“Polar mutagenesis of bacterial transcriptional units using Cas12a”

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Abstract

Bacterial genes are often organized in functionally related transcriptional units or operons. One such example is the *fimAICDFGH* operon, which codes for type I fimbriae in *Escherichia coli*. We tested the hypothesis that markerless polar mutations could be efficiently engineered using CRISPR/Cas12a in the *fim* operon. Cas12a-mediated engineering of a terminator sequence inside the *fimA* gene occurred with efficiencies between 10 and 30%, whilst other types of mutations, such as a 97 bp deletion, occurred with 100% efficiency. Our results showed that some of the obtained mutants, including one with a single base substitution at the *fim* locus, had decreased mRNA levels of *fimA*, suggesting that the regulation of the *fim* operon was disrupted. We corroborated the polar effect of these mutants by phenotypic assays and quantitative PCR, showing up to a 43 fold decrease in expression of genes downstream *fimA*. We believe this strategy could be useful in engineering the transcriptional shut-down of multiple genes in one single step. For bio-production in *E. coli*, this opens the possibility of inhibiting competing metabolic routes.

Short title: Polar mutagenesis with Cas12a

23 Introduction

24

25 The evolutionary success of operons has been attributed to their contribution to the organization of
 26 metabolic pathways, which ultimately allowed organisms to become less dependent on exogenous
 27 sources of organic compounds (1). Importantly this relationship was maintained during evolution
 28 for many metabolic pathways and was probably enhanced by horizontal gene transfer (1). Genes
 29 within operons are co-transcribed from a single promoter but it has been shown that the mRNA of
 30 each open reading frame (ORF) has an independent folding from their neighbouring genes and this
 31 feature directly influences its translation efficiency (2). Other features regulating operon expression
 32 and function in *cis* include attenuators, terminators and processive aniterminators (reviewed in
 33 (3,4)). All these elements of regulation depend on the formation of secondary RNA structures.

34 We asked ourselves if sequences with such a tendency to generate secondary structures could be
 35 easily engineered in the *E. coli* chromosome using CRISPR/Cas technologies. CRISPR/Cas
 36 genome editing in *E. coli* (5), opened the possibility of marker-less genetic engineering with single
 37 base resolution. Therefore, it is possible to now generate multi-scale libraries of mutants in a few
 38 days (6). The objective of the following study was to design a strategy to shut-down the
 39 transcription of entire operons using a synthetic terminator sequence (7) in *E. coli*.

40

41 The possibility of easily controlling entire transcriptional units would serve multiple purposes for the
 42 bio-production of compounds using synthetic metabolic routes. For example one aspect poorly
 43 appreciated in synthetic biology is metabolic burden (8). Protein synthesis has been calculated to
 44 be the most expensive step (ATP-wise) involved in cell growth, compared to the biosynthesis of its
 45 building blocks (amino acids) which uses only a small fraction of ATP (9). Therefore shutting down
 46 the synthesis of unnecessary operons during specific growth conditions could contribute
 47 significantly to balance the burden while expressing heterologous operons. Another example is

48 shutting down or the re-direction of competing metabolic routes (10) during bio-production from
 49 heterologous operons. To that effect, the bioinformatic analysis of competing or converging
 50 metabolic routes will be essential (11). Another interesting application would be the removal of
 51 virulence factors and antibiotic resistance genes as a potential antimicrobial strategy (14). These
 52 type of genes also tend to cluster together in so called pathogenicity islands. Therefore, the
 53 strategy presented in the following study opens another possibility to investigate, with one step, the
 54 effect of competing transcriptional units arranged in operons on metabolic burden and compound
 55 biosynthesis.

56

57 Type one fimbriae are adhesive surface appendages present mainly in *Enterobacteriaceae* that
 58 were first shown to agglutinate red-blood cells (15). They are composed of a main pilin subunit
 59 repeated in the order of hundreds to thousands and are assembled at the surface by an outer
 60 membrane usher. At the tip of the filament the so called minor pilins are assembled in a specific
 61 order. The most distal pilin to the cell (FimH) functions as an adhesin (16,17). In the case of *E. coli*
 62 type I fimbriae the FimH adhesin is inhibited by D-mannose residues and α -methyl-mannoside and
 63 they recognize mannosylated receptors (16–18). Its role in virulence in uropathogenic strains of *E.*
 64 *coli* has been established for a long time (19,20). However, its role in mammalian persistent
 65 intestinal colonization has only been recently shown to be essential for the case of uropathogenic
 66 *E. coli* (21). It has not yet been studied, how each of the several fimbrial systems present in
 67 symbiotic *E. coli* strains (22) contributes to intestinal colonization *in vivo*.

68

69 The *fim* operon is a good system to test an engineered polar effect, for several reasons: Firstly, the
 70 architecture of the *fim* operon is such that the gene coding for the major and most abundant
 71 component of the pilus is the first to be transcribed, followed by the assembly machinery. This
 72 gives the advantage that disruption of the first gene inactivates functionality of the fimbriae
 73 complex and any polar effect would disrupt the function of the subunits important for fimbriae

74 assembly. Secondly, the levels of *fim* transcription are chiefly determined by an invertible element
 75 upstream of the promoter (*fimAp*), which acts as an ON/OFF switch. The regulation of the
 76 mannose-dependent adhesion of type I fimbriae is complex, and has been studied *in vitro* for
 77 decades. It has been shown to be regulated by various genes including NanR and NagC (through
 78 sialic acid and GlcNAc) (23), Lrp, (24,25), H-NS (26) and IHF (27). All these elements mainly
 79 regulate the frequency of inversion of the invertible switch. The molecule (p)ppGpp also regulates
 80 the *fim* switch (28). Therefore, it should be possible to quantify a polar effect in the *fim* operon by
 81 measuring the transcription of genes downstream of *fimA*.

82

83 In this study we introduce a terminator sequence with a strong secondary structure within the *fim*
 84 operon to disrupt its transcription. We showed that this sequence is easy to introduce by
 85 CRISPR/Cas12a engineering and that the assembly machinery of *E. coli*'s type I fimbriae can be
 86 turned off using this polar mutagenesis strategy.

87

88

89 Results

90 Design of *fimA*-targeting crRNA and donor oligos

91

92 We set out to disrupt the *fim* operon using Cas12a to introduce a range of different markerless
 93 mutations in the *fimA* gene, including the insertion of a terminator sequence (Fig. 1). We designed
 94 a CRISPR RNA (crRNA) targeting the *E. coli fimA* gene. To introduce polar and nonpolar
 95 mutations in the *fim* operon we designed two complementary oligos for each mutation to form
 96 dsDNA donor molecules for homologous recombination. The recombination of these oligos
 97 disrupts the binding site of the Cas12a-crRNA complex and permits the positive selection of mutant
 98 cells (29).

