Detailed description of cloning procedures and experimental methods

The generation of plasmids and mutant strains is described below in detail. Strains and plasmids were verified by sequencing after cloning.

Plasmids

pWKS-*csrA*. The complementation vector pWKS-*csrA* was generated by amplification of *csrA* including app. 470 bp of its upstream region using *E. coli* IHE3034 as template and oligonucleotide pair *csrA*-XbaI-s/*csrA*-BamHI-as. The PCR product was digested with XbaI/BamHI and ligated with XbaI/BamHI digested pWKS30 to yield pWKS-*csrA*.

pKD46-*csrA*. The sequence of *csrA* and its promoter were amplified from *E. coli* IHE3034 with oligonucleotides *csrA*-NcoI-s and *csrA*-NcoI-as, digested with NcoI and ligated with NcoI digested pKD46.

Genome modifications

Chromosomal modifications in *E. coli* were carried out using the Lambda Red recombinase system of Datsenko & Wanner (1). Briefly, *E. coli* strains were first transformed with pKD46 (or pKD46-*csrA* for *csrA* modifications), grown in LB medium in the presence of 0.3 % L arabinose to induce expression of the Lambda Red recombinase encoded on pKD46 and then made competent for transformation with respective PCR products carrying the kanamycin or chloramphenicol cassette of pKD4 or pKD3, respectively. For this, oligonucleotides were designed in a way that PCR products carried nucleotide extensions of 50-60 bp homologous to the desired genomic site of manipulation. Clones were selected on agar containing either kanamycin (pKD4) or chloramphenicol (pKD3). After successful genomic manipulation of desired sequences, bacteria were cured from pKD46 (or pKD46-*csrA*) by serial streaking on LB agar and incubation at 37 °C. Successful curing was verified by PCR using oligonucleotide

24   pair pKD46-Seq1/pKD46-Seq2. If required, antibiotic resistance cassettes were removed by

25   transformation with pCP20 (2), which encodes the FLP recombinase that cuts the resistance

26   cassettes at the flanking FRT sites, thus leaving only the FRT site. Cells were cured from pCP20

27   by serial streaking on LB agar and incubation at 37 °C.

28   **E. coli strains M1/5Δ*uvrY*** and **SP15Δ*uvrY*** were obtained by transformation of pKD46

29   carrying *E. coli* M1/5 and SP15 cells with a PCR product comprising the *cat* cassette with

30   extensions homologous to the upstream and downstream regions of *uvrY*. The PCR product was

31   obtained by amplification of the chloramphenicol resistance cassette from pKD3 using primers

32   ABU_*uvrY*::*cat*_Fw and ABU_*uvrY*::*cat*_Rv. Clones were selected on agar containing

33   chloramphenicol.

34   **E. coli M1/5*csrA*51.** This strain was generated to synthesize a CsrA variant with reduced

35   function by shortening of the protein by nine amino acids. It has been shown that the *E. coli*

36   *csrA* gene is hard to manipulate (3). To facilitate chromosomal manipulation of *csrA* we

37   modified the pKD46 plasmid, which is required for genomic modifications by the here-used

38   methodology of Datsenko & Wanner (1), by introducing a *csrA* copy under the control of its

39   native promoter, thus creating plasmid pKD46-*csrA*. Transformation of *E. coli* M1/5 with this

40   plasmid did not only provide the cell with Lambda Red recombinase but also guaranteed

41   expression of a functional CsrA protein during manipulation of the chromosomally encoded

42   *csrA* copy. For manipulation of the genomic *csrA* copy, the kanamycin resistance cassette of

43   pKD4 was amplified using oligonucleotide pair *csrA51*-Del-M15-up and *csrA*-Del-M15-rev.

44   *E. coli* M1/5 pKD46-*csrA* was transformed with the PCR product, which was inserted in a way

45   that a stop codon was placed after nucleotide 153 of the *csrA* sequence. Clones were selected

46   on agar plates containing kanamycin.

47   **E. coli 536-HDM *csrA*51** was generated from *E. coli* 536-HDM as described for *E. coli*

48   M1/5*csrA*51.

49   ***E. coli* M1/5 HPI**⁻, a partial HPI (high pathogenicity island coding for yersiniabactin) mutant

50   (Δ*ybtA-fyuA*), was generated by transformation of M1/5 pKD46 with a PCR product obtained

51   by amplification of the chloramphenicol resistance cassette of pKD3 using oligonucleotides

52   *ybtA*-Del-for and *fyuA*-Del-rev. Clones were selected on agar plates containing

53   chloramphenicol.

