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## What's a SNP between friends: The influence of single nucleotide polymorphisms on virulence and phenotypes of *Clostridium difficile* strain 630 and derivatives.

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1 **What's a SNP between friends: the influence of single nucleotide polymorphisms on**  
2 **virulence and phenotypes of *Clostridium difficile* strain 630 and derivatives**

3

4 **Running Title:** What's a SNP between Friends?

5

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8

9

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19

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21

22 **Keywords:** single nucleotide polymorphism; sporulation; motility; toxin expression;  
23 mutation; Clostron; virulence

24 **Abstract**

25

26 *Clostridium difficile* is a major cause of antibiotic induced diarrhoea worldwide,  
27 responsible for significant annual mortalities and represents a considerable economic  
28 burden on healthcare systems. The two main *C. difficile* virulence factors are toxins A and  
29 B. Isogenic toxin B mutants of two independently isolated erythromycin-sensitive  
30 derivatives (630E and 630 $\Delta$ erm) of strain 630 were previously shown to exhibit  
31 substantively different phenotypes. Compared to 630, strain 630E and its progeny grow  
32 slower, achieve lower final cell densities, exhibit a reduced capacity for spore-formation,  
33 produce lower levels of toxin and are less virulent in the hamster infection model. By the  
34 same measures, strain 630 $\Delta$ erm and its derivatives more closely mirror the behaviour of  
35 630. Genome sequencing revealed that 630 $\Delta$ erm had acquired seven unique Single  
36 Nucleotide Polymorphisms (SNPs) compared to 630 and 630E, while 630E had nine SNPs  
37 and a DNA inversion not found in the other two strains. The relatively large number of  
38 mutations meant that the identification of those responsible for the altered properties of  
39 630E was not possible, despite the restoration of three mutations to wildtype by allelic  
40 exchange and comparative RNAseq analysis of all three strains. The latter analysis revealed  
41 large differences in gene expression between the three strains, explaining in part why no  
42 single SNP could restore the phenotypic differences. Our findings suggest that strain  
43 630 $\Delta$ erm should be favoured over 630E as a surrogate for 630 in genetic-based studies.  
44 They also underline the importance of effective strain curation and the need to genome re-  
45 sequence master seed banks wherever possible.

46

## 47 Introduction

48

49 *Clostridium difficile* is a Gram-positive, anaerobic spore-forming bacterium capable of  
50 causing a range of diseases from mild diarrhoea to potentially fatal toxic  
51 pseudomembranous colitis. The toxigenic effects of *C. difficile* are caused by the activities  
52 of two large, glucosylating toxins. The two toxins are 308kDa (toxin A) and 270kDa (toxin B)  
53 in size<sup>1-3</sup> and are encoded by the chromosomally located genes *tcdA* and *tcdB*,  
54 respectively. Both are cytopathic to cultured cells due to disruption of the cytoskeleton,  
55 although TcdB is thought to be up to 1000-times more potent.<sup>1</sup> Historically, toxin A was  
56 regarded as the main causative agent of the symptoms of *C. difficile* infection (CDI). Pivotal  
57 data was provided by Lyerly et al.<sup>4</sup> who were only able to detect disease when hamsters  
58 were subject to intragastric challenge with purified TcdA alone and not with TcdB. The  
59 latter could, however, cause disease symptoms if prior damage to the mucosa had been  
60 inflicted by co-administration of sub-lethal concentrations of toxin A. Furthermore, co-  
61 administration of both toxins led to more severe disease symptoms. To accommodate  
62 these data, it was generally accepted that both toxins acted in concert to bring about  
63 disease symptoms, with toxin A leading to the initial damage to the colon allowing the  
64 subsequent access of the more potent toxin B.

65 During the 1990's *C. difficile* strains were isolated from symptomatic patients that only  
66 produced toxin B (A-B+)<sup>5,6</sup>. These findings suggested that toxin B, at least in certain strains,  
67 is capable of causing disease without the help of toxin A. It has been reported since that  
68 toxin B, in A-B+ strains, is modified and seems to be an evolutionary hybrid of *C. difficile*  
69 toxin B and *Clostridium sordellii* lethal toxin.<sup>7</sup>

70 With the development of genetic systems, assumptions of the relative importance of  
71 the two toxins could be tested through the creation, and *in vivo* assay, of isogenic mutants  
72 in which production of either toxin had been ablated. Initial findings made by Lyras et al.<sup>8</sup>  
73 appeared to turn the perceived view on its head, through the demonstration that a *tcdA*  
74 mutant producing TcdB alone (A-B+) was capable of causing disease in the hamster model  
75 while a *tcdB* mutant producing only TcdA (A+B-) did not. These data were, however,  
76 almost immediately questioned by a second study conducted in the Minton laboratory<sup>9</sup>  
77 showing that both *tcdA* and *tcdB* *C. difficile* mutants, and therefore TcdA and TcdB alone,  
78 were independently capable of causing disease. Interestingly, a strain has recently<sup>10</sup> been  
79 isolated from a clinical case of CDI, that only produces TcdA (A+B-)

80 The possible reasons for the observed difference in outcomes of the two studies have  
81 been discussed previously.<sup>11</sup> Both studies agree on the virulence potential of toxin B, but  
82 uncertainties remain about the different outcomes concerning the effects of toxin A. In the  
83 work presented here, we have hence focused on comparisons of the parental strains and  
84 the strains only producing toxin A (A+B-). In essence, both sets of mutants were generated  
85 by insertional inactivation of the toxin genes of the *C. difficile* strain 630<sup>12</sup> and, once  
86 created, were tested in the hamster infection model. However, in order to implement the  
87 available gene tools in strain 630 (at the time the only strain for which a genome sequence  
88 was available), it was necessary to first isolate a variant that had become sensitive to  
89 erythromycin, thereby allowing the use of an *ermB* gene as a selective, genetic marker.  
90 Both studies used such an erythromycin-sensitive derivative of strain 630, but they were  
91 independently isolated. In our study (Minton group),<sup>9</sup> we used the strain 630 $\Delta$ *erm*,  
92 isolated in the Mullany laboratory (UCL, London, UK) after 30 repeated subcultures of

93 strain 630 in non-selective media.<sup>13</sup> In parallel, the Rood laboratory (Monash, Australia)  
94 independently isolated the erythromycin sensitive strain JIR8094 (also referred to as 630E),  
95 <sup>14</sup> through an undisclosed number of subcultures of strain 630 in non-selective media. Both  
96 strains are reported to possess the same specific deletion of *ermB*.<sup>13,14</sup>

97 We have previously hypothesised<sup>11</sup> that the different outcomes of the two studies<sup>8,9</sup>  
98 are a direct consequence of the use of the two, independently isolated erythromycin-  
99 sensitive strains, 630 $\Delta$ *erm* and 630E. We suggested that during repeated subculture,  
100 ancillary mutations arose which impacted on the virulence potential of one or other of the  
101 two strains in the presence of different toxin gene alleles. In the current piece of work, we  
102 have set out to test this hypothesis. We have undertaken side-by-side comparisons of  
103 630 $\Delta$ *erm* and 630E, and the A+B- mutant derivatives, in a variety of assays to establish  
104 phenotypic differences. In parallel, we have determined the genome sequences of the  
105 various strains used in the two studies.<sup>8,9</sup> Then, we have used our newly developed allelic  
106 exchange methodologies<sup>15</sup> to correct a number of SNPs in strain 630E back to wild-type  
107 and assessed the consequences. Furthermore we have performed RNAseq experiments  
108 comparing the transcriptome of 630, 630 $\Delta$ *erm* and 630E at three different time points. The  
109 RNA data were related to the whole genome data to draw our final conclusions.

110

## 111 **Results**

### 112 **Generation of a ClosTron insertion in *tcdB* of 630E**

113 Although considered unlikely, the possibility existed that mutants made by the  
114 insertion of a plasmid element carrying *ermB*<sup>14</sup> might behave differently to an equivalent  
115 mutant made by the insertion of a group II intron incorporating *ermB*.<sup>16</sup> Our initial step

116 was, therefore, to create a *tcdB* mutant of strain 630E using Clostron technology.  
117 Accordingly, the Clostron plasmid pMTL007C-E2::Cdi-tcdB-1511a that had previously been  
118 used to generate strain 630 $\Delta$ *erm* A+B-<sup>9</sup> was used to create an equivalent mutant in strain  
119 630E as described.<sup>9</sup> The resulting mutant, 630E A+B-CT, was verified by PCR, Sanger  
120 sequencing and shown by Southern blot to carry a single group II intron insertion (**Fig.**  
121 **S1A**). Parental strains, original A+B- mutants and the newly obtained mutant were tested  
122 for production of toxin A in a Western blot (**Fig. S1B**). As expected all strains produced  
123 toxin A.

124

#### 125 **Phenotypic characterisation of strains**

126 In order to establish whether all strains were phenotypically identical a range of assays  
127 were performed, comparing growth, motility and spore properties. An analysis of growth  
128 rates using the procedure described in Materials and Methods showed that strain 630 $\Delta$ *erm*  
129 and derivatives grew to the highest optical density, closely followed by strain 630 and  
130 630E, and derivatives thereof (**Fig. 1A and 1B**). The data clearly demonstrated that strain  
131 630 $\Delta$ *erm* and its 630 $\Delta$ *erm* A+B- derivative had relatively higher growth rates and achieved  
132 higher optical densities ( $p < 0.0001$ , unpaired t-test at 24 h) than strain 630E and its  
133 derivatives, with strain 630E A+B-CT growing the least (**Fig. 1B**). It was also apparent,  
134 shown by plate motility assay (**Fig. 2**), that strains 630 and 630 $\Delta$ *erm* were motile, while  
135 630E was not. Only 630 and 630 $\Delta$ *erm*, but not 630E, form pseudopod-like structures,  
136 which are characteristic for swarming motility in bacteria.

137 Following the protocols of Burns et al,<sup>17</sup> comparative differences in the numbers of  
138 colony forming units (CFUs) obtained following heat shock were assessed between the

139 strains, as a crude estimate of spore formation.<sup>18</sup> On this basis, strains 630 $\Delta$ *erm* and  
140 630 $\Delta$ *erm* A+B- produced a greater numbers of spores than 630E and its derivatives, which  
141 failed to produce any spores until 72 h. The total number of CFU/mL at this time point was  
142 10<sup>3</sup> times fewer than that obtained with 630 $\Delta$ *erm* or 630 $\Delta$ *erm* A+B- (**Fig. 3A**). Interestingly  
143 parental strain 630 produced very few spores before 72 h, but spore counts increased from  
144 72 h onwards and reached similar levels to strains 630 $\Delta$ *erm* and 630 $\Delta$ *erm* A+B- by the end  
145 of the experiment. The reduction in spore formation may in part be due to the observed  
146 reduction in OD as the 630E strains enter stationary phase, which might also explain their  
147 predilection to flocculate. Indeed comparing percentage sporulation (relative to vegetative  
148 cell count), confirmed the observation that 630 $\Delta$ *erm* and 630 $\Delta$ *erm* A+B- have a higher  
149 sporulation frequency than both 630E (and derivatives) and strain 630.

150 The germination of the 630E strains was also comparatively reduced and did not reach  
151 the same level as that of 630 $\Delta$ *erm* and its progeny. At the last time point (240 min) strains  
152 630E and 630E A+B- reach the same level of CFU/ml as strain 630. The observed delay  
153 could be due to the previously observed reduced cell growth of the 630E strains (**Fig. 3B**).

154

## 155 **Toxin Production**

156 Measurements of the amounts of toxin being produced by 630E and 630 $\Delta$ *erm* and  
157 their derivatives were undertaken using both the *C. DIFFICILE TOX A/B II*<sup>TM</sup> ELISA assay kit  
158 from TechLab, measuring toxin A and B, and kits from TGCbiomics, specifically measuring  
159 either only toxin A or only toxin B. The results of the 72 h time point are shown in **Fig. 4**.

160 As shown in **Fig. 4A**, toxin production of 630, 630 $\Delta$ *erm* or 630 $\Delta$ *erm* A+B- clustered  
161 together, as did toxin production of 630E, 630E A+B- or 630E A+B- CT with the latter three

162 showing no statistical differences between them ( $P > 0.05$ , one-way ANOVA with Fisher's  
163 LSD test). There was, however, a statistically significant difference between the first three  
164 strains (630, 630 $\Delta$ *erm* or 630 $\Delta$ *erm* A+B-) and the second set of three strains (630E, 630E  
165 A+B- or 630E A+B- CT) ( $P < 0.0001$ ). The *C. DIFFICILE TOX A/B II*<sup>™</sup> ELISA does not  
166 differentiate between toxin A and B. In order to be able to quantify each toxin, the kits  
167 from TGCbiomics were used (**Fig. 4B and 4C**). The toxin A ELISA showed significantly higher  
168 production in strain 630 $\Delta$ *erm* compared to 630E ( $p < 0.0016$ ) and also confirmed the  
169 previous observations that strains with impaired *tcdB*, produce more toxin A<sup>8,9</sup> (**Fig. 4B**).  
170 No toxin B production was seen, as expected, in the *tcdB*-mutants. Strains 630 and  
171 630 $\Delta$ *erm*, however, both produced significantly more toxin B than 630E ( $p < 0.0001$ ) (**Fig.**  
172 **4C**).