99

100 To introduce a premature stop codon in the *fimA* gene, we designed dsDNA donor oligos in which
 101 a single bp change disrupted the PAM sequence adjacent to the target of the designed *fimA* crRNA
 102 (crRNA1) and introduced a stop codon (TAA) after 106 bp of the start codon (*fimA*_{A106T}). To
 103 introduce a deletion in the *fimA* (Δ *fimA*) gene, we designed dsDNA donor oligos containing
 104 homology recombination sequences upstream and downstream of the crRNA recognition site to
 105 delete 97 bp introducing a stop codon after 75 bp after the start codon (Fig. 1).

106

107 To engineer a polar mutagenesis strategy, we designed two different donor oligos introducing a
 108 terminator sequence in the *fimA* gene. The first pair of oligos contained the exact same sequences
 109 for homologous recombination as the deletion donor oligos but including the L3S2P56 terminator at
 110 nucleotide position 75 after the start codon (*fimA*_{75::L3S2P56}, called here Term1). The second
 111 donor oligos were similar but the upstream homologous region corresponded to the leader of the
 112 *fimA* gene. Therefore, these oligos introduce a terminator sequence at the start codon
 113 (*fimA*_{1::L3S2P56}, called here Term2).

114

115 **Mutagenesis of the *fimA* gene**

116

117 Terminator sequences have a strong tendency to form secondary structures and therefore we
 118 asked ourselves if such sequences could serve as DNA donors for CRISPR/Cas12a mutagenesis.
 119 To test this we simply electroporated *E. coli* cells carrying the plasmid pKD46-Cas12a (29) with the
 120 annealed dsDNA donor oligos and a plasmid encoding crRNA1. We analysed transformants by
 121 colony PCR using oligos that amplify a segment of the *fimA* gene surrounding the site of crRNA
 122 targeting. For the stop codon mutant (*fimA*_{A106T}) obtained with crRNA1, we analysed five colonies.
 123 The size of the PCR fragment did not appear to change as compared to wild type (WT) *E. coli* (Fig
 124 2). We purified these PCR products and submitted them for Sanger sequencing. Four of the
 125 products showed the specific T->A substitution that introduces a premature stop codon. For the
 126 Δ *fimA* mutant, the PCR fragment showed a noticeable change in migration pattern compared to
 127 WT (Fig 2). Ten out of ten colonies picked showed the deletion (S1 Fig.). For mutants with the
 128 insertion of a terminator sequence only three out of ten and one out of ten colony PCRs showed a
 129 change in migration pattern for the Term1 and Term2 mutations, respectively (Fig. 1 and S1 Fig).
 130 Altogether these results indicate that the engineering of terminator sequences is possible but with
 131 lower success rates compared to the highly efficient engineering of a single base substitution and a
 132 deletion.

133

134 **Phenotypic analysis of *fimA* mutants**

135

136 To characterise the effect of the different mutations, we verified the functionality of the *fim* operon
 137 gene products by a phenotypic assay. We performed agglutination assays of *Saccharomyces*
 138 *cerevisiae* cells (Fig 3A-C). Overnight cultures of WT and *fimA* mutants *E. coli* strains were mixed

139 with a drop of a yeast suspension over a glass plate. With WT *E. coli* this led to agglutination of the
 140 yeast cells, which appeared as white, macroscopically visible aggregates (Fig 3D). Contrastingly,
 141 mixing *Shigella flexneri* M90T, which is known not to produce fimbrial genes when grown in tryptic
 142 soy agar (30), presented no yeast aggregates (Fig 3E). Mixing each of our *fimA* mutants with the
 143 yeast suspension produced no agglutination (Fig 3F-I). There was no difference between the stop
 144 codon mutant (*fimA*_{A106T}), deletion and terminator mutants (Ter1 and Ter2), which suggests that
 145 the disruption of even one base introducing a stop codon was enough to alter the expression of the
 146 *fim* operon.

147

148 **Plasmid trans-complementation of the *fimA* mutants**

149

150 To verify the efficiency of the different mutations in exerting a polar effect, we verified the
 151 functionality of the *fim* operon by a phenotypic assay in WT and mutant strains carrying a plasmid
 152 expressing *fimA* (Fig. 4). Each strain was transformed by the trans-complementing plasmid and an
 153 empty vector as negative control. WT *E. coli* carrying the empty vector agglutinated *S. cerevisiae*
 154 as expected. Similarly, the expression of plasmid encoded *fimA* had no effect on the agglutination
 155 reaction (Fig 4A-B), suggesting that neither of the plasmids had an impact on agglutination. Trans-
 156 complementation of both the stop codon (*fimA*_{A106T}) and deletion *fimA* mutants resulted in yeast
 157 agglutination (Fig 4C-D and Fig 4E-F), suggesting that the mutations did not introduce any effect
 158 on genes downstream of *fimA*, which code for the assembly machinery, and that expression of a
 159 functional copy of this gene rendered these strains agglutination-positive. In contrast, the strains
 160 carrying the empty vector were agglutination-negative. Trans-complementation of both Term1 and
 161 Term2 mutants with the empty vector or the plasmid expressing *fimA*, resulted in no yeast
 162 agglutination (Fig 4G-H and Fig 4I-J). This suggests that the functionality of the *fim* operon was
 163 completely disrupted downstream of *fimA* in these two mutants and suggested that the introduction
 164 of the L3S2P56 terminator functionally disrupted the transcription of the entire *fim* operon.

165

166 **Measurement of mRNA levels of *fimA* and *fimI***

167

168 We wanted to quantify the extent of a polar effect in all the mutants obtained. We used qPCR to
 169 measure the levels of *fimA* and *fimI* mRNA. We designed two sets of primers that allowed for the
 170 estimation of the levels of these two genes (Fig 1B). We performed qPCR using as template cDNA
 171 derived from mRNA obtained from *E. coli* cultures grown in the same conditions as those used for
 172 the phenotypic assays. First, we estimated the amount of the *fimI* mRNA relative to *fimA*. This
 173 represents an unbiased measurement that does not depend on the ON/OFF state of the *fimAp*.
 174 Relative expression of *fimI* in WT *E. coli* was ~2.5 times lower to *fimA* (Table 1). The median
 175 relative expression of *fimI* in the stop and deletion mutants was lower but in the same order of
 176 magnitude (Fig 5A). The median relative expression of *fimI* in the Term1 and Term2 mutants was
 177 considerably lower (Fig 5A). The relative expression fold change for the Term1 and Term2 mutants
 178 was 9 and 43 fold, respectively (Table 1). These results suggest that at least an estimated 9 fold
 179 change in expression is sufficient to disrupt the functionality of the *fim* operon downstream of *fimA*.

180 We wanted to measure the levels of *fimI* and *fimA* mRNA levels with respect to housekeeping
 181 genes. We designed sets of primers for the housekeeping genes *hcaT* and *cysG*, which have been
 182 shown to have stable expression (31). We obtained the average ΔC_t with respect to these two
 183 genes and normalized it to the WT control performed the day of the experiment. The levels of *fimA*
 184 varied in all the mutants as well as the levels of *fimI*. For the stop codon mutant, the deletion
 185 mutant and the Ter1 mutant the levels of *fimA* mRNA were lower than the WT (Fig 5B). The levels
 186 of *fimI* were lower for all the mutants, with the Ter1 and Ter2 mutants having the most severe
 187 defects compared to WT(Fig 5B).

