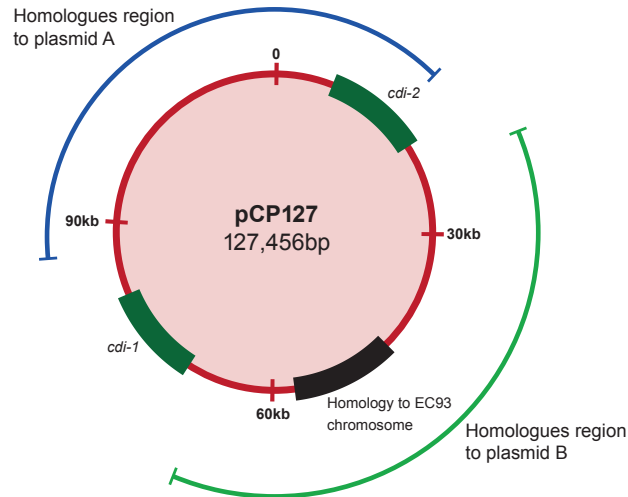


250

251 **Figure S9. EC93 *cdiBAI1* and *cdiBAI2* expression in LB.**

252 A) Relative transcriptional activity, measured as fluorescence of sYFP2 reporter
 253 inserted within the *cdi-1* or *cdi-2* loci in EC93 when cells are grown in LB media
 254 (n=3 biological replicates \pm SEM). B) Quantification of data presented in panel A and
 255 a comparison of *cdi-1* and *cdi-2* expression between LB and M9Glu+casAA media
 256 (M9Glu+casAA expression data taken from Fig. 5BC). Statistical significance was
 257 determined by two-tailed, unpaired t-test (p-value: ***<0.0005).

258



259

260

261 **Figure S10. EC93 pCP127 plasmid homology**

262 Schematic representation of the pCP127 plasmid of EC93 and the regions of

263 homology to plasmid A and plasmid B from an *E. coli* isolate from pigs (GenBank

264 accession no: CP010207.1), indicated by blue and green lines, respectively.

265

266 **References**

- 267 1. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang YJ, et al. PHASTER: a
268 better, faster version of the PHAST phage search tool. *Nucleic acids research*.
269 2016;44(W1):W16-W21. doi: 10.1093/nar/gkw387. PubMed PMID:
270 WOS:000379786800004.
- 271 2. Zhou Y, Liang YJ, Lynch KH, Dennis JJ, Wishart DS. PHAST: A Fast Phage
272 Search Tool. *Nucleic acids research*. 2011;39:W347-W52. doi:
273 10.1093/nar/gkr485. PubMed PMID: WOS:000292325300056.
- 274 3. Willett JLE, Gucinski GC, Fatherree JP, Low DA, Hayes CS. Contact-
275 dependent growth inhibition toxins exploit multiple independent cell-entry
276 pathways. *P Natl Acad Sci USA*. 2015;112(36):11341-6. doi:
277 10.1073/pnas.1512124112. PubMed PMID: WOS:000360994900058.
- 278 4. Chiang SL, Rubin EJ. Construction of a mariner-based transposon for epitope-
279 tagging and genomic targeting. *Gene*. 2002;296(1-2):179-85. doi:
280 10.1016/s0378-1119(02)00856-9. PubMed PMID: 12383515.
- 281 5. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in
282 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*.
283 2000;97(12):6640-5. doi: 10.1073/pnas.120163297. PubMed PMID: 10829079;
284 PubMed Central PMCID: PMCPMC18686.
- 285 6. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in
286 *Escherichia coli* K-12 using PCR products. *P Natl Acad Sci USA*.
287 2000;97(12):6640-5. doi: DOI 10.1073/pnas.120163297. PubMed PMID:
288 WOS:000087526300074.
- 289 7. Datta S, Costantino N, Court DL. A set of recombineering plasmids for gram-
290 negative bacteria. *Gene*. 2006;379:109-15. doi: 10.1016/j.gene.2006.04.018.
291 PubMed PMID: 16750601.
- 292 8. Virtanen P, Waneskog M, Koskiniemi S. Class II contact-dependent growth
293 inhibition (CDI) systems allow for broad-range cross-species toxin delivery
294 within the Enterobacteriaceae family. *Mol Microbiol*. 2019;111(4):1109-25.
295 doi: 10.1111/mmi.14214. PubMed PMID: 30710431; PubMed Central PMCID:
296 PMCPMC6850196.
- 297 9. Morse RP, Nikolakakis KC, Willett JLE, Gerrick E, Low DA, Hayes CS, et al.
298 Structural basis of toxicity and immunity in contact-dependent growth inhibition

- 299 (CDI) systems. *P Natl Acad Sci USA*. 2012;109(52):21480-5. doi:
300 10.1073/pnas.1216238110. PubMed PMID: WOS:000313627700071.
- 301 10. Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA. Contact-
302 dependent inhibition of growth in *Escherichia coli*. *Science*.
303 2005;309(5738):1245-8. Epub 2005/08/20. doi: 10.1126/science.1115109.
304 PubMed PMID: 16109881.
- 305 11. Ghosh A, Baltekin O, Waneskog M, Elkhalfa D, Hammarlof DL, Elf J, et al.
306 Contact-dependent growth inhibition induces high levels of antibiotic-tolerant
307 persister cells in clonal bacterial populations. *EMBO J*. 2018;37(9). doi:
308 10.15252/emboj.201798026. PubMed PMID: 29572241; PubMed Central
309 PMCID: PMC5920241.
- 310 12. Ruhe ZC, Wallace AB, Low DA, Hayes CS. Receptor polymorphism restricts
311 contact-dependent growth inhibition to members of the same species. *MBio*.
312 2013;4(4). doi: 10.1128/mBio.00480-13. PubMed PMID: 23882017; PubMed
313 Central PMCID: PMC3735181.
- 314