54   ***E. coli* M1/5 HPI**⁻ *csrA*51 was generated from *E. coli* M1/5 HPI⁻ as described for *E. coli* M1/5

55   *csrA*51.

56   ***E. coli* strains SP15 Δ*csrB*, *E. coli* SP15 Δ*csrC*, *E. coli* SP15 Δ*csrB* Δ*csrC*.** lacking either

57   the small regulatory RNA gene *csrB*, or *csrC* or both were generated by recombineering. The

58   *csrB* gene was replaced by a *cat* cassette, whereas *csrC* was exchanged by a *tet* cassette.

59   Clones were selected on agar plates containing chloramphenicol or tetracycline, respectively.

60

Construction of *venus* reporter strains and plasmids

62   **pUC-P*lacUV5*-*venus*.** This plasmid was generated in order to serve as a template for PCR

63   reactions used in allelic replacements (1) to construct various chromosomally based *venus*

64   reporter gene strains for *clbQ*, and *ybtA* (see below). The plasmid with pUC18 as a backbone

65   contained the constitutive *lacUV5* promoter, a sequence encoding an artificial 5'UTR leader

66   derived from pTXB1 (lacking the LacI binding site), a modified *venus* sequence as a reporter

67   gene and a chloramphenicol resistance cassette to enable selection of clones. The sequence of

68   the artificial 5'UTR leader (from hereon designated artificial leader and abbreviated AL) was

69   chosen because it neither did exhibit any binding site for transcriptional regulators nor did its

70   transcript harbour a CsrA binding motif. Sequences of P*lacUV5*, AL and *venus* were fused by

71   an overlap PCR. A first PCR product was obtained by amplification of the translational fusion

72   of *rplL* and the *venus* gene from pMB*rplL-venus* using oligonucleotides *venus*-fus-s and

73   *venus*-fus-as introducing a downstream SalI restriction site. A second PCR product containing

74   the P*lacUV5,* the artificial 5'UTR leader sequence and a portion of the *rplL-venus* (from hereon

75   *venus*) sequence was yielded by template-free PCR using oligonucleotides P*lacUV5*-s and

76   P*lacUV5*-as. This way, an AatII restriction site was introduced upstream of P*lacUV5*. The PCR

77   products were then fused using oligonucleotides P*lacUV5*-s/*venus*-fus-as and the resulting

78   amplicon was digested with AatII and SalI and ligated with AatII/SalI digested pMB*rplL-venus*,

79   leading to plasmid pUC-P*lacUV5-venus.*

80   <u>*clbQ-venus* fusions</u>

81   A set of chromosomally based *clbQ-venus* fusions was constructed using allelic replacement

82   described by Datsenko & Wanner (1). PCR products with extensions homologous to the *clbQ*

83   locus were generated with pUC-P*lacUV5-venus* as a template.

84   **E. coli M1/5 P*clbQ-clbQ*'-'*venus*.** A translational fusion of the *clbQ* start codon and the *venus*

85   sequence was obtained by amplification of the *venus* sequence and the chloramphenicol

86   resistance cassette with oligonucleotides *clbQ*-TLN1-*venus*-s and *clbQ-cat*-as. *E. coli* M1/5

87   pKD46 was transformed with the resulting PCR product, *venus-cat*, to yield *E. coli* M1/5

88   P*clbQ-clbQ*'-'*venus.* This strain carried the native *clbQ* promoter as well as the native *clbQ*

89   5'UTR but the *venus* sequence instead of *clbQ*.

90   **E. coli M1/5 P*clbQ-clbQ*'-'*venus csrA*51** was generated from *E. coli* M1/5 P*clbQ-clbQ*'-'*venus*

91   as described above for *E. coli* M1/5*csrA*51.