188

189 We were puzzled by the small but measurable changes of mRNA levels in the stop and deletion

mutants in *fimA* mRNA (Fig. 5B). To further investigate this phenomenon, we decided to create two different stop codon mutants. We co-introduced in *E. coli* plasmids encoding crRNA2 and crRNA3 (Fig 1) with donor template oligos introducing a stop codon instead of the PAM sequence into *E. coli* carrying the plasmid pKD46-Cas12a. For crRNA2 the donor oligos introduced the mutations T103A, T104A (*fimA*_{T103A,T104A}) to disrupt the PAM sequence. For crRNA3 the donor oligo introduced the mutations A158T, A159T, A160T, C161A, C162A (*fimA*_{A158T,A159T,A160T,C161A,C162A}). Two clones of each of these mutants were selected and analysed by qPCR measurements of *fimI* and *fimA*. The median relative expression of *fimI* to *fimA* in these two new mutants was also lower but in the same order of magnitude of mutants carrying the allele *fimA*_{A106T} (Fig 5A, orange and yellow circles). This result suggests that the reduced levels of *fimA* expression are consistent in all three stop codon mutants. Moreover, these results indicate that the lower *fimA* mRNA levels are not caused by an off-target effect of crRNA1.

202

203 **Observation of a *fimA* terminator mutant by AFM**

204

We wanted to observe the formation of fimbriae by Atomic Force Microscopy (AFM) and agglutination in previously described favourable conditions. Fluorescence microscopy measurements have shown an increase of fimbriae in static cultures of *E. coli* strain MG1655 (32). We grew WT *E. coli* and the Ter2 mutant expressing *fimA* from a plasmid on static cultures to observe if there was any formation of fimbriae. To prepare the AFM samples in a way that it introduced the least amount of manipulations, we diluted the static culture in deionized water to an OD600 of 0.05 and we immobilized a drop of this suspension on freshly cleaved mica for 20 minutes. After the incubation period the mica was gently washed with more deionized water. In the wild type strain expressing plasmid-encoded *fimA*, we observed bacteria showing the classical type I fimbriae (Fig. 6A and 6A inset). However in the Ter2 mutant expressing plasmid-encoded *fimA* we did not observe any formation of fimbriae (Fig. 6A). In some cells, only the formation of flagella

could be observed (Fig.6A inset). To confirm the absence of functional fimbriae in this mutant, we repeated the yeast agglutination assay after static growth conditions (Fig. 6B). As a negative control we incubated this time the wild type strain with α -methyl-mannoside. The wild type strain expressing plasmid-encoded *fimA* was agglutination-positive as shown in Fig. 4. However using the same strain pre-incubated with α -methyl-mannoside completely abolished agglutination. This confirms that agglutination is mediated by the mannose-dependent type I fimbriae encoded in the *fim* operon. Agglutination by the Ter2 mutant expressing plasmid-encoded *fimA* did not complement agglutination after having grown in static conditions and the agglutination reaction appeared as the α -methyl-mannoside negative control. All these results confirm that the Ter2 mutant has a *fim* negative phenotype even when *fimA* is expressed in *trans*.

Table 1. Relative levels of *fimI* to *fimA* mRNA.

Strain	Median Δ ct (<i>fimI</i> - <i>fimA</i>)*	Median Δ ct (<i>fimA</i> - <i>fimI</i>)	~Fold change inhibition
WT	0.39	2.6	1
Stop codon	0.10	9.8	4
Δ <i>fimA</i>	0.17	5.8	2
Term1	0.04	23.3	9
Term2	0.009	111.4	43

Table1. Relative levels of *fimI* transcript with respect to *fimA*. The median Δ Ct values are shown as in Fig 5A. The fold change was calculated by dividing the median Δ Ct (*fimI* – *fimA*) of each mutant by the median value in the wild type.

232 Discussion

233

234 CRISPR/Cas technologies have become part of routine work in many laboratories. For the
 235 synthetic biology field these techniques opened the possibility of quickly engineer chromosomal
 236 loci. For biotechnological purposes, it also opens the possibility to decrease the use of plasmids to
 237 produce heterologous enzymes. Plasmids can be lost and impose extra metabolic burdens and
 238 regulatory burdens that have to be taken into account when fine-tuning bio-production (reviewed in
 239 [\(8\)](#)). One proposed approach is to iteratively test the effect of engineered mutations in strains to
 240 optimize this process [\(33\)](#). However the effect of a mutation in a system often carries unpredictable
 241 or poorly controlled consequences. In *E. coli*, 63% of its genes are organized in operons [\(34\)](#) and
 242 40% are organised in uber operons [\(35\)](#). In both of these organisation levels, genes tend to be
 243 related by functional conservation across bacterial genomes [\(35\)](#). The possibility to
 244 transcriptionally shut-down with one step a whole operon could contribute to the quick design and
 245 testing of strains with more predictable outcomes if regulons and uber operons were taken into
 246 account.

247

248 In this study we used an artificial terminator sequence to shut down the transcription of the *fim*
 249 operon. This designed artificial sequence has an extended secondary structure that has been
 250 shown to improve transcriptional termination [\(7\)](#). We did not know whether such secondary
 251 structures could impede the recombination process in the context of the donor homology
 252 recombination oligonucleotides used for CRISPR/Cas genome engineering. The predictable
 253 outcome from inserting this sequence in the *fim* operon is the premature transcriptional termination
 254 of the genes coding for the type I fimbriae assembly machinery. Our results indeed showed a
 255 reduction of up to 43 fold in the expression of the *fim* machinery as measured by the expression of
 256 *fimI*.

257

258 The efficiency of genome engineering strains has been shown to depend on the efficiencies of
 259 gRNAs to direct Cas9 DNA cleavage and donor oligos (6). Using the same crRNA (crRNA1) to
 260 generate several mutations at the same target sequence we saw that each mutation was produced
 261 with efficiencies ranging from 10% to 100%. However the donor oligonucleotides varied in
 262 homology arms and secondary structure. Therefore our results suggest that, as suspected, these
 263 two parameters are important for bacterial genome engineering. Despite these differences we
 264 could readily find mutants with inserted terminator sequences. Among the 582 terminator
 265 sequences characterised by Chen *et al.*, we decided to use an artificial terminator sequence
 266 (L3S2P56) that showed to have low recombination rates (7), which would ensure more stability
 267 within the *E. coli* chromosome. However we did not know whether this would decrease the
 268 recombination mediated by homology arms upstream and downstream of the terminator sequence.
 269 Our results show that this sequence was easily engineered using the Cas12a-encoding plasmids
 270 designed by Yan *et al.* (29). These plasmids are easy to use and allow multiple rounds of
 271 mutagenesis similar to previously reported Cas9 approaches (6). Cas12a also brings the
 272 advantage that it processes its own crRNAs, which we think will allow certain flexibility in the future
 273 when dealing with organisms with poorly characterised promoter sequences. All together this study
 274 shows that it is possible to shut down the expression of a whole operon using an artificial
 275 terminator. This approach could be complementary to other approaches that allow the
 276 transcriptional repression of multiple genes such as the use of multiple gRNAs for transcriptional
 277 repression (12) or the deletion of large genomic stretches (13). The use of Cas12a also facilitated
 278 genome engineering, because its PAM sequence NTTT (AAAN in the bottom strand) can be easily
 279 mutated to introduce a stop codon. Indeed here we showed that a single base substitution
 280 (*fimA*_{A106T}) disrupted PAM recognition and allowed the selection of *fimA* mutant strains.