173

#### 174 **Whole genome sequencing**

175 To establish whether strains 630E and 630 $\Delta$ *erm*, and derivatives, contained any  
176 additional changes to the *ermB* gene deletion, relative to the parent strain 630, the  
177 following strains were sequenced using Next Generation Sequencing platforms: 630E A+B-,  
178 630E A+B- CT on Illumina HiSeq (GATC, Germany) and 630 $\Delta$ *erm* A-B on a Roche 454  
179 (Deepseq, University of Nottingham, UK) and the data compared to the published genome  
180 of the parental strain 630<sup>12</sup> and previously sequenced 630 $\Delta$ *erm* $\Delta$ *pyrE*.<sup>19</sup> We used a  
181 frequency of 70% as a cut-off for SNP calling and found multiple SNPs, InDels and other  
182 minor changes, both common and unique to 630, 630E and 630 $\Delta$ *erm*. In total, two SNPs in  
183 coding regions with non-synonymous changes were found that were common to all three  
184 strains (in CD630\_11900, encoding an acyl-CoA N-acyltransferase where SNP changes

185 phenylalanine133 to leucine and in CD630\_13880, a pseudo gene where a frameshift is  
186 introduced). In addition to these, we found in both 630 and 630 $\Delta$ erm strains three SNPs  
187 (two in intergenic regions and one in a coding region of CD630\_2667, encoding the BC  
188 domain of a glucose PTS, changing valine228 to isoleucine). 630 $\Delta$ erm had seven unique  
189 changes compared to 630 and 630E (including six non-synonymous SNPs in coding  
190 regions), while 630E had eleven SNPs (with nine non-synonymous SNPs in coding regions)  
191 not found in the other two strains. SNPs were confirmed by Sanger sequencing and  
192 thereafter by RNAseq data (see below). Indeed, the SNPs found in the DNA-seq data were  
193 validated by using the RNA-seq sequence reads mapped on the genome sequence with  
194 Bowtie2<sup>20</sup> with each position being checked using Tablet<sup>21</sup>. A complete list of SNPs and  
195 other small changes are indicated in **Table 1** and in supplementary **Table S1**. During the  
196 preparation of this manuscript a new sequence of 630 was published by Riedel *et al.*<sup>22</sup>. We  
197 incorporated their data into **Table 1** (and **Table S1**). Overall this new sequence shows very  
198 few disparities to the original one. However two SNPs found in our data were attributed to  
199 mistakes in the original sequence (in CD630\_17670 and CD630\_31561). Another paper was  
200 recently published by van Eijk *et al.*,<sup>23</sup> resequencing 630 $\Delta$ erm. Overall there are very few  
201 discrepancies between their data and our findings, confirming the quality of both data sets.  
202 We have incorporated their findings into **Table S1**.

203 It may be assumed, that during the repeated subculture of strain 630 undertaken in  
204 the Mullany<sup>13</sup> and Rood<sup>14</sup> laboratories, sub-populations within the culture were isolated  
205 carrying SNPs. However, it seems improbable that the two SNPs (**Table 1**), common to all  
206 three strains, arose independently. Rather we hypothesise that these SNPs might be  
207 sequencing mistakes. This theory gains weight through the new sequencing data by Riedel

208 *et al.*<sup>22</sup>. Two SNP changes which we identified originally between the published 630  
209 sequence and our data were confirmed by Riedel et al to also be the sequence of their 630  
210 seed stock. Unfortunately the genome announcement<sup>22</sup> does not state the exact source of  
211 their 630 strain. As mentioned above another three SNPs were only found in 630 and  
212 630 $\Delta$ *erm*, two of these are in intergenic regions which showed no expression in our  
213 RNAseq experiment, and the third is located in a PTS gene in 630 and 630 $\Delta$ *erm* (position  
214 3080703, Val<sub>228</sub>Ile). Rather than having occurred independently it is more likely that these  
215 SNPs arose in the Mullany laboratory, subsequent to provision of chromosomal DNA to the  
216 Sanger Centre for determination of the 630 genome sequence<sup>12</sup>, and before the strain 630  
217 was passaged to obtain 630 $\Delta$ *erm*. At the time, *C. difficile* strains in the Mullany laboratory  
218 were routinely stored at 4°C as Robertson's Cooked Meat stocks, as opposed to being  
219 frozen at -80°C in 10% glycerol (A.R. Roberts, personal communication). On this basis, the  
220 traditional microbiological practice of using Robertson's Cooked Meat to curate strains  
221 might not be ideal as strains are not entirely dormant and genome changes can occur over  
222 time. The SNPs that were found to be unique to 630 $\Delta$ *erm* and 630E (n=8 and n=11,  
223 respectively) can be assumed to have been accrued at some point after the two 630  
224 populations diverged, that is when the strain was sent to the Rood laboratory. It is most  
225 likely, although not certain, that the majority, if not all of the strain-specific SNPs arose  
226 during the repeated subculture experiments undertaken to isolate the *ermB* deletion  
227 strains 630E and 630 $\Delta$ *erm*.

228 Changes specific to 630 $\Delta$ *erm* include SNPs in three intergenic regions, which all have  
229 been determined with a coverage of over 150 and 100 % frequency (see **Table 1**). The  
230 other five changes comprise four non-synonymous SNPs and an insertion. The insertion

231 has previously been reported by Rosenbusch *et al.*<sup>24</sup> and was confirmed by van Eijk *et al.*<sup>23</sup>  
232 and is an 18 bp duplication in *spo0A*, the master regulator of sporulation. This insertion  
233 might be responsible for the reduced sporulation frequency seen in strain 630 and also in  
234 630E and derivatives, which do not carry this duplication (which does not have this  
235 duplication) (**Fig. 3A**). The SNPs have been found in the following genes: CD630\_08260,  
236 encoding a ferric uptake regulator (*perR* homologue) (Thr<sub>41</sub>Ala); CD630\_19070, encoding  
237 an alcohol dehydrogenase homologue (*eutG*) (Gly<sub>252</sub>Glu); and CD630\_35630, encoding a  
238 transcriptional regulator of the GntR family (Ala<sub>91</sub>Val).

239 In contrast, strain 630E contains a larger number of non-synonymous SNPs including  
240 changes that result in nonsense mutations and in one case the inversion of a small  
241 segment of DNA preceding a flagella operon. We found two changes in intergenic regions,  
242 one with 100 % frequency and a coverage of 94 (position 3528736); the other at a much  
243 lower frequency (41 %), but confirmed a 150 bp inversion by Sanger sequencing in the  
244 promoter region of *flgB*, the first gene in a F3 flagella operon (early flagella genes). Non-  
245 synonymous SNPs were found in CD630\_07610, encoding a putative RNA helicase  
246 (Asp<sub>136</sub>Tyr); CD630\_14040, encoding an oligopeptide transporter (Glu<sub>536</sub>Gly);  
247 CD630\_20270, encoding a hydrolase (Gly<sub>373</sub>Glu); CD630\_29430, encoding a phage  
248 replication protein (Asn<sub>210</sub>Asp); and CD630\_33790, encoding a conjugative transposon  
249 protein (Glu<sub>63</sub>Asp). Finally, there is another SNP at position 3034953, in gene CD630\_26270  
250 (Gly<sub>68</sub>Cys), encoding a conserved hypothetical protein. Interestingly the new genome  
251 sequence from Eijk *et al.*<sup>23</sup> suggested an “A” at position 3034953 in contrast to the earlier  
252 annotation suggesting “C”. The new annotation is in line with our RNAseq data (**Table S1**)  
253 and taken into account our sequencing data (**Table 1**) suggests that this is indeed a

254 mutation in 630E and was miss-annotated in the original sequence. In one instance the  
255 nucleotide substitution resulted in the creation of a nonsense, stop codon, and as a  
256 consequence a severe, premature truncation of the encoding protein. Thus, the stop codon  
257 introduced into CD630\_12740 encoding a topoisomerase I (*topA*) homologue (Gln<sub>386</sub>\*)  
258 truncated the protein from 695 amino acids to 385 amino acids. Conversely, in the case of  
259 the glucose PTS operon *ptsG-BC*, the conversion of the stop codon of *ptsG-B* gene  
260 (CD630\_26670) to a Glu codon (\*<sub>524</sub>Glu) resulted in its fusion to the coding region of the  
261 immediately downstream *ptsG-C* gene.

#### 262 **Virulence testing of 630Δ*erm*, 630E and mutants using an *in vivo* model**

263 In order to confirm previous data and to rule out differences in experimental set up in  
264 different laboratories, the virulence of 630E and derivatives was assessed using the  
265 hamster infection model in our laboratory (University of Nottingham) as previously  
266 described.<sup>9</sup>

267 **Figure 5** shows the times from infection to endpoint (in days) for the hamsters  
268 infected with 630E, 630E A+B- and 630E A+B- CT. For comparative purposes data for  
269 infection with 630Δ*erm* and 630Δ*erm* A+B- from a previous study<sup>9</sup> is also included. The  
270 latter emphasises the fact that all eight hamsters infected with strain 630Δ*erm* were  
271 colonised and succumbed to *C. difficile* disease (with an average time of 3.25 days from  
272 infection to endpoint). This is in direct contrast to what is observed with 630E where of the  
273 five animals successfully infected, only three were colonised till the respective endpoints  
274 and of these, two succumbed to disease (at day two and six). Two animals lost colonisation  
275 after days 15 and 18, respectively.

276 In our previous study<sup>9</sup> seven, of the eight animals infected with 630 $\Delta$ erm A+B- (as also  
277 shown in **Fig. 5**), succumbed to disease with an average time to death of colonised  
278 hamsters being just under two days. One animal showed no signs of disease until the  
279 experimental endpoint, but was found not to have been colonised. Here of the 11 animals  
280 infected with the equivalent mutant of strain 630E (630E A+B-), only two animals  
281 succumbed to CDI (on day two and nine). Four animals in this group were never colonised,  
282 one lost colonisation after day three and the others were colonised till endpoint. Six  
283 animals were infected with 630E A+B- CT, and of these two hamsters developed infection  
284 (day three and five). Two of the surviving animals lost colonisation after day 15 and 18  
285 respectively. (**Fig. 5** and **Table S2**).

286 The difference between the average time to death of all hamsters administered  
287 630 $\Delta$ erm and 630 $\Delta$ erm A+B- was found not to be statistically significant (one-way ANOVA,  
288  $p=0.5355$ ) (results from Kuehne *et al.*<sup>9</sup>). Similarly, the differences between the average  
289 times to death of all animals administered 630E, 630E A+B- and 630E A+B- CT was not  
290 statistically significant (one-way ANOVA,  $p=0.8919$ ). In contrast, the difference between  
291 the 630 $\Delta$ erm strain (and derivative) and the 630E strain (and derivatives) was statistically  
292 significant (one-way ANOVA,  $p>0.0001$ ).

293

#### 294 **Correction of SNPs in strain 630E**

295 In view of the large number of SNPs present in strain 630E, it was impractical to  
296 change them all back to the 630 parental sequence. We therefore selected just three  
297 specific mutations present in 630E and converted them back to the sequence present in  
298 the parental strain, 630.

299 Our principal target was to remove the stop codon from within the topoisomerase I  
300 gene, CD630\_12740, as this enzyme plays a central role in the regulation of DNA negative  
301 supercoiling and its inactivation is likely to result in extensive pleiotropic effects. Indeed, in  
302 some bacteria its inactivation is lethal.<sup>25-27</sup> Moreover, bacterial genes related to  
303 pathogenesis and virulence have been shown to be sensitive to *topA* mutation in *E. coli*,<sup>28</sup>  
304 *S. flexneri*,<sup>29</sup> *Yersinia enterocolitica*<sup>30</sup> and *Salmonella*.<sup>31</sup> We therefore converted the “T”  
305 nucleotide at position 1480649 in 630E back to an “A” nucleotide, thereby removing the  
306 nonsense stop codon and allowing the production of full length native topoisomerase  
307 enzyme.