92   **E. coli M1/5 P*lacUV5-clbQ*'-'*venus*.** To construct a *clbQ* leader fusion with the constitutive

93   *lacUV5* promoter, the native *clbQ* 5'UTR and *venus* as reporter, first the *clbQ* promoter of

94   *E. coli* M1/5 was exchanged by the constitutive P*lacUV5*. Therefore, P*lacUV5* from

95   pUC-P*lacUV5-venus* was amplified with oligonucleotides *Kan*-P*lac*-fus-s and

96   *clbQ*-P*lacUV5*-as. The resulting PCR product was fused downstream of the FRT site-flanked

4

97   kanamycin resistance cassette previously amplified from pKD4 using *clbQ*-P*lacUV*5-s and

98   *Kan*-P*lac*-fus-as. The resulting PCR product, *Kan*-P*lacUV*5, carried nucleotide extensions

99   homologous to the upstream region of the *clbQ* transcription start. After transformation of

100   *E. coli* M1/5 (pKD46) this amplicon and subsequent selection on kanamycin containing agar

101   plates the resulting strain, *E. coli* M1/5 Kan-P*lacUV*5-*clbQ*, was first transformed with pKD46

102   and afterwards with the *venus-cat* PCR sequence as described for *E. coli*

103   M1/5 P*clbQ-clbQ*'-'*venus*, leading to *E. coli* M1/5 *Kan*-P*lacUV*5-*clbQ*'-'*venus*. Transformation

104   with pCP20 led to the removal of the kanamycin resistance cassette and the final strain was

105   designated to *E. coli* M1/5 P*lacUV*5-*clbQ*'-'*venus.*

106   **E. coli M1/5 P*lacUV*5-*clbQ*'-'*venus csrA*51** was generated from *E. coli* M1/5 P*lacUV*5-*clbQ*'-

107   '*venus* as described above for *E. coli* M1/5*csrA*51.

108   **E. coli M1/5 P*lacUV*5-*clbQ**'-'*venus*** was a derivative of *E. coli* M1/5 P*lacUV*5-*clbQ*'-'*venus*

109   except that the sequence encoding the putative CsrA binding site within *clbQ* 5'UTR was

110   changed from <u>ACAA</u>GGA to <u>TTAT</u>GGA. With oligonucleotides *clbQ*-TLN-TTAT-*venus*-s

111   and *clbQ-cat*-as the *venus-cat* sequence from pUC-P*lacUV*5-*venus* was amplified with

112   extensions homologous to the region upstream and downstream of *clbQ*, respectively,

113   introducing the modified sequence in the upstream extension. *E. coli* M1/5 *Kan*-P*lacUV*5-*clbQ*

114   carrying pKD46 was transformed with this PCR amplicon and afterwards treated with pCP20

115   to remove the kanamycin resistance cassette, resulting in the desired strain *E. coli* M1/5

116   P*lacUV*5-*clbQ**'-'*venus.*

117   **E. coli M1/5 P*lacUV*5-*clbQ**'-'*venus csrA*51** was generated from *E. coli* M1/5 P*lacUV*5-

118   *clbQ**'-'*venus* as described above for *E. coli* M1/5*csrA*51.

119   **E. coli M1/5 P*clbQ*-AL-'*venus*.** This strain carried a fusion of the *clbQ* promoter with the

120   sequence encoding the artificial leader and the *venus* sequence of pUC-P*lacUV*5-venus being

121 amplified with oligonucleotides *clbQ*-TXN-*venus*-s and *clbQ*-*cat*-as. Transformation of

122 *E. coli* M1/5 (pKD46) with this product yielded the desired strain.

123 **E. coli M1/5 P*clbQ*-AL-'*venus* *csrA*51** was generated from *E. coli* M1/5 P*clbQ*-AL-'*venus* as

124 described above for *E. coli* M1/5*csrA*51.

125 <u>*ybtA-venus* fusions</u>

126 Genome modifications. At first, a leader fusion of *ybtA* with the *venus* gene as a reporter was

127 generated in the bacterial chromosomal using the method of Datsenko & Wanner (1).

128 Chromosomal insertion of *venus* was performed in a way, that only the start codon of *ybtA*

129 remained.

130 **E. coli M1/5 M1/5 P*ybtA*-*ybtA*'-'*venus*.** In more detail, the *venus* encoding sequence attached

131 to the chloramphenicol resistance cassette (*venus-cat*) of pUC-P*lacUV5-venus* was amplified

132 with primer pairs *ybtA*-TLN-*venus*-s2/*ybtA*-*cat*-as. *E coli* M1/5 cells harbouring plasmid

133 pKD46 for homologous recombination were transformed with the PCR product, plated on

134 chloramphenicol containing agar and the resulting mutant strain M1/5 P*ybtA*-*ybtA*'-'*venus* was

135 selected.