281

282 The *fim* operon was an ideal study case because the phenotype is macroscopically observable and
 283 the characterisation of the *fimA* mutants by yeast agglutination produced easy to interpret results.
 284 Moreover, the extent of fimbriation is directly correlated to the state of the switch inside *fimAp* (32),

therefore for every *fimA* transcript observed the presence of a *fimI* transcript reflects the polarity of the mutants generated here. Indeed, insertion of a terminator sequence abolished the trans-complementation by a plasmid-encoded *fimA* gene. Less predictable phenotypes were obtained by measuring the relative expression of *fimI* to *fimA* in typical laboratory conditions. Three out the four mutants that were generated in this study, both polar and nonpolar, had reduced median levels of *fimA* transcript and of the ratio *fimI/fimA* transcripts. This includes a single base substitution that introduces a stop codon (*fimA*_{A106T}). Several possible explanations exist to accommodate this fact. For example, it is known that ribosome binding protects mRNAs from RNase E degradation (36). Therefore, one possible explanation would be that the premature stop codon introduced in *fimA*_{A106T} and in the deletion mutant induces the premature release of ribosome (37,38) and at the same time exposes the *fimA* transcript to degradation. Another more complicated involves the stalling of ribosomes during translation, which may induce mRNA cleavage next to or at a stop codon (39). An initial cleavage event could lead to further degradation by classical mRNA decay pathways. However this could only make sense if the folding of the *fimA* mRNA would be compromised and stalling would be induced by the mutations designed here. This can also be put in the context of the recent discovery that the mRNA of individual genes within operons have a unique secondary structure (2). Deletions smaller than a whole gene could affect the folding of an individual genes' mRNA within an operon. Another possibility would be that there would be some kind of positive feedback on type I fimbriae expression or functionality. On the conditions that we assayed *fimA* expression, that is rich medium at 37 C, switching off dominates on *fimAp* (40). Therefore, any kind of feedback would quickly result in *fimA* expression inhibition. Another possibility would be that the mutation introduced in this study interferes with a differential stability within *fim* genes' transcripts, which is an important element for other fimbrial systems (e.g, the *pap* fimbrial operon from uropathogenic *E. coli*) (41). One final possibility is that expression of a functional type I fimbriae system confers a growth advantage (32,42), although this has only been proposed for static cultures. Alternatively, the peptide products of the stop codon mutants (~35 amino acids long and hydrophobic) could also be partially toxic and induce the selection of cells with the *fim* switch in the off state. Although this would not apply to the terminator insertion at

position 1 (Ter2) mutant, for which there should not be any partial *fimA* peptide product. Further studies will be needed to clarify the role of the three stop codon mutants and deletion on the expression of *fimA*. However, our results show that even a single nucleotide substitution can have subtle but measurable changes at the transcriptional level within an operon when measured in typical laboratory conditions.

The chromosome of *E. coli* encodes multiple fimbrial systems. However, in the conditions used in this study, only the *fim* type I fimbriae are functional (22). The results shown in this study open the possibility of quickly generating *E. coli* strains in which each fimbrial operon is inactivated. This could be useful to see if any of these systems is essential under a particular growth condition. Another future direction of interest is to characterise the effect of other *fimA* stop codon mutants on the stability of its transcript. It would be crucial to determine if the effects observed here are gene specific or can occur in many other premature stop codon mutants. This is important in the light that introducing a premature stop codon is the easiest form to disrupt a gene using markerless techniques such as Cas9- or Cas12a-mediated mutagenesis.

327

In summary, in this study we show that Cas12a can be used to introduce markerless insertions in *E. coli* in one step and that this insertion can have strong secondary structures such as terminator sequences without excessively compromising the efficiency of the process. This approach allows the transcriptional shut-down of whole operons as demonstrated for the case of the *fim* type I fimbriae system. More importantly, this strategy can be used to easily test the importance of entire operons for the physiology of *E. coli* or its effect in the design of novel or artificial biosynthetic pathways.

335 **Materials and Methods**

336

337 **Cas12a Mutagenesis.** To design Cas12a crRNA targeting *fimA* with no off-targets in *E. coli*

MG1655 (43), we used the webtool Chop-Chop (44) and crRNAs ranking 1, 2 and 4 were selected due to its closeness to the start codon. To introduce all the mutations designed in this study we used the plasmids designed by Yan *et al.* (29) and followed their protocol with some modifications. Cloning of crRNAs was done by annealing forward and reverse oligos at 95 °C and ramping the temperature down at 2.55°C/min. The oligos were then phosphorylated using polynucleotide kinase (ThermoFisher). The plasmid pAC-crRNAred (29) was digested with BsaI-HF (New England Biolabs), dephosphorylated with FastAP (ThermoFisher) and ligated to the annealed oligos. Donor oligos were treated in the same way as crRNA oligos and their annealing was verified as suggested by König *et al.* (45). The night before the experiment we grew *E. coli* MG1655 carrying plasmid pKD46-Cas12a at 30 °C starting from a glycerol stock. The day of the experiment, 100 µl of overnight culture were transferred to a new agar plate with 0.2% arabinose and 4 h later electrocompetent cells were prepared using the “rapid” protocol (46). These cells were co-transformed by plasmids carrying one crRNA and an annealed pair of donor oligos. As a control transformation we used a crRNA targeting the gene coding for GFP. Colony PCR was then performed on transformants for genotyping. Each colony was resuspended in 20 µl water and boiled for 5 min. One µl of this sample was used as template for PCR. Agarose gel electrophoresis or Sanger sequencing was followed to determine the number of mutants. At least two independent colonies of each mutant were selected for further analysis. After mutagenesis was confirmed by sequencing, the strains were cured from the plasmids following the recommendations of Yan *et al.* (29). We observed that this process had a 100% efficiency by growing the cells at 37 °C in 5% sucrose in very rich medium such as YT2X. Glycerol stocks were then prepared for each clone. The structure of the L3S2P56 terminator was calculated with the Vienna websuite (47) and visualised with Forna (48).