308 As a second target we elected to correct the inversion of DNA upstream of the F3  
309 flagella operon. As strain 630E is non-motile, and as the inverted region encompasses the  
310 non-coding region immediately upstream of the *flgB* gene, it is likely to have disrupted the  
311 promoter responsible for both *flgB* expression and the genes in the downstream operon.  
312 The inversion is therefore likely to be the principal cause of the loss of motility in 630E.  
313 Furthermore, factors affecting flagella expression can also influence toxin expression  
314 levels.<sup>32, 33</sup>

315 Finally, we sought to correct the fusion of the two PTS components *ptsG-B* and *ptsG-C*,  
316 by resurrection of the stop codon of *ptsG-B* through the conversion of the “C” nucleotide  
317 at position 3079815 back to an “A” nucleotide. As glucose is known to affect toxin  
318 production, through catabolite repression,<sup>34, 35</sup> it was reasoned that this particular SNP  
319 could be affecting toxin expression, and therefore virulence.

320 The plasmids carrying the 630 wildtype alleles necessary for the correction of the  
321 three targeted SNPs were assembled as described in Materials and Methods and then used

322 to effect the replacement of the 630E mutant alleles by allelic exchange.<sup>15</sup> To verify that  
323 the mutant clones obtained were correct, each targeted region was amplified by PCR using  
324 appropriate oligonucleotide primers and the DNA fragments obtained subjected to Sanger  
325 sequencing on both DNA strands. In every case, clones carrying the desired 'corrected'  
326 sequence were obtained. The new strains were named after the genes or regions that  
327 were corrected, namely 630E\_*topA*, 630E\_CD2667 and 630E\_*flgB*, respectively.

328 To assess the effects of the changes on the characteristics of the mutant strains,  
329 growth rate, sporulation and germination, motility, and *in vitro* cytotoxicity and toxin  
330 production (ELISA) were measured. None of the three corrected mutants exhibited any  
331 difference in growth rate compared to the parental strain 630E (data not shown). Similarly,  
332 sporulation and germination remained unaffected (data not shown). Toxicity testing  
333 revealed no difference to 630E using the *C. DIFFICILE TOX A/B II*<sup>™</sup> ELISA assay kit from  
334 TechLab (**Fig. 4A**). To quantify toxin A and toxin B individually the ELISA kits from  
335 TGCbiomics were used to assay 630E\_*topA* and 630E\_CD2667 (**Fig. 4B**). No differences  
336 were measured for toxin A, but the strain 630E\_*topA* showed significantly higher levels of  
337 toxin B than the parental strain 630E.

338

### 339 **Transcriptomic comparison of 630, 630Δ*erm* and 630E**

340 RNA was extracted from strains 630, 630Δ*erm* and 630E at 6, 14 and 24 h and used in  
341 an RNAseq experiment as described in Materials and Methods. The Principal Component  
342 Analysis (PCA) (**Fig. 6**) showed that strain 630Δ*erm* and 630E are closely correlated on a  
343 transcriptional level which is significantly separated from 630. While this result implies that  
344 both strains are fundamentally different to the parental strain, it does not indicate that the

345 differences to 630 are the same for both strains. The analysis depicted by the Venn  
346 diagram (**Fig. 7**) confirms the results of the PCA, showing that the majority of differentially  
347 expressed genes are observed comparing 630 $\Delta$ *erm* and 630E to 630. From a total of 1337  
348 differentially expressed genes (**Table S3** contains all the genes differentially expressed  
349 along the growth and also comparisons between the strains), only 139 were common  
350 between all three strains. A total of 345 were common between 630E and 630 $\Delta$ *erm*, 60  
351 were common between 630 and 630 $\Delta$ *erm* and 58 were common between 630 and 630E.

352 Most of the 345 genes differentially expressed in both, 630 $\Delta$ *erm* and 630E, were either  
353 up or down-regulated in the same way highlighting again how distinct the two strains are  
354 from the parental strain 630 (**Table S3**). In TY medium used for the transcriptomic  
355 experiments, known as a non-optimal for spore production, a total of 44 sporulation genes  
356 were differentially expressed in both 630 $\Delta$ *erm* and 630E, and all of these were  
357 downregulated at 14 and 24 h compared to 6 h. No further differentially expressed  
358 sporulation genes appeared in 630 $\Delta$ *erm*, however, our analysis showed a further 22  
359 sporulation genes, of which 21 were downregulated, in 630E. Amongst these was the  
360 master regulator of sporulation *spo0A*. Nine genes classed as stress-related are  
361 differentially expressed in all three strains (five upregulated), with a further three in 630  
362 (all upregulated), seven in 630 $\Delta$ *erm* (four upregulated) and 16 in 630E (11 upregulated).  
363 Nine genes related to secretion are down regulated in 630E and one gene related to type  
364 IV pili is upregulated. In comparison only one secretion gene (putative pilus assembly  
365 ATPase) is differentially expressed only in 630 $\Delta$ *erm* (down regulated) and none in 630.  
366 Metabolism is also highly differentially regulated in the three strains. 90 genes were  
367 uniquely, differentially expressed in 630E, 50 in 630 $\Delta$ *erm* and 30 in 630. In particular the

368 amino acid metabolism stands out for 630E with the majority of genes being  
369 downregulated. (**Table S3**).

370 RNAseq data can be used to independently corroborate genome re-sequencing data.  
371 Thus, it was apparent that those changes identified by CLC Bio as being present with a  
372 frequency of 70% or less, except for CD630\_20102, were not real accordingly to the  
373 RNAseq analysis (**Table S1**). This increases the confidence in disregarding changes  
374 identified by NGS with a low frequency. In most cases, the SNPs and Indels identified by  
375 NGS were confirmed by the RNAseq analysis, with the following exceptions: for SNPs in  
376 630E we found two disagreements notably in CD630\_33790 and CD630\_29430, which both  
377 had 100 % frequency and a high coverage (around 200 reads) in the DNA sequence  
378 analysis, but only low coverage in the RNAseq experiment. Due to the low coverage of  
379 these regions during the RNAseq experiment, which is indicative of low or no expression  
380 under the examined conditions, a sequencing error cannot be excluded. For the SNP in  
381 CD630\_12740 the RNA coverage corroborated the genomic data for 630E, but was in  
382 disagreement with the genomic data for 630 and 630 $\Delta$ *erm*. For three SNPs in 630 $\Delta$ *erm*  
383 similar scenarios were observed. CD630\_19070 had very low RNA coverage, CD630\_35650  
384 showed ambiguous RNA data with low coverage for 630E. CD630\_08260, the *perR*  
385 homologue, had convincing DNA data, with frequencies of 98-100 % and coverage of at  
386 least a 100 which was corroborated for 630 $\Delta$ *erm* by RNAseq coverage.

387 In terms of actual expression data (**Table S1**), CD630\_07610 (the RNA helicase),  
388 CD630\_14040 (oligopeptide transporter), CD630\_20270 (hydrolase) and CD630\_29430  
389 (phage replication protein) all showed differential expression in 630E compared to the  
390 other two strains, with the first two showing reduced expression and the latter two an

391 increase. CD630\_29430, however, also showed an increase in expression in 630 at the  
392 later time point. Changes in CD630\_26670 in 630 $\Delta$ *erm* and 630E both seem to lead to  
393 severely reduced expression. CD630\_12740 (*topA*) only showed differential expression at  
394 24 h in 630 $\Delta$ *erm* and CD630\_12140 (*spo0A*) expression was severely reduced in 630E.

395

## 396 **Discussion**

397 Previously, two studies<sup>8,9</sup> have attempted to use isogenic mutants defective in the  
398 production of either toxin A or toxin B to determine the relative importance of these two  
399 virulence factors in CDI using the hamster infection model. However, despite generating  
400 essentially equivalent A+B-insertion mutants in ostensibly the same strain of *C. difficile*  
401 (630), contradictory outcomes were obtained in terms of the importance of toxin A. Thus,  
402 a *tcdB* mutant created in the one study<sup>8</sup> producing only TcdA did not cause disease in the  
403 hamster, whereas the equivalent ClosTron mutant made in our laboratory (Minton group)<sup>9</sup>  
404 remained virulent. The work undertaken here has provided compelling evidence that the  
405 reason for the observed conundrum resides in the use of two different erythromycin-  
406 sensitive derivatives of strain 630.

407 Here we have shown that both erythromycin-sensitive derivatives, 630E<sup>14</sup> and  
408 630 $\Delta$ *erm*<sup>13</sup> carry a significant number of SNPs compared to the published sequence.  
409 Moreover, it is clear that whilst the phenotypic properties of 630 $\Delta$ *erm* and its mutant  
410 derivatives closely resemble that of the parent strain 630, strain 630E and its progeny  
411 exhibit substantive differences. Thus, whereas latter strains exhibit reduced growth rates,  
412 are less proficient in spore formation and are non-motile, 630 $\Delta$ *erm* strains mirror the  
413 behaviour of the 630 parental strain with respect to these phenotypes. Furthermore, 630E

414 strains produce reduced amounts of toxin and both struggle to colonise hamsters, and  
415 once colonised, animals are less likely to succumb to disease. In short, 630E and its  
416 derivatives (i.e., 630E A-B+ and 630E A+B- CT) are less virulent than 630 $\Delta$ *erm* and its  
417 mutant counterparts (i.e., 630 $\Delta$ *erm* A+B-).

418       The altered properties of 630E and its derivatives are undoubtedly a consequence of  
419 the observed SNPs. However, the substantive number of changes involved makes it  
420 difficult to assign any particular SNP to a specific alteration in the observed phenotype,  
421 particularly as a combination of mutagenic changes could be responsible. Whilst it is now  
422 possible to make precise changes to the genome using allelic exchange methodologies<sup>15</sup> it  
423 is not practically feasible to make all of the sequential rational changes needed to  
424 definitively identify the mutation(s) responsible for a particular phenotype. As such, we  
425 only corrected three specific SNPs that we reasoned may be making a significant  
426 contribution. The outcomes of these experiments only emphasised the difficulty of such an  
427 undertaking, and served to highlight the dangers involved in making assumptions. Thus,  
428 whilst it seemed reasonable to assume that the DNA inversion within the promoter region  
429 of the flagella operon was likely to have caused the observed non-motile phenotype, this  
430 surprisingly proved not to be the case. Re-inversion of the 150 bp region failed to restore  
431 motility. Clearly other SNPs are at least partly responsible for the observed lack of motility.  
432 Singling out any other SNP as the culprit would in the absence of experimental evidence be  
433 counterproductive.

434       Equally negative was the observed outcome of correcting the mutation in  
435 CD630\_12740 that results in a truncation of the encoded topoisomerase I enzyme. Given  
436 this enzyme controls DNA supercoiling, and given that its mutation in certain bacteria is

437 either a lethal event <sup>25-27</sup> and/or is involved in the regulation of virulence factors, <sup>28-30</sup> it  
438 seemed likely that its presence would result in pleiotropic effects that could have  
439 contributed to the observed phenotypic changes. However, its correction, with the  
440 exception of a measurable increase in toxin B levels, seemingly had no effects on the  
441 behaviour of the strain, at least for those properties measured. The reasons are not clear.  
442 In other bacteria, mutations of *topA* are only isolated if compensatory mutations arise  
443 elsewhere in the genome. <sup>26</sup> Whether any of the other SNPs present in 630E (eg., the RNA  
444 helicase mutation) are negating the effects of the TopA truncation is currently unknown.

445 To understand the differences observed further, we analysed the transcriptome of  
446 630, 630 $\Delta$ *erm* and 630E, comparing expression at 6 h to 14 h and to 24 h (**Table S3**). The  
447 data corroborated the phenotypic analysis, showing vastly different transcriptomes for all  
448 three strains. While 630E and 630 $\Delta$ *erm* cluster together in the PCA (**Fig. 6**), this only  
449 highlights how different the two strains really are from the progenitor. The analysis clearly  
450 shows that the three strains are very different from each other and also serves as an  
451 explanation as to why the change of a single SNP could not restore any given phenotype.  
452 Overall 630E seems the most divergent with many genes differentially expressed involved  
453 in metabolism and regulation (**Table S3**). Additionally 32 genes grouped under the  
454 descriptor 'cell factor', many of which play a role in energy metabolism, are differentially  
455 expressed in 630E, with only six of these being upregulated. In contrast out of 19 genes in  
456 630, 11 are upregulated and, out of 10 in 630 $\Delta$ *erm*, six were upregulated. The number of  
457 genes downregulated in energy metabolism in 630E might relate to the growth differences  
458 seen between the strains.