136 <u>Plasmids</u>

137 **pBAD33*.** A derivative of the medium copy vector pBAD33 devoid of the *araC* gene as well

138 as the *araBAD* promoter, designated pBAD33*, was constructed to create plasmid-encoded

139 *ybtA-venus* fusions. To generate this vector, pBAD33 was digested with SacI and EcoRV,

140 treated with Mung Bean Nuclease (MBN) and religated. Cloning of the different *ybtA-venus*

141 fusions into this vector is described below.

142 **pBAD33-*csrA*.** To obtain fusion plasmids that contained *csrA* for complementation,

143 SacI/EcoRV digested pBAD33 was ligated with the *csrA* gene including app. 280 bp of its

144   upstream region previously cut from pWKS-*csrA*. The resulting plasmid was then digested with

145   ZraI and EagI, treated with MBN and religated, yielding pBAD33-*csrA*.

146   **pBAD33-*venus*-*csrA*.** This plasmid, carrying a promoterless *venus* gene as well as the *crsA*

147   gene with its own promoter, was generated by amplification of *venus* from pUC-P*lacUV5*-*venus*

148   using primer pair *venus*-XbaI and MK67, subsequent digestion of the PCR product with XbaI

149   and HindIII and ligation with XbaI/HindIII digested pBAD33-*csrA*.

150   **pP*ybtA*-*ybtA*'-'*venus*2 and pP*ybtA*-*ybtA*'-'*venus*2-*csrA*.** These plasmids represent fusions of

151   the *ybtA* promoter, the *ybtA* 5'UTR and *venus* as a reporter. Since the *ybtA* promoter region does

152   not only contain the *ybtA* promoter but also the divergently oriented *ybtP* promoter, a

153   transcription terminator had to be introduced upstream of the *ybtA* promoter to prevent

154   expression from the *ybtP* promoter. Therefore, the *rrnB* terminator was cloned into pBAD33*

155   and pBAD33-*csrA*. The terminator was amplified from pBAD33 using primer pair

156   *rrnB*-XbaI-for/*rrnB*-SacI-rev2, digested with XbaI and SacI and ligated with XbaI/SacI

157   digested pBAD33* or pBAD33-*csrA* leading to pBAD33-*rrnB* or pBAD33-*csrA*-*rrnB*,

158   respectively. A sequence harbouring the *ybtA* promoter region, the *ybtA* 5'UTR as well as the

159   *venus* gene, was amplified from *E. coli* M1/5 P*ybtA*'-'*ybtA*-*venus* using primer pair *ybtA*-TLN-

160   XbaI and MK67, digested with XbaI and SphI and ligated with XbaI/SphI pBAD33-*rrnB*. The

161   resulting plasmid pP*ybtA*-*ybtA*'-'*venus*1 contained a translational fusion of the first three

162   nucleotides of *ybtA* and the *venus* gene. In an additional step, the *ybtA* sequence was extended

163   from three to twelve nucleotides in a restriction-free cloning strategy (Unger et al., 2010) using

164   pP*ybtA*-*ybtA*'-'*venus*1 as a template and *ybtA*-long-for and *ybtA*-long-rev as primers, yielding

165   plasmid pP*ybtA*-*ybtA*'-'*venus*2. Plasmid pP*ybtA*-*ybtA*'-'*venus*2-*csrA* was generated by digestion

166   of pP*ybtA*-*ybtA*'-'*venus2* with XbaI and SphI and cloning of the P*ybtA*-*ybtA*'-'*venus* sequence

167   into XbaI/SphI digested pBAD33-*csrA*-*rrnB*.

168   **pP*ybtA*-*ybtA**'-'*venus*2.** The sequence encoding the putative CsrA binding motif within the

169   *ybtA* leader of pP*ybtA*-*ybtA*'-'*venus*2 was modified on DNA basis from <u>ACAG</u>GGA to

170   <u>TTAT</u>GGA using the respective plasmids as templates with the primer pair

171   *ybtA*-TLN-TTAT-for/*ybtA*-TLN-TTAT-rev by restriction-free cloning. The resulting plasmid

172   was designated pP*ybtA*-*ybtA**'-'*venus*2.