361

Agglutination assay. To observe yeast agglutination, wild type or mutant MG1655 was grown overnight for 16 h on Luria agar plates as described before (49). A 1 µl plastic loop was used to take a colony from the plate and mixed with 200 µl of a *Saccharomyces cerevisiae* suspension at

365 OD₆₀₀ 5 prepared from refrigerated baker's yeast (Lidl Sverige). The loop was used to gently mix
 366 the two cell populations and the appearance of white flocculated aggregates could be observed for
 367 the wild type strain in less than 30 seconds. *Shigella flexneri* 5A M90T was used as a negative
 368 control (50). To perform this assay from static cultures, 10 µl from strains grown overnight in static
 369 broth (20 ml tryptic soy broth) were mixed with 200 µl of a *Saccharomyces cerevisiae* suspension
 370 at OD₆₀₀ 5. In the case of the wild type strain expressing plasmid-encoded *fimA*, the agglutination
 371 took approximately 2-5 min under this conditions.. As a negative control, 10 µl of a 3% (w/v)
 372 solution of α-methyl-mannoside was added to a 10 µl suspension of wild type *E. coli* and pre
 373 incubated for 1 minute before agglutination.

374

375 **RNA isolation.** To isolate RNA we used the modified method described by Blomberg *et al.* (51)
 376 with one further modification. Namely, we grew cells in the same conditions as for agglutination
 377 assays (overnight on Luria agar plates) on a lawn. The equivalent to half a plate was scraped and
 378 dissolved immediately in DEPC treated water with 10% SDS, 20 mM sodium acetate pH 4.8 and
 379 10 mM EDTA and flash frozen in liquid nitrogen. Frozen cells were thawed and lysed by incubating
 380 the samples for 5 min at 65 °C. Total RNA was extracted by the hot-phenol method (51). Residual
 381 DNA was digested by DNase I (ThermoFisher; 1 U/µg RNA, 60 min, 37 °C) in the presence of
 382 RNase inhibitor (Ribolock, ThermoFisher Scientific; 0.1 U/µl) followed by a cleaning step using
 383 phenol/chloroform/isoamyl alcohol (25:24:1) and precipitation with ethanol, 0.1 M sodium acetate
 384 pH 5.5 and 20 µg of glycogen (ThermoFisher). Removal of residual DNA was verified by PCR
 385 using *fimA* oligos. Complementary DNAs (cDNAs) were prepared from each RNA sample using the
 386 RevertAid kit (ThermoFisher) according to the manufacturer instructions. As a control of DNA
 387 contamination we prepare for each sample a sample without reverse transcriptase (-RT sample).

388

389 **qPCR analysis.** To perform qPCR we used SYBR Green master mix (ThermoFisher) according to
 390 the manufacturer instructions. To analyse the expression of *fimA*, *fimI*, *hcaT* and *cysG*, we

391 designed specific oligos using primer BLAST (Table 2). We used an iCycler iQ5 (Bio-Rad) and for
 392 each gene cDNAs generated from bacteria grown on plates and as a control we produced a
 393 sample without reverse transcriptase (-RT) using the exact same RNA. We verified that for each
 394 qPCR experiment we did not find any detectable signal in the -RT sample. Denaturation curves
 395 were also acquired to verify the presence of a single product. We performed these controls for
 396 every single biological replicate. To verify the linearity of each primer we diluted the cDNA obtained
 397 from wild type *E. coli*, which always contained the highest amounts of *fimA* and *fimI* and verified
 398 that 10 times dilutions steps had a slope close to -3.3. To analyse qPCR data we used the iQ5
 399 optical system software (Bio-Rad) obtained the ΔCt from the suggested values of the software. We
 400 verified in any case manually that changes in the threshold did not affect or bias the results. Each
 401 measurement was quantified using the following formula: $\Delta Ct = 2^{(ct1-ct2)}$, where *ct* is the threshold
 402 crossing value at which the SYBR green fluorescent signal appears. In the case of the *fimI* to *fimA*
 403 ratio reported in Fig. 5A we simply report the ΔCt without any transformation. Although *Ct* values
 404 varied across experiments, the *fimI* to *fimA* ΔCt was, in our hands, consistent across experiments
 405 within one order of magnitude. To normalize the data of *fimA* and *fimI* against housekeeping genes
 406 we obtained the ΔCt of each sample against the *ct* value of *cysG* and *hcaT* and the average of the
 407 two values was obtained. The average ΔCt values of each sample was normalized against the ΔCt
 408 of the wild type strain as a percentage. The ratio of the *fimI* to *fimA* expression was obtained by
 409 dividing their normalized ΔCt values and expressed as a percentage.

410

411 **AFM.** To prepare AFM samples, punch-holed 1 cm pieces of muscovite mica were immobilized on
 412 metal supports using double sided tape. The static cultures were diluted ten times in ultrapure
 413 milliQ water. A drop of 100 μ l from the diluted bacterial suspension was afterwards placed on the
 414 mica right after cleaving it with laboratory tape. The suspension was incubated for 20 minutes to
 415 allow adhesion of bacteria and the liquid was exchanged three times with ultrapure milli Q water.
 416 The samples were placed in the chemical hood and left to dry for a minimum of 5 min. The
 417 presence of bacteria on the mica was verified using an optical system mounted on the AFM

418 (Bruker) and a random area was selected for imaging. The AFM imaging was performed in a
 419 MultiMode8 instrument (Bruker) in force tapping mode using the ScanAsyst software at a rate of 1
 420 Hz and using ScanAsyst tips with a nominal spring constant of 0.4 N/m. Images shown in Fig. 6
 421 depict the error signal generated by the piezo (J scanner).

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423

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431 References

- 432 1. Fondi M, Emiliani G, Fani R. Origin and evolution of operons and metabolic pathways.
433 Research in Microbiology. 2009 Sep 1;160(7):502–12.
- 434 2. Burkhardt DH, Rouskin S, Zhang Y, Li G-W, Weissman JS, Gross CA. Operon mRNAs are
435 organized into ORF-centric structures that predict translation efficiency. Elife.
436 2017;6:e22037.
- 437 3. Merino E, Yanofsky C. Transcription attenuation: a highly conserved regulatory strategy
438 used by bacteria. Trends in Genetics. 2005 May 1;21(5):260–4.
- 439 4. Goodson JR, Winkler WC. Processive Antitermination. Microbiology Spectrum. 2018 Oct
440 5;6(5):RWR-0031-2018.
- 441 5. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial
442 genomes using CRISPR-Cas systems. Nature biotechnology. 2013 Mar;31(3):233–239.
- 443 6. Zerbini F, Zanella I, Fraccascia D, König E, Irene C, Frattini LF, et al. Large scale validation
444 of an efficient CRISPR/Cas-based multi gene editing protocol in *Escherichia coli*. Microbial
445 cell factories. 2017;16(1):68.
- 446 7. Chen Y-J, Liu P, Nielsen AAK, Brophy JAN, Clancy K, Peterson T, et al. Characterization of
447 582 natural and synthetic terminators and quantification of their design constraints. Nature
448 Methods. 2013 Jun;10(7):659–664.
- 449 8. Wu G, Yan Q, Jones JA, Tang YJ, Fong SS, Koffas MAG. Metabolic Burden: Cornerstones
450 in Synthetic Biology and Metabolic Engineering Applications. Trends in Biotechnology.
451 2016 Aug 1;34(8):652–64.
- 452 9. Russell JB, Cook GM. Energetics of bacterial growth: balance of anabolic and catabolic
453 reactions. Microbiol Mol Biol Rev. 1995 Mar 1;59(1):48–62.
- 454 10. Solomon KV, Moon TS, Ma B, Sanders TM, Prather KLJ. Tuning Primary Metabolism for
455 Heterologous Pathway Productivity. ACS Synth Biol. 2013 Mar 15;2(3):126–35.
- 456 11. Kuwahara H, Alazmi M, Cui X, Gao X. MRE: a web tool to suggest foreign enzymes for the
457 biosynthesis pathway design with competing endogenous reactions in mind. Nucleic Acids