459            Interestingly 22 genes involved in sporulation are differentially expressed, 21 of these  
460 downregulated, in 630E versus one in 630 and two in 630 $\Delta$ *erm*. This is consistent with the  
461 observed delay in sporulation and reduced amount of spores produced by 630E. Secretion  
462 also seems most affected in 630E, with 10 genes differentially expressed, compared to  
463 none in 630 and one in 630 $\Delta$ *erm*. A general defect in secretion could affect the secretion  
464 of certain virulence or adhesion factors. Furthermore, 33 cell wall genes are differentially  
465 expressed in 630E (compared to 14 each in 630 $\Delta$ *erm* and 630). This may also contribute to  
466 the observed colonisation deficiencies. Mobile elements are, however, more differentially  
467 transcribed in 630 and 630 $\Delta$ *erm* (15 each) versus 630E (nine). As in many cases different  
468 pathways were affected, we propose that this could at least in part explain the different  
469 adaptability and virulence of the two strains. In both strains many regulators were  
470 differentially affected providing a further basis for the observed phenotypic variation  
471 between strains.

472

## 473 **Conclusion**

474            Our study has established that the parental strains (630E and 630 $\Delta$ *erm*) used in the  
475 two previous studies, that explored the relative roles of toxin A and toxin B in disease,<sup>8,9</sup>  
476 are phenotypically and genetically distinct. Here we also reveal that the three strains (630,  
477 630 $\Delta$ *erm* and 630E) have vastly different transcriptomes, which no doubt lead to the  
478 different phenotypes observed. This immense diversity also underlines our finding that by  
479 restoring just one SNP, the entire transcriptome cannot be changed. The presence of SNPs  
480 in strain 630E significantly affects its transcriptome which in turn has a significant impact  
481 on growth, sporulation and finally virulence of this strain in the hamster model of infection

482 under the conditions tested. Data (such as motility, toxicity and virulence) obtained with  
483 strain 630 $\Delta$ *erm* reflects more accurately the behaviour of the parent strain 630. As such, it  
484 may be concluded that 630 producing toxin A alone will cause disease in the hamster. As a  
485 consequence, toxin A should remain a target for the rational development of effective  
486 countermeasures against *C. difficile*.

487 This study has also highlighted a number of issues that need to be borne in mind in the  
488 future. At a specific level, if researchers wish to undertake genetic-based studies with  
489 strain 630, then the use of strain 630 $\Delta$ *erm* should be favoured over strain 630E. At a more  
490 fundamental level, researchers need to effectively curate their strains to prevent the  
491 inadvertent isolation of SNPs. Ideally, master seed banks need to be established as frozen  
492 glycerol stocks. Moreover, the genome of the stored strain should be re-sequenced as part  
493 of the storage process whenever a strain is received from external sources, regardless of  
494 whether it has been re-sequenced in the sending laboratory.

495

## 496 **Materials and Methods**

### 497 **Bacterial strains and routine culture conditions**

498 Bacterial strains and plasmids used in this study are listed in **Table 2**. *E. coli* was  
499 cultured aerobically at 37°C with shaking at 200 rpm in LB medium with chloramphenicol  
500 supplementation (25 µg/ml) where appropriate. *C. difficile* was cultured in TY (tryptose  
501 yeast) medium supplemented with thiamphenicol (15 µg/ml) where appropriate. When  
502 needed, *C. difficile* strains were plated on BHIS agar (Brain Heart Infusion agar [Oxoid]  
503 supplemented with 5 mg/ml yeast extract [Oxoid] and 0.1% [wt/vol] cysteine  
504 [Calbiochem]) supplemented with d-cycloserine (250 µg/ml), cefoxitin (8 µg/ml) [Oxoid]

505 (BHIScc). Fluorocytosine selections were carried out on *C. difficile* minimal medium  
506 (CDMM) as described previously.<sup>15</sup> All *C. difficile* cultures were incubated at 37°C  
507 anaerobically in an anaerobic MACS1000 workstation (Don Whitley, Yorkshire, UK).

#### 508 **Mutant nomenclature**

509 For the sake of simplicity, *C. difficile* strains that carried a *tcdA* insertional mutant were  
510 referred to as A-, those carrying a *tcdB* mutant as B-, and those strains carrying a mutation  
511 in both genes as A-B-. To avoid any ambiguity, if the gene was not inactivated it was  
512 referred to as A+ or B+, as appropriate. Thus, a *tcdA* mutant of 630 $\Delta$ *erm* constructed using  
513 ClosTron technology as described,<sup>9, 16</sup> was designated 630 $\Delta$ *erm* A-B+. The equivalent  
514 mutant in 630E constructed through the insertion of a replication-deficient plasmid,  
515 according to the method of O'Connor et al.,<sup>14</sup> was designated 630E A-B+. When ClosTron  
516 technology was used in 630E, this was clarified by adding a 'CT' suffix, 630E A-B+CT to the  
517 strain designation.

518

#### 519 **Whole genome sequencing and bioinformatics**

520 Genomic DNA from strains 630E A+B-, 630E A+B-CT and 630 $\Delta$ *erm* A+B- was prepared  
521 by phenol:chloroform extraction. 630E A+B-, 630E A+B-CT, 630 $\Delta$ *erm*( $\Delta$ *pyrE*) were  
522 sequenced on Illumina highseq (GATC, Germany) and 630 $\Delta$ *erm* A+B- on a Roche 454  
523 (Deepseq, Nottingham, UK) and the data compared to the published genome of 630<sup>11,19</sup>  
524 using CLC genomic workbench. All raw sequencing data have been deposited in the  
525 sequence read archive (SRA) under the study name PRJNA304508. The accession number is  
526 SRP066836. The sequencing data for 630 $\Delta$ *erm* $\Delta$ *pyrE* had been obtained previously<sup>19</sup> and  
527 with no additional changes, other than the *pyrE* deletion, present compared to 630 $\Delta$ *erm*,

528 were used to analyse the parental strain 630 $\Delta$ *erm*. We used a frequency of 70% as a cut-off  
529 for SNP calling. SNPs, InDels and inversions were confirmed by amplifying a few hundred  
530 base pairs up- and downstream of the area of interest (primers are listed in **Table S4**) and  
531 the amplicon was Sanger sequenced (Source BioScience, UK). This confirmation was done  
532 on all strains including the parental strains (630, 630 $\Delta$ *erm*, 630E) and the derivatives  
533 (630 $\Delta$ *erm* A+B-, 630E A+B-, 630E A+B-CT).

534

#### 535 **Correction of SNPs and reversal of 150-bp region within the flagellar operon**

536 Using the method described by Cartman et al.<sup>15</sup> we “corrected” the two SNPs and an  
537 inversion in 630E to the 630 $\Delta$ *erm* genotype. A stretch of DNA corresponding to  
538 approximately 500 bp either side of the area to be altered was synthesised by Biomatik  
539 and cloned into plasmid pMTL-SC7315 $\lambda$ 2.3. This vector was transformed by  
540 electroporation into *E. coli* CA434 cells<sup>36</sup> and subsequently conjugated into 630E. Single  
541 crossover colonies were identified as those growing faster on plates containing  
542 thiamphenicol. Following overnight incubation on CDMM containing 5-fluorocytosine,  
543 colonies were incubated on BHIScc plates with and without thiamphenicol. Those strains  
544 that had lost the plasmid (both wildtype and double crossover) were unable to grow on  
545 thiamphenicol. SNP corrections were confirmed by PCR (Primers see **Table S4**) and Sanger  
546 sequencing (Source BioScience, UK).

547

#### 548 **ClosTron mutagenesis**

549 A *tcdB* mutant was generated in the 630E background according to the published  
550 method,<sup>16</sup> using the same plasmid that was used to generate the equivalent mutant in

551 630 $\Delta$ erm. <sup>9</sup> This newly created strain was referred to as 630E A+B- CT.

552

### 553 **Southern and Western blot**

554 The Southern and Western blot were performed as described in Kuehne et al., 2010. <sup>9</sup>

555

### 556 ***In vivo* testing of mutants**

557 *In vivo* testing was carried out in Syrian Golden hamsters (Charles River, Germany) as

558 previously described. <sup>9</sup> Briefly, clindamycin was administered orally on day -five to render

559 the animals susceptible to infection. On day zero, 10,000 spores were administered orally.

560 Animals were assessed for signs of CDI (weight loss, wet tail, lethargy, lack of response to

561 stimulus) six times a day for the first five days, and once daily for the following 14 days. At

562 this point animals that failed to display signs of CDI were euthanised. Faecal pellets were

563 collected daily from day zero to endpoint, homogenised and plated on *C. difficile* fructose

564 agar (CDFA). *C. difficile* colonies were sub-cultured onto BHIS agar and the genotype was

565 established by PCR (primers in **Table S4**) followed by Sanger sequencing (results in **Table**

566 **S2**). At the experimental endpoint, part of the caecum of each animal was collected. This

567 was also used to plate on CDFA to verify colonisation.

568

### 569 ***In vitro* testing of mutants**

570 **Growth curves:** To assess the effects of SNPs and “corrected” SNPs/inversion on the

571 growth characteristics of all strains, we performed growth curve experiments over 24 h. A

572 180  $\mu$ L volume of TY medium was inoculated with 20  $\mu$ L of an overnight culture in 96-well

573 plates and incubated for 24 h in a GloMax-Multi Microplate Multimode Reader (Promega,

574 USA). Samples were shaken every h and OD<sub>600</sub> measurements were taken immediately  
575 after.

576 **Motility assays:** 2xYTG (tryptone (1.6%), yeast (1%), NaCl (0.4%), Gelzan (0.24%)[Sigma-  
577 Aldrich] and glucose (0.5%)) agar was utilised. 25 mL were poured into each petri dish and  
578 let to solidify at room temperature for 15 min. The plates were then dried at 37°C for 30  
579 min. The plates were placed into the anaerobic cabinet 24 h before use. 2 µL volumes of  
580 overnight culture were 'dropped' onto each plate. Plates were incubated anaerobically for  
581 48 h. Motility was assessed by eye and the plates photographed.

582 **Sporulation and germination assays:** Sporulation and germination assays were carried out  
583 as previously described.<sup>17,18</sup> Briefly, for the sporulation assay cultures were grown for five  
584 days, with 2 X 500 µl samples taken at 0, 24, 48, 72, 96 and 120 h. One sample from each  
585 time point was heated to 65°C for 30 min while the other sample was kept at room  
586 temperature. After this time, samples were serially diluted from 10<sup>0</sup> to 10<sup>-7</sup> in PBS. 3 X 20µl  
587 of each dilution was spotted onto BHIS plates containing 0.1% taurocholic acid and were  
588 incubated for 24 h. The following day, colonies were counted and CFU/mL were calculated.  
589 A 630Δ*erm spo0A*::CT mutant strain (containing a ClosTron insertion in the *spo0A* gene<sup>37</sup>),  
590 which is unable to form spores, was used as a negative control.

591 Germination was measured as a function of the ability of a germinated spore to  
592 outgrow in the absence of taurocholate. Spore stocks were prepared as previously  
593 described by Heeg *et al.*, 2012<sup>38</sup> and stored at -20°C. The optical density of spore  
594 suspensions (OD<sub>600</sub>) was adjusted to 1.0 and 450 µl was used per measurement. This  
595 equated to approximately 2.5 X 10<sup>7</sup> spores. Spore suspensions were heat treated at 60°C  
596 for 25 min to kill any remaining vegetative cells and then centrifuged and resuspended in

597 BHIS with the germinant taurocholic acid (0.1%) in a total volume of 20 ml. Samples were  
598 taken at 5, 10, 20, 30, 60, 90, 120, 180 and 240 min, briefly centrifuged, washed and  
599 resuspended in phosphate-buffered saline (PBS), the samples were then diluted and plated  
600 on plain BHIS agar. Plates were incubated for 24 h before the CFUs were enumerated.  
601 Colonies that grew on these plates were considered to be germinated vegetative bacteria.

602

603 **Toxin A/B ELISA:** ELISA assays were performed using the *C. DIFFICILE TOX A/B II*<sup>™</sup> kit from  
604 TechLab and the TGC-E002-1-separate detection of *C. difficile* toxins A and B kit from  
605 TGCbiomics according to the manufacturers' instructions. Cultures of *C. difficile* were  
606 grown in TY medium without glucose for 72 h, at which time 1 ml samples were taken,  
607 centrifuged and the supernatant filter-sterilised and used for the ELISAs. A 1:2 dilution was  
608 used for the toxin B ELISA kit from TGCbiomics. To quantify toxins a standard curve with  
609 pure toxin (the native antigen company) was established for the TechLab ELISA and also  
610 Toxin A ELISA from TGCbiomics.