173   **pP*lacUV5*-AL-*venus*.** A plasmid carrying a fusion of the *lacUV5* promoter, a sequence

174   encoding the 5' untranslated region without CsrA binding motif (artificial leader, AL) and the

175   *venus* sequence and was designed to be used as a control for CsrA-independent regulation of

176   various *ybtA*- and *fyuA*-*venus* fusions. In a first step, the P*lacUV*-AL fusion with a partial venus

177   sequence was amplified from template vector pUC-P*lacUV*5-*venus* with primers

178   *Kan*-P*lac*-fus-s and *venus*-Seq-as. In a second step, this PCR product served as a megaprimer

179   and plasmid pP*lacUV*5-*ybtA*'-'*venus*1 as a template in a restriction-free cloning approach as

180   described above to generate pP*lacUV*5-AL-*venus*.

181   **pP*ybtA*-AL-*venus*.** This plasmid carried a fusion of the *ybtA* promoter, a sequence encoding

182   the 5' untranslated region without CsrA binding motif (artificial leader, AL) and the *venus*

183   sequence. A megaprimer pair was generated via PCR using pP*lacUV*5-AL-*venus* as a template

184   and primers *ybtA*-TXN-for and *venus*-Seq-as2. Next, these megaprimers were used to replace

185   the *ybtA* 5'UTR of plasmid p*PybtA*-*ybtA*'-'*venus*2 by the AL using restriction-free cloning as

186   described above, leading to pP*ybtA*-AL-*venus*.

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188

Megalocytosis and γH2AX assays

HeLa cells, maintained by serial passage in DMEM supplemented with 10 % FCS and non-essential amino acids at 37 °C and 5 % CO_2, were used to demonstrate the cytotoxic effect of colibactin on mammalian cells. Colibactin has been shown to induce double strand breaks, which leads to cell cycle arrest and therefore formation of megalocytotic cells (4).

For assays to demonstrate megalocytosis induced by colibactin-producing bacteria, 2.5×10^4 HeLa cells per well were seeded in a 24-well cell culture plate. To examine the extent of DNA double strand breaks using serine139-phosphorylated H2AX, γH2AX, induced by bacteria synthesizing colibactin, 1.25×10^5 HeLa cells were dispersed in a 6-well cell culture plate. The next day, bacterial overnight cultures were used to infect HeLa cells with a multiplicity of infection (MOI) of 200 in interaction medium (DMEM, 5 % FCS, 25 mM HEPES). Four hours post infection, cells were washed 4-8 times with 1x Hank´s Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, Wesel, Germany) containing 100 µg mL^{-1} gentamycin and further incubated at 37 °C and 5 % CO_2 in DMEM with 10 % FCS, non-essential amino acids and 200 µg mL^{-1} gentamycin (600 µg mL^{-1} gentamycin for cells infected with *E. coli* strains carrying the *csrA*51 mutation).

For demonstration of megalocytosis, cells were washed twice with 1x HBSS 72 h post infection, afterwards fixed with methanol, stained with 10 % Giemsa stain (Sigma-Aldrich, Munich, Germany) and then used for microscopy.

For γH2AX detection, cells were washed eight hours post infection with ice-cold phosphate buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4) and immediately suspended and lysed with sample buffer (32.9 mM Tris, 15 % (v/v) glycerol, 0.5 % sodium dodecyl sulfate, pH 6.8). Total protein in cell extracts was quantified using BCA reagent (Thermo Fisher Scientific). 4-5 µg total protein were first separated by SDS-PAGE using 4-20 % Mini-Protean-TGX gels (Bio-Rad, Munich, Germany) and then transferred onto a

215   polyvinyl difluoride (PVDF) membrane. For the detection of γH2AX antibody anti-γH2A.X

216   (phospho S139) antibody (Abcam, Cambridge, UK) and as secondary antibody Peroxidase

217   AffiniPure goat anti-rabbit IgG (H+L) (Dianova, Hamburg, Germany) were used. Alternatively,

218   anti-phospho-Histone H2A.X (Ser139), clone EP854(2)Y (Merck-Millipore, Darmstadt,

219   Germany) served as a primary antibody for detection of γH2AX. As a control, β-actin was

220   detected with anti-beta actin [AC-15] antibody (HRP) (Abcam). As substrate for horseradish

221   peroxidase Bio-Rad Clarity Western ECL-substrate was used (Bio-Rad). Blots were analysed

222   using the ChemiDoc XRS+ system (Bio-Rad).

223

224   **N-myristoyl-D-asparagine (C14-Asparagine) quantification**

225   Cultures in 9.5 mL DMEM-Hepes (Gibco) inoculated with 500 µL o/n LB culture were used

226   for lipid extraction. After 24 h, the OD_{600} was measured and the culture was centrifuged for

227   5 min at 5000 × g and 4 °C. Pellets were resuspended in 500 µL HBSS and frozen at -80 °C

228   until lipid extraction.