- Res. 2016 Jul 8;44(W1):W217–25.
12. Cress BF, Toparlak ÖD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, et al. CRISPathBrick: modular combinatorial assembly of type II-A CRISPR arrays for dCas9-mediated multiplex transcriptional repression in *E. coli*. ACS synthetic biology. 2015;4(9):987–1000.
13. Su T, Liu F, Gu P, Jin H, Chang Y, Wang Q, et al. A CRISPR-Cas9 Assisted Non-Homologous End-Joining Strategy for One-step Engineering of Bacterial Genome. Scientific Reports. 2016 Nov 24;6(1):1–11.
14. Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, et al. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. Nat Biotechnol. 2014 Nov;32(11):1146–50.
15. Duguid JP, Smith IW, Dempster G, Edmunds PN. Non-flagellar filamentous appendages (“fimbriae”) and haemagglutinating activity in *Bacterium coli*. The Journal of pathology and bacteriology. 1955;70(2):335–348.
16. Jones CH, Pinkner JS, Roth R, Heuser J, Nicholes AV, Abraham SN, et al. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. Proceedings of the National Academy of Sciences. 1995;92(6):2081–2085.
17. Krogfelt KA, Bergmans H, Klemm P. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. Infection and immunity. 1990;58(6):1995–1998.
18. Collier WA, De Miranda JC. [Bacterial hemagglutination. III. Mannose inhibition of *E. coli* hemagglutination]. Antonie Van Leeuwenhoek. 1955;21(2):133–40.
19. Hultgren SJ, Porter TN, Schaeffer AJ, Duncan JL. Role of type 1 pili and effects of phase variation on lower urinary tract infections produced by *Escherichia coli*. Infection and immunity. 1985;50(2):370–377.
20. Wright KJ, Seed PC, Hultgren SJ. Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. Cellular microbiology.

- 2007;9(9):2230–2241.
21. Spaulding CN, Klein RD, Ruer S, Kau AL, Schreiber HL, Cusumano ZT, et al. Selective depletion of uropathogenic *E. coli* from the gut by a FimH antagonist. *Nature*. 2017 22;546(7659):528–32.
22. Korea C-G, Badouraly R, Prevost M-C, Ghigo J-M, Beloin C. *Escherichia coli* K-12 possesses multiple cryptic but functional chaperone–usher fimbriae with distinct surface specificities. *Environmental Microbiology*. 2010;12(7):1957–77.
23. Sohanpal BK, El-Labany S, Lahooti M, Plumbridge JA, Blomfield IC. Integrated regulatory responses of fimB to N-acetylneuraminic (sialic) acid and GlcNAc in *Escherichia coli* K-12. *PNAS*. 2004 Nov 16;101(46):16322–7.
24. Gally DL, Rucker TJ, Blomfield IC. The leucine-responsive regulatory protein binds to the fim switch to control phase variation of type 1 fimbrial expression in *Escherichia coli* K-12. *Journal of Bacteriology*. 1994 Sep 1;176(18):5665–72.
25. Kroner GM, Wolfe MB, Freddolino PL. *Escherichia coli* Lrp regulates one-third of the genome via direct, cooperative, and indirect routes. *Journal of bacteriology*. 2019;201(3):e00411–18.
26. O’gara JP, Dorman CJ. Effects of local transcription and H-NS on inversion of the fim switch of *Escherichia coli*. *Molecular Microbiology*. 2000;36(2):457–66.
27. Eisenstein BI, Sweet DS, Vaughn V, Friedman DI. Integration host factor is required for the DNA inversion that controls phase variation in *Escherichia coli*. *PNAS*. 1987 Sep 1;84(18):6506–10.
28. Åberg A, Shingler V, Balsalobre C. (p) ppGpp regulates type 1 fimbriation of *Escherichia coli* by modulating the expression of the site-specific recombinase FimB. *Molecular microbiology*. 2006;60(6):1520–1533.
29. Yan M-Y, Yan H-Q, Ren G-X, Zhao J-P, Guo X-P, Sun Y-C. CRISPR-Cas12a-Assisted Recombineering in Bacteria. *Appl Environ Microbiol*. 2017 Sep;83(17):00947-17.
30. Bravo V, Puhar A, Sansonetti P, Parsot C, Toro CS. Distinct mutations led to inactivation of

- type 1 fimbriae expression in *Shigella spp.* PLoS ONE. 2015;10(3):e0121785.
31. Zhou K, Zhou L, Lim Q 'En, Zou R, Stephanopoulos G, Too H-P. Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. BMC Mol Biol. 2011 Apr 23;12:18.
32. Blomfield IC, McClain MS, Princ JA, Calie PJ, Eisenstein BI. Type 1 fimbriation and fimE mutants of *Escherichia coli* K-12. Journal of Bacteriology. 1991 Sep 1;173(17):5298–307.
33. Van Dien S. From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. Current Opinion in Biotechnology. 2013 Dec 1;24(6):1061–8.
34. Cao H, Ma Q, Chen X, Xu Y. DOOR: a prokaryotic operon database for genome analyses and functional inference. Brief Bioinform. 2019 Jul 19;20(4):1568–77.
35. Che D, Li G, Mao F, Wu H, Xu Y. Detecting uber-operons in prokaryotic genomes. Nucleic Acids Res. 2006 Apr 1;34(8):2418–27.
36. Richards J, Belasco JG. Obstacles to Scanning by RNase E Govern Bacterial mRNA Lifetimes by Hindering Access to Distal Cleavage Sites. Molecular Cell. 2019 Apr 18;74(2):284-295.e5.
37. Laurberg M, Asahara H, Korostelev A, Zhu J, Trakhanov S, Noller HF. Structural basis for translation termination on the 70S ribosome. Nature. 2008 Aug;454(7206):852–7.
38. Scolnick E, Tompkins R, Caskey T, Nirenberg M. Release factors differing in specificity for terminator codons. Proc Natl Acad Sci U S A. 1968 Oct;61(2):768–74.
39. Hayes CS, Sauer RT. Cleavage of the A Site mRNA Codon during Ribosome Pausing Provides a Mechanism for Translational Quality Control. Molecular Cell. 2003 Oct 1;12(4):903–11.
40. Gally DL, Bogan JA, Eisenstein BI, Blomfield IC. Environmental regulation of the fim switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: effects of temperature and media. Journal of bacteriology. 1993;175(19):6186–6193.
41. Båga M, Göransson M, Normark S, Uhlin BE. Processed mRNA with differential stability in