611

612 **RNAseq:** Total RNA was extracted from 2 independent biological replicates of 630,  
613 630 $\Delta$ *erm* and 630E strains at 3 time points (18 samples). Bacteria were grown in TY broth  
614 medium after 6, 14 and 24 h as previously described.<sup>35</sup> The mRNA was treated with  
615 MicroExpress kit (Ambion). For oriented RNA-seq library construction, the Truseq  
616 stranded RNA seq Illumina kit was used according to manufacturer's instructions before  
617 sequencing using the Illumina HiSeq 2500 machine. Sequencing reads were mapped using  
618 Bowtie<sup>39</sup> to the reannotated 630 reference genome<sup>40</sup> complemented with the known  
619 ncRNA.<sup>41</sup> Statistical analyses were performed on each strand coverage count with DESeq2

620 <sup>42</sup> using the 6 h value as a reference for reporting the expression data of 14 and 24 h. A  
621 gene was considered differentially expressed when the fold change was > 2 and the *P value*  
622 was < 0.05.

623 The RNA-seq data discussed in this publication have been deposited in NCBI's Gene  
624 Expression Omnibus database under the accession no. GSE72006

625 RNA-seq coverage visualization is available through the COV2HTML software:<sup>43</sup>

|                  |          |   |
|------------------|----------|---|
| 630              | 14H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-20">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-20</a> |
|                  | 24H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-22">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-22</a> |
| 630 $\Delta$ erm | 14H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-24">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-24</a> |
|                  | 24H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-26">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-26</a> |
| 630E             | 14H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-28">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-28</a> |
|                  | 24H - 6H | <a href="http://mmonot.eu/COV2HTML/visualisation.php?str_id=-30">http://mmonot.eu/COV2HTML/visualisation.php?str_id=-30</a> |

626

### 627 **Graphs and statistical analyses**

628 All graphs were generated and statistical analyses were performed using GraphPad  
629 PRISM 6.02. Statistical analysis comprised either 2-way ANOVA for multiple comparisons or  
630 unpaired t test for pairwise comparisons. All experiments were carried out in triplicate  
631 unless stated otherwise.

632

### 633 **Ethics statement**

634 This work was reviewed and approved locally by the Animal Welfare and Ethical  
635 Review Body (formerly the Ethical Review Committee) at the University of Nottingham and  
636 performed under a project licence (PPL 40/3590) granted under the Animal (Scientific

637 Procedures) Act, 1986, by the UK Home Office. The work was performed in accordance  
638 with the NC3R<sup>s</sup> ARRIVE guidelines.<sup>44</sup>

639

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646 Medical Research Council (UK).

647

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770

771

772

773 **Figure legends**

774

775 **Figure 1.** Growth curves of strain 630 and its derivatives. A. 630 (630), 630 $\Delta$ *erm* and 630E were  
776 grown in TY-broth for 24 h in a 96 well plate reader. The optical density at 600 nm was measured  
777 every 30 min. B. This graph shows the same growth as A. and in addition the growth of derivatives  
778 630 $\Delta$ *erm* A+B-, 630E A+B- and 630E A+B- CT.

779

780 **Figure 2.** Motility assays. The assay was carried out by inoculating overnight cultures onto motility  
781 agar plates and incubating anaerobically for 48 h. Strains 630, 630 $\Delta$ *erm* and 630E were compared  
782 for their ability to swarm.

783

784 **Figure 3.** Sporulation and Germination. A. Sporulation over 120 h comparing heat treated CFUs of  
785 strains 630, 630 $\Delta$ *erm*, 630 $\Delta$ *erm* A+B-, 630E, 630E A+B- and 630E A+B- CT with a non-sporulating  
786 control (*spo0A*). B. The extent of germination of the indicated strains was measured over 250 min  
787 as the ability of germinated spores to form colonies on plates lacking taurocholate.

788

789 **Figure 4.** Toxin ELISAs. A. The *C. DIFFICILE TOX A/B II*<sup>™</sup> ELISA assay kit from TechLab was used to  
790 measure combined toxin A and B in strains 630, 630 $\Delta$ *erm*, 630 $\Delta$ *erm* A+B-, 630E, 630E A+B-, 630E  
791 A+B- CT, 630E\_*topA*, 630E\_*flgB* and 630E\_CD2667 grown in TY for 72 h. B. and C. Toxin ELISAs  
792 TGcbiomics, measuring the toxins separately were used to quantify toxin A (B) and toxin B (C)  
793 produced by strains 630, 630 $\Delta$ *erm*, 630 $\Delta$ *erm* A+B-, 630E, 630E A+B- and 630E A+B- CT grown in TY  
794 for 72 h. Statistics were performed using one-way ANOVA with Fisher's LSD test.

795

796 **Figure 5.** Infection to endpoint in the Hamster infection model. Groups of Golden Syrian Hamsters  
797 were challenged with *C. difficile* 630E (5 hamsters), 630E A+B- (11 hamsters) and 630E A+B- CT (6  
798 hamsters). The graph represents the time from inoculation to endpoint. The maximal duration of  
799 the experiment was set to 20 days. Animals represented in open symbols, have not been colonized  
800 despite challenge or lost colonization before day 20. Details can be seen in **Table 1**. The dotted line  
801 separates this experiment from data obtained by Kuehne et al. <sup>9</sup>, which are represented here as a  
802 comparator. In that study 8 hamsters were infected with *C. difficile* 630 $\Delta$ *erm* and another 8  
803 hamsters with 630 $\Delta$ *erm* A+B-.

804

805 **Figure 6.** Principal Component Analysis (PCA). The Principal Component Analysis (PCA) visualizes  
806 the variance of the data in a single graph. The axis represent the two largest variances of the data;  
807 PC1 accounts for 42% and PC2 accounts for 21%, that means that 63% of the total variance of the  
808 dataset is explained in this graph. The third component accounts for less than 10% and further  
809 components have a value that falls rapidly. The PCA represents the RNAseq data (at three different  
810 time points, six, 14 and 24 h) in duplicate for 630 (blue), 630 $\Delta$ *erm* (green) and 630E (orange). The  
811 different time points are represented as dots in the different shades of the respective colour as  
812 indicated in the colour legend.

813

814 **Figure 7.** Venn Diagram representing differentially expressed genes in the three different strains.  
815 The diagram summarises the output from the RNAseq data, comparing strains 630, 630 $\Delta$ *erm* and  
816 630E. It depicts all differentially expressed genes and shows how many genes are differentially  
817 expressed in all strains, in two of the strains or are unique to just one strain.

818

**Table 1. Single Nucleotide Polymorphisms (SNPs) and other changes found after re-sequencing.**

| Gene                             | Description                           | Position | 630 <sup>12</sup> | 630 <sup>22</sup> | 630 | C   | F   | 630 $\Delta$ erm           | C   | F   | 630E | C   | F   | AA        |
|----------------------------------|---------------------------------------|----------|-------------------|-------------------|-----|-----|-----|----------------------------|-----|-----|------|-----|-----|-----------|
| <b>630E</b>                      |                                       |          |                   |                   |     |     |     |                            |     |     |      |     |     |           |
| CD630_07610                      | Putative ATP-dependent RNA helicase   | 933139   | G                 | -                 | -   |     |     | -                          |     |     | T    | 199 | 100 | Asp136Tyr |
| CD630_14040                      | Putative oligopeptide transporter     | 1626977  | A                 | -                 | -   |     |     | -                          |     |     | G    | 187 | 100 | Glu536Gly |
| CD630_20270                      | N-carbamoyl-L-amino acid hydrolase    | 2339506  | G                 | -                 | -   |     |     | -                          |     |     | A    | 73  | 100 | Gly373Glu |
| CD630_26670                      | PTSG-BC                               | 3079815  | A                 | -                 | -   |     |     | -                          |     |     | C    | 165 | 100 | *524Glu   |
| CD630_26270                      | Hypothetical protein                  | 3034953  | C                 | A                 | A   |     |     | A                          |     |     | -    | 156 | 100 | Gly68Cys  |
| CD630_33790                      | conjugative transposon protein        | 3951559  | C                 | -                 | -   |     |     | -                          |     |     | A    | 307 | 100 | Glu63Asp  |
| CD630_12740                      | <i>topA</i>                           | 1480649  | C                 | -                 | -   |     |     | -                          |     |     | T    | 146 | 100 | Gln386*   |
| CD630_29430                      | Putative phage replication protein    | 3422569  | T                 | -                 | -   |     |     | -                          |     |     | C    | 195 | 100 | Asn210Asp |
| IG                               | Intergenic region                     | 309208   | -                 | -                 | -   |     |     | -                          |     |     | INV  | 129 | 41  |           |
| IG                               | Intergenic region                     | 3528736  | G                 | -                 | -   |     |     | -                          |     |     | T    | 94  | 100 |           |
| <b>630<math>\Delta</math>erm</b> |                                       |          |                   |                   |     |     |     |                            |     |     |      |     |     |           |
| CD630_19070                      | <i>eutG</i>                           | 2209236  | G                 | -                 | -   |     |     | A                          | 127 | 97  |      |     |     | Gly252Glu |
| CD630_35650                      | GntR family transcriptional regulator | 4166495  | G                 | -                 | -   |     |     | A                          | 182 | 100 | -    |     |     | Ala91Val  |
| IG                               | Intergenic region                     | 2937176  | C                 | -                 | -   |     |     | A                          | 173 | 100 | -    |     |     |           |
| IG                               | Intergenic region                     | 3005866  | T                 | -                 | -   |     |     | G                          | 156 | 100 | -    |     |     |           |
| IG                               | Intergenic region                     | 3591103  | G                 | -                 | -   |     |     | A                          | 211 | 100 | -    |     |     |           |
| CD630_12140                      | <i>spo0A</i>                          | 1413057  | -                 | -                 | -   |     |     | AGAATGTA<br>GGAAATAT<br>AG | 112 | 40  | -    |     |     | Insertion |
| CD630_08260                      | Ferric uptake regulator               | 1000995  | A                 | -                 | -   | 100 | 100 | G                          | 153 | 100 | -    | 170 | 98  | Thr41Ala  |
| <b>630</b>                       |                                       |          |                   |                   |     |     |     |                            |     |     |      |     |     |           |
| CD630_32450                      | <i>prdR</i>                           | 3797112  | C                 | -                 | T   | 117 | 100 | -                          |     |     | -    |     |     | Glu261Lys |

|   |   |         |                  |   |   |     |     |   |     |     |   |     |     |  |           |
|---|---|---------|------------------|---|---|-----|-----|---|-----|-----|---|-----|-----|--|-----------|
| CD630_02050                                       | Transcription antiterminator,<br>PTS operon regulator | 268934  | G                | - | T | 123 | 97  | - |     |     | - |     |     |  | Gly165Cys |
| <b>630 and 630<math>\Delta</math>erm</b>          |   |         |                  |   |   |     |     |   |     |     |   |     |     |  |           |
| CD630_2667  | PTSG-BC   | 3080703 | C                | - | T | 75  | 100 | T | 141 | 100 | - |     |     |  | Val228Ile |
| IG  | Intergenic region                                     | 2203033 | A                | - | T | 105 | 100 | T | 174 | 99  |   |     |     |  |           |
| IG  | Intergenic region                                     | 4007463 |                  |   | C | 10  | 100 | C | 62  | 100 |   |     |     |  |           |
| <b>630 and 630<math>\Delta</math>erm and 630E</b> |   |         |                  |   |   |     |     |   |     |     |   |     |     |  |           |
| CD630_11900                                       | acyl-CoA N-acyltransferase                            | 1391850 | T                | - | C | 118 | 100 | C | 133 | 100 | C | 143 | 100 |  | Phe133Leu |
| CD630_13880                                       | pseudo  | 1607453 | INS <sup>1</sup> | - | T | 119 | 94  | T | 175 | 94  | T | 124 | 88  |  | Thr16fs   |
| <b>Mistake in original sequence</b>               |   |         |                  |   |   |     |     |   |     |     |   |     |     |  |           |
| CD630_17670                                       | <i>gapB</i>   | 2044514 | C                | G | G |     |     | G |     |     | G |     |     |  |           |
| CD630_31561                                       | pseudo  | 3686535 | INS <sup>1</sup> | A | A |     |     | A |     |     | A |     |     |  |           |

The table shows the Single Nucleotide Polymorphism (SNP) changes in 630 $\Delta$ erm and 630E compared to the reference 630<sup>12</sup> and also the new annotation by Riedel *et al* (The column 'Gene' represents the gene (or intergenic region (IG)) in which the change occurs, the column 'position' indicates the exact nucleotide position of the change.).<sup>22</sup> It also contains SNP frequency (F) and genomic coverage (C) as well as the resultant amino acid change (AA). No change from the original 630 annotation<sup>12</sup> is represented by a dash (-).