229   Pellets were crushed in Lysing Matrix A tubes (MP biomedicals, Santa Ana, California, USA)

230   with a Precellys instrument (5 m/s; 2 x 30 s) (Ozyme, Montigny le Bretonneux, France) after

231   addition of 5 µL internal standard (IS) mixture (Deuterium-labeled compounds: 400 ng/mL).

232   After two crush cycles (5 m/s, 30 s), 0.3 mL of cold methanol (MeOH) was added and samples

233   were centrifuged at 1016 × g for 15 min (4 °C). HLB plates (OASIS® HLB 2 mg, 96-well plate,

234   Waters, Ireland) were used for solid phase extraction of lipids after being conditioned with 0.5

235   mL MeOH and 0.5 mL H_2O/ MeOH (90:10, v/v). Samples' volumes were brought to 2 mL with

236   water and then loaded on the plate at a flow rate of about one drop per 2 s. After complete

237   loading, columns were washed with 0.5 mL H_2O/ MeOH (90:10, v/v) and dried under

238   aspiration. Lipids were eluted with 750× µL MeOH and extracts were submitted to two series

239   of evaporation under N_2, then resuspended in a final volume of 10 µL MeOH for quantification

240   of C14-Asn.

241   The quantification of C14-Asn was performed by the MetaToulLipidomics Facility (INSERM

242   UMR1048, Toulouse, France). They developed a quantification assay by high-performance

243   liquid chromatography tandem mass spectrometry analysis (LC-MS/MS) (5). Briefly a high-

244   performance liquid chromatography (HPLC, Agilent 1290 Infinity) was designed to separate

245   the metabolites using a C18 column. The HPLC system was coupled online to an Agilent 6460

246   triple quadrupole MS for detection and quantification (Agilent Technologies). Analyses were

247   performed in Multiple Reaction Monitoring in optimal conditions. Peak detection, integration

248   and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent

249   Technologies).

250

251   **<u>DNA crosslinking assay</u>**

252   The assay was performed as previously described (6). Linearized plasmid DNA was produced

253   by digestion of pUC19 plasmid with BamHI (New England Biolabs). For bacteria-DNA

254   interactions, 6×10^6 CFU were cultivated in 500 µL micro tube under static condition at 37 °C.

255   After 3.5 h of cultivation, 400 ng of linearized DNA and EDTA of a final concentration of 1mM

256   were added and further incubated for 30 min. Following centrifugation for 5 min at $5,000 \times g$

257   to pellet bacteria, the DNA present in the supernatants was purified using the PCR Purification

258   Kit (Qiagen) according to the manufacturer's recommendations.

259   A denaturing 1% agarose gel was prepared in a 100 mM NaCl and 2 mM EDTA solution

260   (pH 8.0). The gel was then soaked overnight in an alkaline running buffer solution (40 mM

261   NaOH and 1 mM EDTA, pH ~12.0). 100 ng of each DNA sample were loaded on the denaturing

262   agarose gel. The gel was run for 45 min at 1 V/cm and then for 2 h at 2 V/cm and then

263   neutralized for a total of 45 min in a 100 mM Tris pH 7.4 buffer solution containing 150 mM

264   NaCl, changed frequently. The gel was stained with GelRed and revealed with UV exposure

265   using the ChemiDoc Imaging System (BioRad).

266

Reporter gene measurements

Fluorescence of the yellow fluorescent protein Venus was measured to determine the expression

levels of various *E. coli* M1/5 *clbQ-* and *ybtA-venus* fusion strains.

For *clbQ-venus* reporter gene assays, 50 mL glucose-free M9 medium with pyruvate and casein

hydrolysate were inoculated to an OD_{600} of 0.05 from overnight cultures of respective strains

as well as their controls (corresponding strains without the cloned *venus* gene). For strains

carrying plasmids 100 µg mL^{-1} ampicillin was added. During growth at 37 °C under shaking

conditions, samples were drawn to determine the OD_{600} for monitoring of growth and to

measure fluorescence. Samples were centrifuged and washed once with phosphate buffered

saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium

hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4) and resuspended in

PBS. 150 µl of each sample were transferred into a black 96-well polystyrene microplate with

transparent and flat bottom (Greiner Bio-One, Bad Nenndorf, Germany) and fluorescence was

determined in a Tecan Infinite 200 Reader (Tecan Group Ltd., Männedorf, Switzerland) with

following parameters: 10 s shaking with an amplitude of two, absorption was measured at

595 nm (10 nm bandwidth) and five flashes (pulses), fluorescence was excited at 485 nm

(20 nm bandwidth) and emitted at 535 nm (25 nm bandwidth) with 50x amplification, 10

flashes and 20 µs of integration time.