- the regulation of *E. coli* pilin gene expression. *Cell*. 1988 Jan 29;52(2):197–206.
42. Old DC, Duguid JP. Selective Outgrowth of Fimbriate Bacteria in Static Liquid Medium. *Journal of Bacteriology*. 1970 Aug 1;103(2):447–56.
43. Guyer MS, Reed RR, Steitz JA, Low KB. Identification of a sex-factor-affinity site in *E. coli* as $\gamma\delta$. In: Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press; 1981. p. 135–140.
44. Labun K, Montague TG, Gagnon JA, Thyme SB, Valen E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res*. 2016 Jul 8;44(W1):W272–6.
45. König E, Zerbini F, Zanella I, Fraccascia D, Grandi G. Multiple Stepwise Gene Knockout Using CRISPR/Cas9 in *Escherichia coli*. *Bio-protocol*. 2018;8(2):e2688.
46. Gonzales MF, Brooks T, Pukatzki SU, Provenzano D. Rapid Protocol for Preparation of Electrocompetent *Escherichia coli* and *Vibrio cholerae*. *JoVE (Journal of Visualized Experiments)*. 2013 Oct 8;(80):e50684.
47. Gruber AR, Lorenz R, Bernhart SH, Neuböck R, Hofacker IL. The Vienna RNA Websuite. *Nucleic Acids Res*. 2008 Jul 1;36(suppl_2):W70–4.
48. Kerpedjiev P, Hammer S, Hofacker IL. Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. *Bioinformatics*. 2015 Oct 15;31(20):3377–9.
49. Müller CM, Åberg A, Strasevičiene J, Emödy L, Uhlin BE, Balsalobre C. Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP. *PLoS pathogens*. 2009;5(2):e1000303.
50. Sansonetti PJ, Kopecko DJ, Formal SB. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infection and immunity*. 1982;35(3):852–860.
51. Blomberg P, Wagner EG, Nordström K. Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically in vivo and in vitro by RNase III. *The EMBO journal*. 1990;9(7):2331–2340.

566 **Table 2 Strains used in this study.**

567

Name	Strain	Reference
Wild type	<i>Escherichia coli</i> MG1655	Guyer <i>et al.</i>
<i>S. flexneri</i>	<i>Shigella flexneri</i> 5A M90T	Sansonetti <i>et al.</i>
Stop1	<i>Escherichia coli</i> fimA _{106T}	This study
Stop2	<i>Escherichia coli</i> fimA _{T103A,T104A}	This study
Stop3	<i>Escherichia coli</i> fimA _{A158T,A159T,A160T,C161A,C162A}	This study
fimA	<i>Escherichia coli</i> fimA ₇₆₋₁₇₂	This study
Term1	<i>Escherichia coli</i> fimA _{75::L3S2P56}	This study
Term2	<i>Escherichia coli</i> fimA _{1::L3S2P56}	This study

569 **Table 3. Oligos used in this study.**

570

Name	Sequence	Annotation
17-0001	GGCAATCGTTGTTCTGTGCGG	FimA forward
17-0002	GAAGGTCGCATCCGCATTAG	FimA reverse
17-0003	GGCTCTGTCCCTCAGTTCTACAGCGGCTCTGGCCGCTGC CtaaGACAGGTTTCGTACCGCATCGCTGGCACAGGAAGGA GCAAC	FimaA deletion donor oligo forward
17-0004	GTTGCTCCTTCCTGTGCCAGCGATGCGGTACGAACCTGT CttaGGCAGCGGCCAGAGCCGCTGTAGAACTGAGGGACA GAGCC	FimA deletion donor oligo reverse
17-0005	TGCCACGACGGTTAATGGTGGGACCGTTCACTTTTAAGG GGAAGTTGTTAACGCCGCTTGCGCAGTTGAT	fimA mutant 1 stop codon crRNA1
17-0006	ATCAACTGCGCAAGCGGCGTTAACTTCCCCTTAAAA GTGAACGGTCCCACCATTAAACCGTCGTGGCA	fimA mutant 1 stop codon crRNA1
17-0007	CTGCCACGACGGTTAATGGTGGGACCGTTCACTAAAAAG GGGAAGTTGTTAACGCCGCTTGCGCAGTTGA	fimA mutant 2 stop codon crRNA2
17-0008	TCAACTGCGCAAGCGGCGTTAACTTCCCCTTTTATAG TGAACGGTCCCACCATTAAACCGTCGTGGCAG	fimA mutant 2 stop codon crRNA2
17-0009	GCCGCTTGCGCAGTTGATGACGGCTCTGTTGATCTTTAA GTTTCAAGTTAGGACAGGTTTCGTACCGCATCGC	fimA mutant 3 stop codon crRNA3
17-0010	GCGATGCGGTACGAACCTGTCTTAAGTAACTTAAAGAT CAACAGAGCCTGCATCAACTGCGCAAGCGGC	fimA mutant 3 stop codon crRNA3
17-0013	TTAGAAATAGTTTTTTTGAAGGAAAGCAGCCTCGGTACC AAATTTTCGAAAAAGACGCTGAAAAGCGTCTTTTTTCG TTTTGGTCCGACAGGTTTCGTACCGCATCGCTGGCACAGG	fimA terminator2 at ATG forward
17-0014	CCTGTGCCAGCGATGCGGTACGAACCTGTTCGGACCAAAA CGAAAAAAGACGCTTTTTCAGCGTCTTTTTTCGAAAATTT GGTACCGAGGCTGCTTTCTTTCAAAAACTATTTCTAA	fimA terminator2 at ATG reverse
17-0015	CCTGTGCCAGCGATGCGGTACGAACCTGTTCGGACCAAAA CGAAAAAAGACGCTTTTTCAGCGTCTTTTTTCGAAAATTT GGTACCGAGGGCAGCGGCCAGAGCCGCTGTAGAACTGAG	fimA terminator2 inside forward
17-0016	CCTGTGCCAGCGATGCGGTACGAACCTGTTCGGACCAAAA CGAAAAAAGACGCTTTTTCAGCGTCTTTTTTCGAAAATTT GGTACCGAGGGCAGCGGCCAGAGCCGCTGTAGAACTGAG	fimA terminator2 inside reverse
17-0017	tagataAGGGGAAGTTGTTAACGCCGCTT	fimA crRNA2 BsaI fw
17-0018	agacAAGCGGCGTTAACTTCCCCTTa	fimA crRNA2 BsaI rev
17-0019	tagataATCAACAGAGCCTGCATCAACTGC	fimA crRNA3 BsaI fw
17-0020	agacGCAGTTGATGACAGGCTCTGTTGATa	fimA crRNA3 BsaI rev
17-0021	tagataAAGTGAACGGTCCCACCATTAAACC	fimA crRNA1BsaI fw
17-0022	agacGGTTAATGGTGGGACCGTTCACTTa	fimA crRNA1 BsaI rev
17-0042	CTGGAACCGTAACGCAGACT	FimA 2 Fw for terminator genotyping
17-0043	AGGCAACAGCGGCTTTAGAT	FimA 2 Rv for terminator genotyping
18-0012	ATCTTTTGGGGGAAACTGTGC	qPCR fimA Fw 90 bp recognizes all mutants
18-0013	ATTTCTAAATCGACATGGGCAGT	qPCR fimA Rv 90 bp recognizes all mutants
17-0067	TGCTGCCAATGTTTGCTCTG	fimI qPCR Fwd 233pb
17-0068	TTCACTCACCACCGTGCTAC	fimI qPCR Rev
17-0073	tacgCCAAGCTTTGAAAGGAAAGCAGCATGAAAATTAAA AC	fimA-FW cloning pSU19 HindIII EcoRI
17-0074	gccagtGAATTCTTATTGATACTGAACCTTGAAGGTCTG	fimA-rv cloning pSU19 HindIII EcoRI
17-0077	tagatCGTCGCCGTCCAGCTCGACCAGGA	crRNA BsaI GFP control fw
17-0078	agacTCTGGTCGAGCTGGACGGCGACGa	crRNA BsaI GFP control rv