**Table 2. Strains and plasmids used in this study.**

| <b>Name</b>   | <b>Description</b>   | <b>Source</b>                 |
|---|--|-------------------------------|
| <b>Bacterial strains</b>                                |  |                               |
| <i>E. coli</i> TOP 10                                   | F– <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> )<br>Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ ( <i>ara</i><br><i>leu</i> ) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str <sup>R</sup> ) <i>endA1</i> <i>nupG</i> | Invitrogen                    |
| <i>E. coli</i> CA434                                    | Conjugation donor  | Williams et al. <sup>36</sup> |
| <i>C. difficile</i> 630                                 | Wild-type  | Brendan Wren                  |
| <i>C. difficile</i> 630Δ <i>erm</i>                     | Erythromycin sensitive strain of <i>C. difficile</i> 630   | Hussain et al. <sup>13</sup>  |
| <i>C. difficile</i><br>630Δ <i>erm</i> (Δ <i>pyrE</i> ) | <i>C. difficile</i> 630Δ <i>erm</i> containing a deletion in<br>the <i>pyrE</i> gene   | Ng et al. <sup>19</sup>       |
| <i>C. difficile</i> 630E                                | Erythromycin sensitive strain of <i>C. difficile</i> 630   | Lyras et al. <sup>8</sup>     |
| <i>C. difficile</i> 630Δ <i>erm</i><br>A+B-             | <i>C. difficile</i> 630Δ <i>erm</i> <i>tcdB</i> -1511a::intron <i>ermB</i>   | Kuehne et al. <sup>9</sup>    |
| <i>C. difficile</i> 630E A+B-                           | <i>C. difficile</i> 630E   | Lyras et al. <sup>8</sup>     |
| <i>C. difficile</i> 630E A+B-<br>CT                     | <i>C. difficile</i> 630E <i>tcdB</i> -1511a:: intron <i>ermB</i>   | This study                    |
| <i>C. difficile</i> 630E_ <i>topA</i>                   | <i>C. difficile</i> 630E   | This study                    |
| <i>C. difficile</i> 630E_ <i>flgB</i>                   | <i>C. difficile</i> 630E   | This study                    |
| <i>C. difficile</i><br>630E_CD2667                      | <i>C. difficile</i> 630E   | This study                    |
| <b>Plasmids</b>   |  |                               |
| pMTL007C-E2: <i>tcdB</i> -<br>1511a                     | Clostron plasmid containing retargeted region<br>to <i>tcdB</i> at IS 1511 (antisense oriented) for <i>C.</i><br><i>difficile</i> 630Δ <i>erm</i> or 630E  | Kuehne et al. <sup>9</sup>    |
| pMTL-   | pMTL-SC7315λ2.3 containing 1,000 bp  | This study, based on          |

|                                   |  |  |
|-----------------------------------|--|--|
| SC7315λ2.3:: <i>topA</i>          | homology arms to change nucleotide 1480649<br>from T to C in 630E  | Cartman et al. <sup>15</sup>                         |
| pMTL-<br>SC7315λ2.3:: <i>flgB</i> | pMTL-SC7315λ2.3 containing 1,156 bp<br>homology arms to reverse the inversion<br>upstream of <i>flgB</i> in 630E | This study, based on<br>Cartman et al. <sup>15</sup> |
| pMTL-<br>SC7315λ2.3::CD2667       | pMTL-SC7315λ2.3 containing 1,000 bp<br>homology arms to change nucleotide 3079815<br>from C to A in 630E         | This study, based on<br>Cartman et al. <sup>15</sup> |

## Supporting Information

**Figure S1.** Southern and Western blot of 630E A+B- CT. A. Southern blot showing the single Clostron insertion to create 630E A+B- CT (mutant 1, 2 and 3). The Clostron plasmid pMTL007C-E2:*tcdB*-1511a was used as a positive control and the parental strain 630E as a negative control. The probe used binds to the intron insertion. B. Western blot using an anti-TcdA antibody to detect production of toxin A in the supernatant of 630, 630 $\Delta$ *erm*, 630 $\Delta$ *erm* A+B-, 630E, 630E A+B- and 630E A+B- CT after 96 h.

**Table S1. Genomic and transcriptomic differences between 630, 630 $\Delta$ *erm* and 630E.**

SNPs, insertions (INS) and inversion (INV) are shown in this table as well as the corresponding RNAseq coverage. Changes between 630, 630 $\Delta$ *erm* and 630E are represented compared to the published genome of 630, the new 630 genome by Riedel *et al.*<sup>22</sup> and also the newly published genome of 630 $\Delta$ *erm*<sup>23</sup>.

See attached excel file: S1\_Table

**Table S2. Colonisation of hamsters with *C. difficile* strains.**

Colonisation of hamsters with 630E, 630E A+B- and 630E A+B- CT was confirmed by plating faecal and caecal samples on CDFA and by extraction genomic DNA and PCR of the toxin A and B genes.

| Strain given | Colonisation        | 630 PCR | <i>tcdA</i> | <i>tcdB</i> | Succumbed to CDI |
|--------------|---------------------|---------|-------------|-------------|------------------|
| 630E         |                     | Caecum  | +           | +           |                  |
| 630E         | Colonised to Day 18 | Faecal  | +           | +           |                  |
| 630E         | Colonised to Day 15 | Faecal  | +           | +           |                  |
| 630E         |                     | Caecum  | +           | +           | CDI              |
| 630E         |                     | Caecum  | +           | +           | CDI              |
| 630EA+B-     |                     | Caecum  | +           | -           | CDI              |
| 630EA+B-     | Not colonised       | Faecal  | -           | -           |                  |
| 630EA+B-     | Not colonised       | Faecal  | -           | -           |                  |
| 630EA+B-     | Not colonised       | Faecal  | -           | -           |                  |
| 630EA+B-     | Not colonised       | Faecal  | -           | -           |                  |

|              |                     |        |   |    |     |
|--------------|---------------------|--------|---|----|-----|
| 630EA+B-     |                     | Caecum | + | -  |     |
| 630EA+B-     |                     | Caecum | + | -  | CDI |
| 630EA+B-     |                     | Caecum | + | -  |     |
| 630EA+B-     | Colonised to Day 3  | Faecal | + | -  |     |
| 630EA+B-     |                     | Caecum | + | -  |     |
| 630EA+B-     |                     | Caecum | + | -  |     |
| 630E A+B- CT |                     | Caecum | + | CT | CDI |
| 630E A+B- CT |                     | Caecum | + | CT | CDI |
| 630E A+B- CT | Colonised to Day 15 | Faecal | + | CT |     |
| 630E A+B- CT | Colonised to Day 18 | Faecal | + | CT |     |
| 630E A+B- CT |                     | Caecum | + | CT |     |
| 630E A+B- CT |                     | Caecum | + | CT |     |

**Table S3. Transcriptomic differences between 630, 630Δerm and 630E.**

Differentially expressed (DE) genes, comparing RNAseq data from 6 h to 14 h and 24 h in 630, 630Δerm and 630E. The genes are classed into cell factor, cell growth, cell wall, fermentation, membrane transport, amino acid metabolism, carbon metabolism, cofactor metabolism, lipid metabolism, nucleic acid metabolism, mobile elements, motility, operons, regulation, anaerobic respiration, secretion, sporulation, stress, translation, unknown and virulence factors. Downregulated genes are coloured in green and upregulated genes in red.

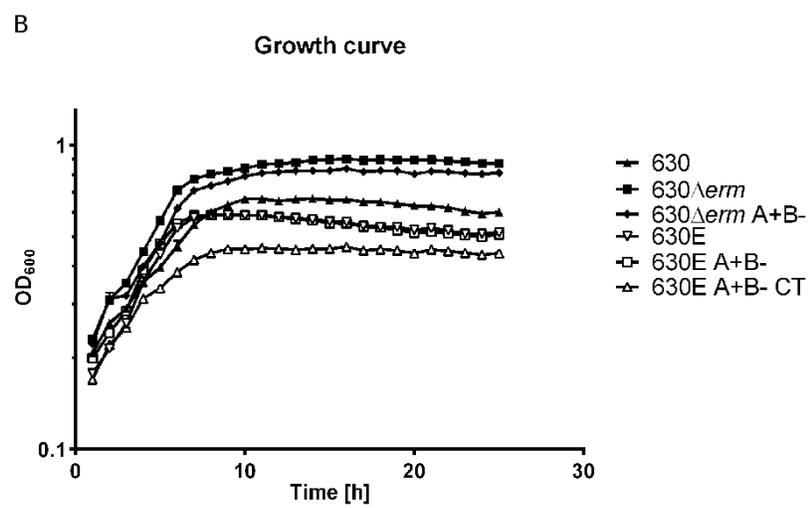
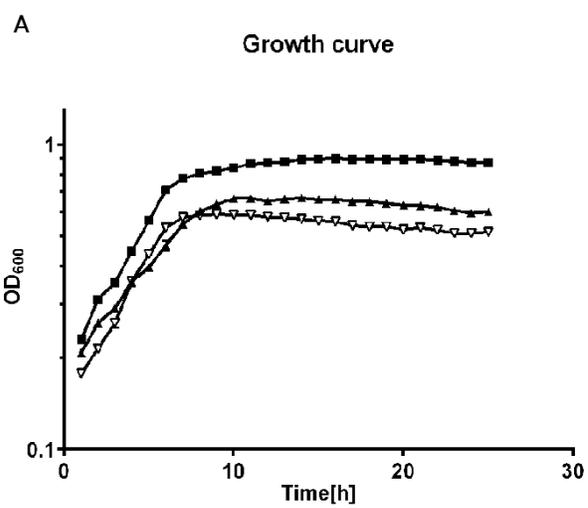
See attached excel file: S3\_Table

**Table S4. Primers used in this study.**

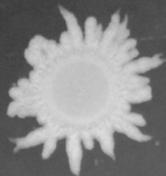
| Primer name | Sequence (5'-3')                   | Explanation or SNP target (where applicable) |
|-------------|------------------------------------|--|
| topAMC1     | GATGCACAACAGGCAAGAAGAGTGC          | To screen for chromosomal change             |
| topAMC2b    | CCCGATTGTAAAACAACACTAGACCAATTATG   | To screen for chromosomal change             |
| flgBMC1b    | CTATCAAATACAGATGGAAGTTGTGGTG       | To screen for chromosomal change             |
| flgBMC2c    | CGAGCATATGATTCTAACGTAGATACATTGAATG | To screen for chromosomal change             |