E. coli M1/5 strains carrying plasmids with *ybtA-venus* fusions were grown in glucose-free M9

medium with pyruvate and casein hydrolysate in the presence of 100 µM 2,2'-dipyridyl to

induce iron limitation, and 15 µg mL^{-1} chloramphenicol. For each strain (including a strain with

a promoterless *venus* gene as negative control) two wells of 150 µl fresh medium were

inoculated to an OD_{600} of 0.05 from overnight cultures. Cells were grown for 23 h in a Tecan

Infinite 200 Reader (Tecan Group Ltd.) with a shaking amplitude of 2 and the parameters as

291 described for *clbQ-venus* fusion strains, except that absorbance and fluorescence were

292 determined in 15 min-intervals with a 3-min shaking pause during each interval. For the

293 measurement of fusion strains containing the *ybtA* or *lacUV5* promoter fused to the sequence

294 encoding an artificial leader and the *venus* gene a 40x amplification of the fluorescence signal

295 was applied.

296 Raw data were first corrected for autofluorescence of PBS (*clbQ-venus*) or M9 medium (*ybtA-*

297 *venus*), respectively and the resulting values were then normalized to the OD_{595}. The calculated

298 fluorescence/OD_{595} values of the negative control strains were subtracted from that of the

299 respective fluorescent strains to correct for autofluorescence of bacteria.

300

301 **<u>RNA electrophoretic mobility shift assays (RNA EMSA)</u>**

302 Biotin-labelled and unlabeled RNA oligonucleotides representing the 5' untranslated leader

303 transcripts of *clbQ*, *ybtA* and *phoB* as a negative control (7) were purchased from metabion

304 GmbH (Planegg/Steinkirchen, Germany). Purified recombinant CsrA protein of *E. coli* (catalog

305 # MBS1176282) was purchased from MyBioSource. EMSA were carried out using the

306 LightShift™ Chemiluminescent RNA EMSA Kit (Thermo Fisher Scientific). Briefly, 10 µl

307 reaction mixtures containing biotin-labeled RNA and increasing amounts of purified CsrA in

308 incubation buffer (10 mM HEPES, 20 mM potassium chloride, 1 mM magnesium chloride,

309 1 mM DTT, 10 µg yeast tRNA (Thermo Fisher Scientific), 40 units RNaseOUT™ (Thermo

310 Fisher Scientific), pH 7.3) were prepared and incubated for 30 min at 37 °C to enable protein

311 binding to RNA. For competitive EMSA, non-labeled RNA was added to the labelled RNA.

312 After incubation, the samples were immediately loaded on precast native 8 % Tris Borate

313 EDTA polyacrylamide gels (anamed Elektrophorese GmbH, Groß-Bieberau/Rodau, Germany)

314 and run for 90 min 0.5x TBE and 80 V. Afterwards, samples were transferred onto a positively

315 charged nylon membrane in 0.5x TBE and 300 mA for 37 min, which was followed by RNA

316 crosslinking to the membrane using UV light (2x with 1,200 units). Biotinylated RNA was

317   detected with the chemiluminescent detection module (Thermo Fisher Scientific) according to

318   the manufacturer´s protocol and blots were analyzed in a ChemiDoc XRS+ System (Bio-Rad,

319   Munich, Germany).

320

321   **Yersiniabactin quantification**

322   The amount of yersiniabactin produced by various *E. coli* strains was quantified using the

323   *Salmonella* indicator strain WR1542 carrying the plasmid pACYC5.3L (gift from W. Rabsch,

324   Wernigerode). This plasmid carries all necessary genes of the high pathogenicity island for

325   uptake of yersiniabactin, *fyuA*, *ybtA*, *irp6*, *irp7* and *irp8*. Moreover, the plasmid harbors a

326   transcriptional fusion of the *fyuA* promoter with the luciferase gene as a reporter allowing the

327   detection of yersiniabactin-dependent upregulation of the *fyuA* promoter, thus indirect

328   quantification of yersiniabactin. The method has been previously described in detail (8).

329

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