571

Figure 1

Fig 1. Design of Cas12a-dependent mutagenesis of the *fimAICDFGH* operon. A) The *E. coli* *fim* operon is transcribed from a promoter (*fimAp*) containing an invertible element (grey dotted line) and *fimA* codes for the major fimbrial subunit, which was targeted by Cas12a. B) Enhanced view of the *fim* transcript from the +1 transcriptional start site. The regions amplified for qPCR analysis in the *fimA* and *fimI* genes are shown together with the homologous positions to the three crRNAs used in this study. A stop codon was introduced to substitute the PAM sequence to generate a stop codon mutant (*fimA*_{A106T}). C) Scheme of the 97 bp *fimA* deletion (Δ *fimA*) generated with a dsDNA donor oligo bearing 40 bp of homology. D) Scheme of the 54 bp *fimA* terminator insertion generated with a dsDNA donor oligo bearing a terminator sequence surrounded by 30 bp of homology. Two different insertions were generated: the first was generated at the site of the deletion shown in C (*fimA*_{75::L3S2P56}) and the second at the position of the start codon (*fimA*_{1::L3S2P56}) of the *fimA* gene. E) Secondary structure prediction of the L3S2P56 terminator used to create the mutants shown in D.

Figure 2

Fig. 2. Detection of *fimA* mutants by electrophoresis of PCR products. Lanes 1, 3, 5, show a 514 bp PCR product generated by primers that amplify a segment of the wild type (WT) or mutant (Mut) *fimA* gene. Lane 2 shows the unchanged migration pattern of the PCR product amplifying the stop codon mutant (*fimA*_{A106T}). Lanes 4 and 6 show the PCR product generated after amplifying DNA from the deletion mutant (Δ *fimA*, 420 bp) and the internal terminator insertion (Ter1, 474 bp), respectively. Lanes 7 show a 988 bp PCR product generated by primers that amplify a segment of wild type *fimA* including the leader sequence and lane 8 shows the 870 bp PCR product generated with DNA from the second terminator insertion (Ter2).

Supplementary Figure 1

Supplementary Fig 1. Efficiency of *fimA* mutant generation with Cas12a. A) 10/10 *E. coli* colonies co-transformed with crRNA1 and the donor oligo carrying a 97 bp *fimA* deletion tested positive for a mutation as detected by colony PCR. B) 3/10 *E. coli* colonies co-transformed with crRNA1 and the donor oligo carrying a 54 bp *fimA* terminator insertion tested positive as detected by colony PCR (marked with *). C) 1/10 *E. coli* colonies co-transformed with crRNA1 and the donor oligo carrying a 54 bp terminator insertion at the *fimA* start codon tested positive as detected by colony PCR (marked with *).

Figure 3

Fig 3. Agglutination assay of *fimA* mutants. A) A suspension of *Saccharomyces cerevisiae* was spread on glass plate. B) The strain to be tested was mixed. The FimH fimbriae subunit recognizes mannose residues on the surface of yeast cells. C) The polyvalent binding of fimbriae to cells causes an agglutination chain reaction, which is macroscopically visible. Agglutination reaction for the following strains: D) WT *E. coli*, E) *Shigella flexneri* M90T (negative control), F) Stop codon mutant (*fimA*_{A106T}), G) Δ *fimA*, H) *fimA*_{75::L3S2P56} and I) *fimA*_{1::L3S2P56}. Scale bar 2 cm.

Figure 4

Fig 4. Plasmid complementation of *fimA* mutants. *fimA* was expressed from a plasmid under the *lac* promoter (orange). The empty vector, pSU19, was used as negative control. Agglutination reaction for the following strains: A) WT *E. coli* + empty vector, B) WT *E. coli* + *fimA* plasmid, C) *fimA*_{A106T} + empty vector, D) *fimA*_{A106T} + *fimA* plasmid, E) Δ *fimA* + empty vector, F) Δ *fimA* + *fimA* plasmid, G) Term1 (*fimA*_{75::L3S2P56}) + empty vector, H) Term1(*fimA*_{75::L3S2P56}) + *fimA* plasmid, I) Term2 (*fimA*_{1::L3S2P56}) + empty vector, J) Term2 (*fimA*_{1::L3S2P56}) + *fimA* plasmid. Scale bar 2 cm.

Figure 5

Fig 5. Quantitative PCR analysis of *fimA* and *fimI* expression. Two PCR product were used to quantify the expression of *fimA* and *fimI* (see scheme in Fig. 1). A) *fimI* expression normalized to *fimA* expression. Each data point represents a biological replicate and the median is shown (—). The error bars show the 25th and 75th percentile. B) Same data as in A) but represented as median ΔCt expression of *fimA* and *fimI* normalized to the housekeeping genes *hcaT* and *cysG* and expressed as percent of the wild type. Orange and yellow data points represent the stop codon mutants generated with crRNA1 and crRNA2 as in A. The median ratio of the normalized *fimI*/*fimA* expression is shown. The error bars show the 25th and 75th percentile. The labels represent the following mutants: Stop (*fimA*_{A106T}), deletion mutant ($\Delta fimA$), Ter1 (*fimA*_{75::L3S2P56}), Ter2 (*fimA*_{1::L3S2P56}).

Figure 6

Figure 6. Atomic force microscopy (AFM) and agglutination of static cultures. A) AFM imaging of wild-type *E. coli* + *fimA* plasmid grown in static conditions. A cell showing fimbriae is shown in the inset. B) AFM imaging of Term2 mutant of *E. coli* (*fimA*₁::L3S2P56) + *fimA* plasmid grown in static conditions. A cell with flagella is shown as an inset. C) Yeast agglutination assay of wild-type *E. coli* + *fimA* plasmid grown in static conditions. D) Yeast agglutination assay of of wild-type *E. coli* + *fimA* plasmid grown in static conditions in the presence of α -methyl-mannoside. E) Yeast agglutination assay of Term2 mutant of *E. coli* (*fimA*₁::L3S2P56) + *fimA* plasmid grown in static conditions. Scale bars: A-B) 4 μ m. A-B) insets 1 μ m. C-E) 2 cm.