|                          |                                       |   |
|--------------------------|---------------------------------------|---|
| CD2667MC1                | GTCACCTTATGAGTGAAGTTTGTAAATAAATGTGG   | To screen for chromosomal change  |
| CD2667MC2b               | GCAAGAGCTGCTGCTGGAAGAC                | To screen for chromosomal change  |
| JRP3441 <sup>8</sup>     | GTTACCAGGAATACAACCAGAC                | To test for interrupted <i>tcdB</i>   |
| JRP2839 <sup>8</sup>     | CGGCCAGCCTCGCAGAGCAG                  | To test for interrupted <i>tcdB</i>   |
| JRP3442 <sup>8</sup>     | GCACTTGCTTGATCAAAGCTCC                | <i>toxA</i> specific PCR, positive for all strains (630E, 630E A+B-, 630E A+B-CT) |
| JRP2342 <sup>8</sup>     | CCGGAATTCGCTCTATTGGACTAGACCGTTG       | <i>toxA</i> specific PCR, positive for all strains (630E, 630E A+B-, 630E A+B-CT) |
| Cdi-tcdB-F1 <sup>9</sup> | TGATAGTATAATGGCTGAAGCTAATGCAGATAATGG  | To test for <i>tcdB</i> ClosTron insertion  |
| Cdi-tcdB-R1 <sup>9</sup> | CTTGCATCGTCAAATGACC ATAAGCTAGCC       | To test for <i>tcdB</i> ClosTron insertion  |
| 268934_F                 | TGTCAAGTGAATTAGAAAAGAAACCA            | 268934  |
| 268934_R                 | AAGTGAGCCGTGTTTTGAAAA                 | 268934  |
| 309208_F                 | GCCAGTTGCCAAAAAGAGTC                  | 309208  |
| 309208_R                 | GGCATAGCATCATTTAGTGTTTC               | 309208  |
| MCSNP11                  | CGGGAAAAACAGCTGCTTTTAGTATCCC          | 933139  |
| MCSNP12                  | CCTCTTGCTTGTAATCTCCTACCAATTC          | 933139  |
| 10000995_F               | GATGAAGAAGTTGTTTGCAAT                 | 10000995  |
| 10000995_R               | CCTACTTGGCTACACCTTTTACA               | 10000995  |
| 1391850_F                | TCTGTCATTTGGAAAGGATGAA                | 1391850   |
| 1391850_R                | TCTGTACTTGCTTTTGATATACTTGGA           | 1391850   |
| Spo0A_F1                 | GGCATAGCTAAGGATGGAATTG                | 1413057   |
| Spo0A_R1                 | GGAGTAGAGGAAAAGTTGACACAA              | 1413057   |
| 1480649_F                | GCTTCAACAAGAAGGAGCAAA                 | 1480649   |
| 1480649_R                | TGCTGGTGGTTGTGTAATAATG                | 1480649   |
| 1607453_F                | TTGAAGGTGTAACCTCAGTTGTAGG             | 1607453   |
| 1607453_R                | TCCAAATAAAAGTCTATGAAAATGAA            | 1607453   |
| 1626977_F                | TGGTGGTAGCAAAAACGAAA                  | 1626977   |
| 1626977_R                | TGCCATTGAATTTGTTGCAG                  | 1626977   |
| 2044514_F                | CATACTAAATGAGGGGTAAAATAAAGA           | 2044514   |
| 2044514_R                | TTTTTCTGCCTTTTCTCTTTGTG               | 2044514   |
| MCSNP1                   | GTTCTGTAATACCTTTTTCTTTAGCTATTTTAATTGC | 2203033   |
| MCSNP2                   | GTTACCGATATTATAGGCAAACTGCCC           | 2203033   |
| 2209236_F                | CCATTAGTGAGTGATGATTTACTTCC            | 2209236   |
| 2209236_R                | GCAAGTTTTGCTATTTCTCTTTCTT             | 2209236   |
| 2320410_F                | GTCCTGGTAGGAATTAATCTAACG              | 2320410   |
| 2320410_R                | TGCTTTCACAAATGCTTTTCG                 | 2320410   |
| MCSNP13                  | GAAAGATGAATTTCTATCCATCTTCATCAAATGTGG  | 2339506   |
| MCSNP14                  | GGGGCTGTTGACCTTGGACCC                 | 2339506   |

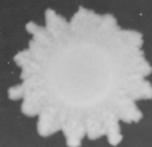
|           |  |         |
|-----------|--|---------|
| MCSNP3    | CCTTTGATGTCTAGTTAATTTCTTCACTTATTTTAAGC     | 2937176 |
| MCSNP4    | GGAAAACCAGCAAAAGCTTGTATTATGATTCCC          | 2937176 |
| 3005866_F | AATATAATCCCAACCTTCCAAA                     | 3005866 |
| 3005866_R | TTTGTTGAAGATTAGTGGTGATTG                   | 3005866 |
| MCSNP5    | GTATATTTTTCTCTAGCTTTATCTCCATCAGGG          | 3034953 |
| MCSNP6    | GGAAAGGATAAACCCAGGTATAGTGCC                | 3034953 |
| 3079815_F | CCCGCTTTTACTTCATCTCC                       | 3079815 |
| 3079815_R | GCATCAGAGATTTTGATTGCTTT                    | 3079815 |
| MCSNP7    | CCTGCTACAAATTTTTCTTTCTGGC                  | 3080703 |
| MCSNP8    | CTGCTTATCTTTATAAAAAGTTTATAAAAATTGAATTACCTC | 3080703 |
| MCSNP15   | CTTCATATTGAGTGAAAGTCTGATTGAAGTTAGC         | 3422569 |
| MCSNP16   | CTCAACCGTGTGCCGTTTTCCCG                    | 3422569 |
| 3526888_F | CTCTTTCCTGCATTCCCAAG                       | 3526888 |
| 3526888_R | TTGTTGAGCAGATATAAAATCCCA                   | 3526888 |
| MCSNP17   | CAATCTATTCAAAGATAAACTATAGTACTTCTTCTAC      | 3528736 |
| MCSNP18   | CCTACTCCTTTAGGTGTGAGATGG                   | 3528736 |
| 3591103_F | GGCACTAGCTGCTCCTAATAAA                     | 3591103 |
| 3591103_R | CCATATACCCCTATCCCTCCTT                     | 3591103 |
| 3686535_F | TCTTCCAAGCTTTACCTGTTTG                     | 3686535 |
| 3686535_R | GCTCTGTCCAGTTAATTG                         | 3686535 |
| 3797112_F | TGCTCCTGTAATGCACCTG                        | 3797112 |
| 3797112_R | CTGTAAAATACAAGTCACTCATTCCAA                | 3797112 |
| MCSNP19   | CCGTTCCAGACTGTTCAATGCTCC                   | 3951559 |
| MCSNP20   | CCTAAGTAGTAGTTACTGGCAACAGCAC               | 3951559 |
| MCSNP9    | GCACCCTTAATAACTTGACCAGTAAAAAGG             | 4007463 |
| MCSNP10   | CGCCCGAAGCCGATTATCTAACC                    | 4007463 |
| 4166495_F | GCATCAAGTAAGTATTTATGCTCTTCA                | 4166495 |
| 4166495_R | TGAACTTGGATAATTACAAGCCATT                  | 4166495 |



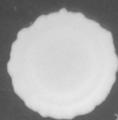
A  
630



B  
630 $\Delta$ erm

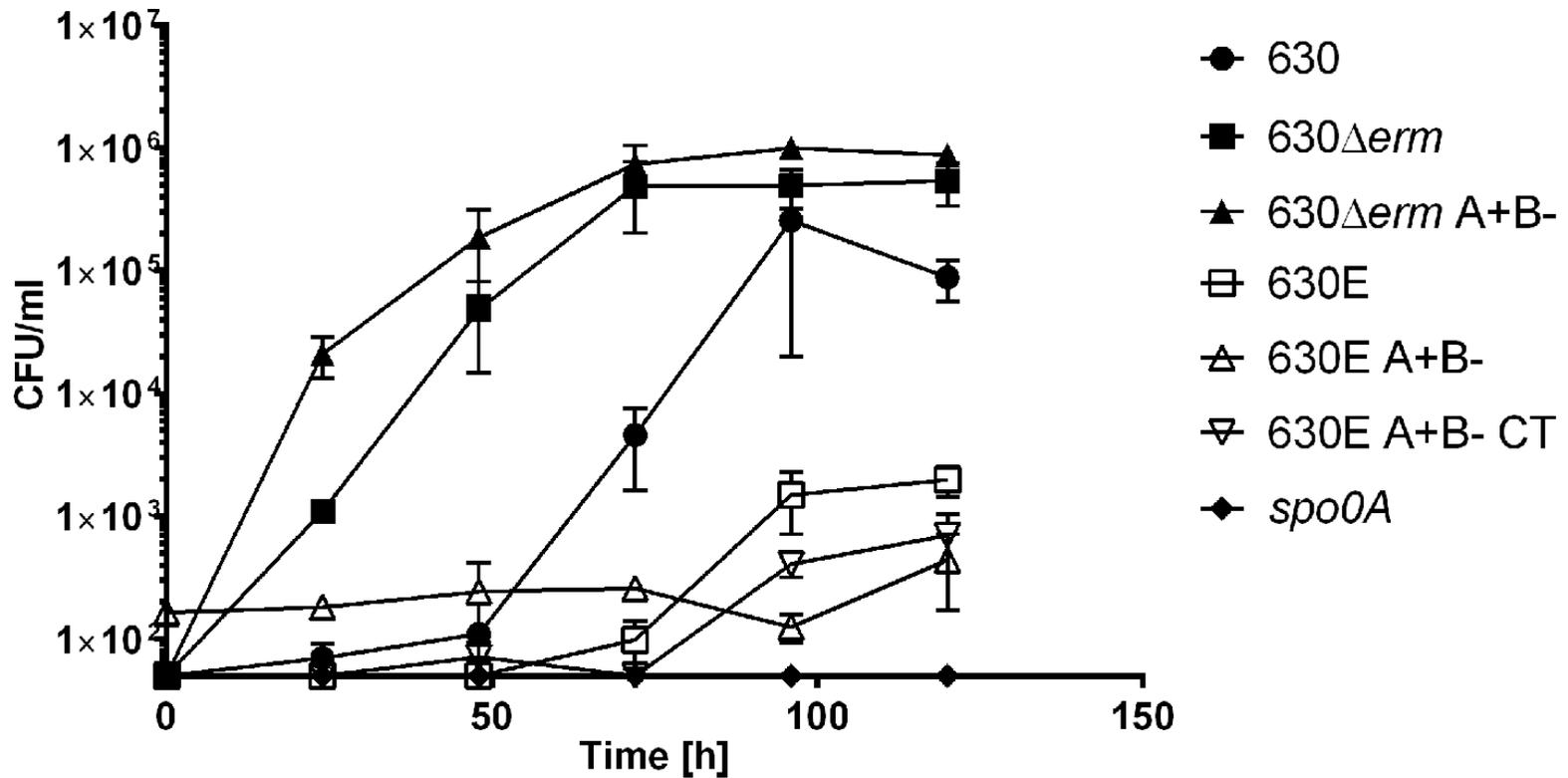


C  
630E



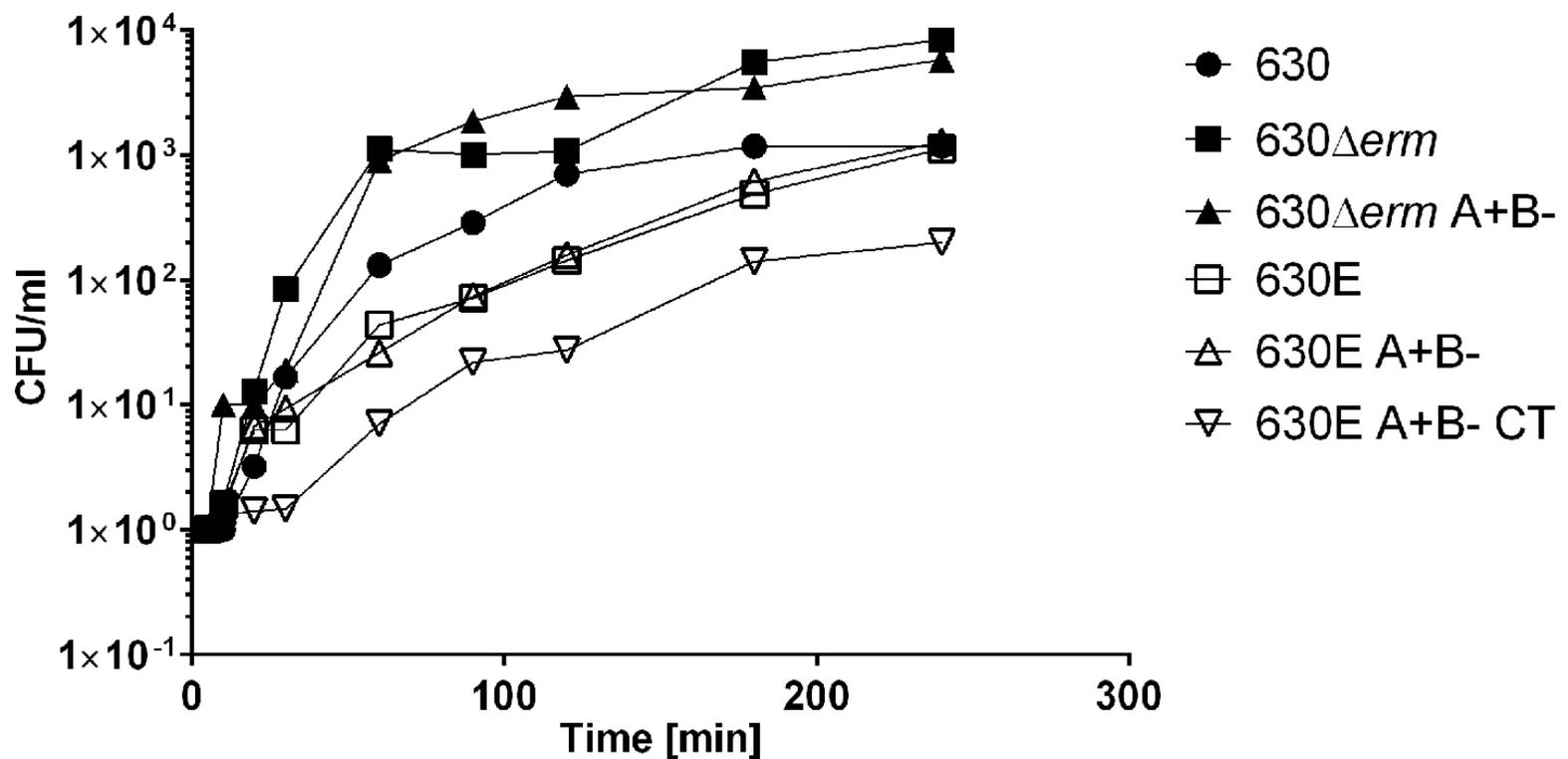
A

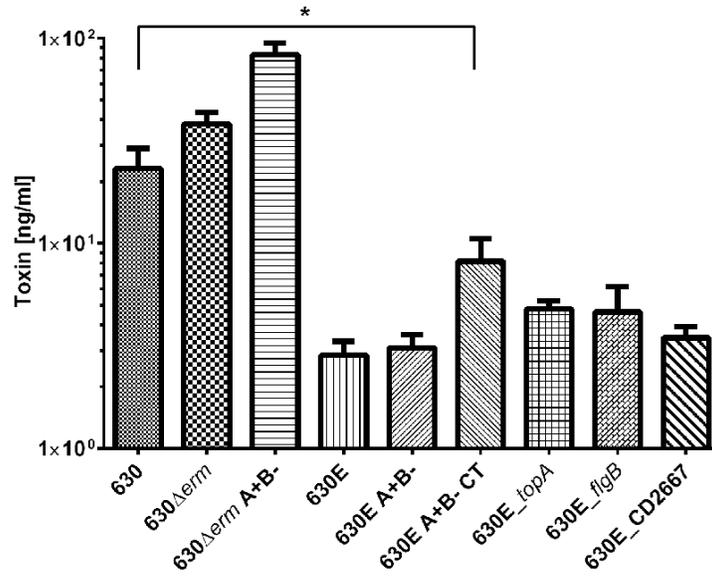
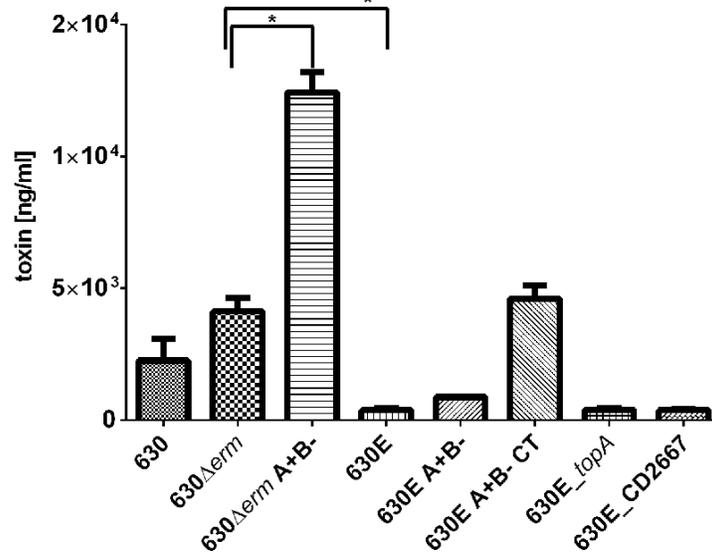
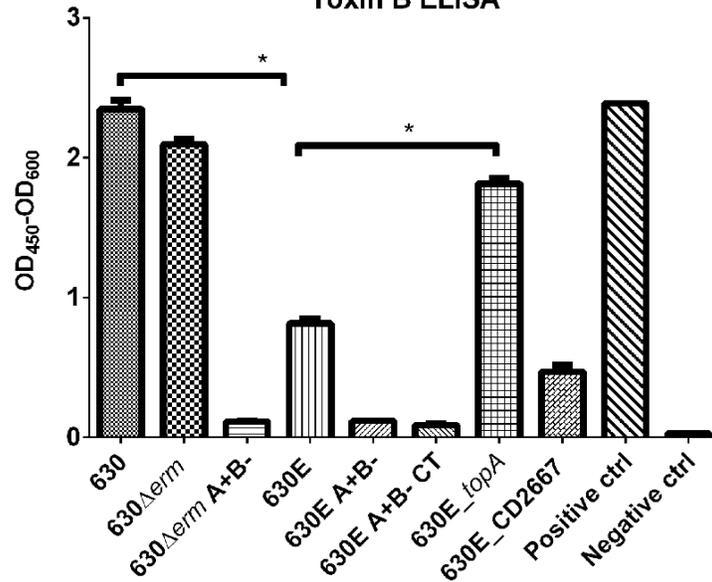
## Sporulation



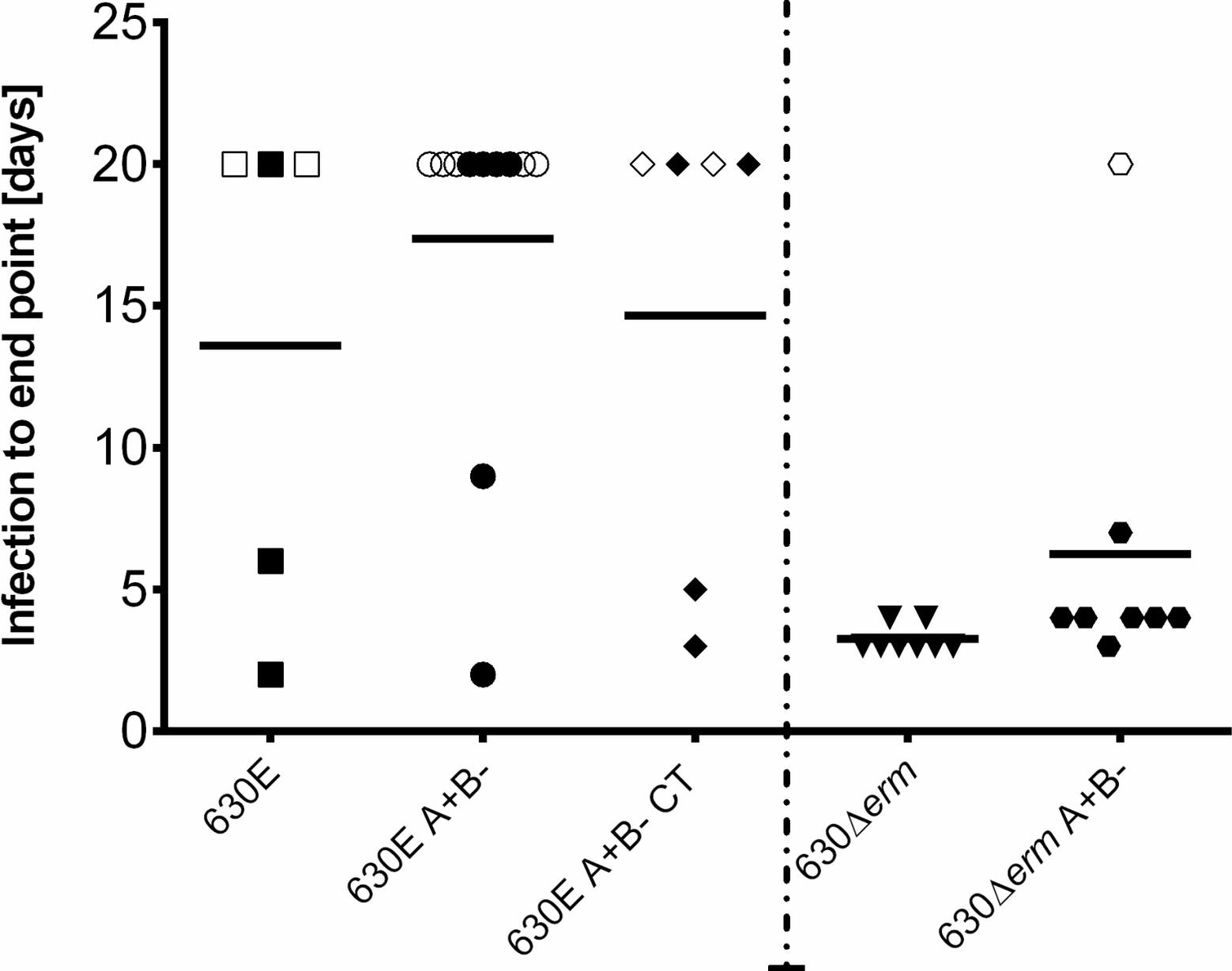
B

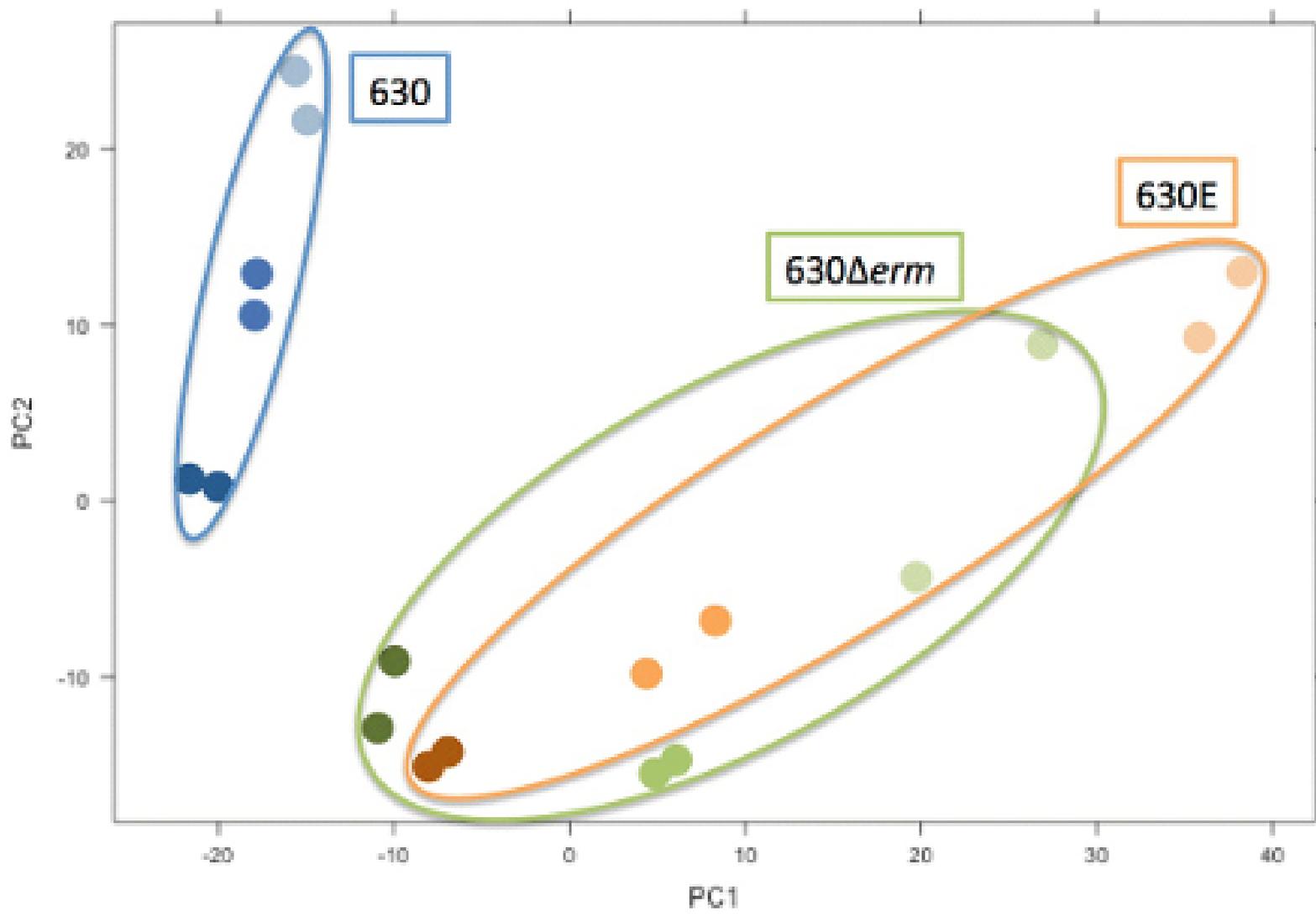
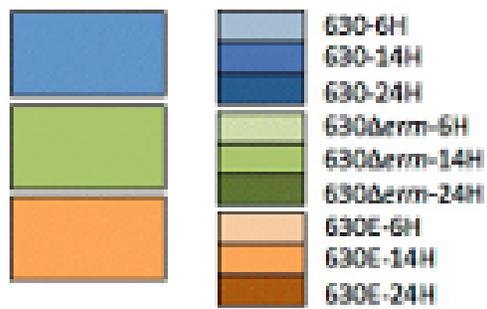
## Germination

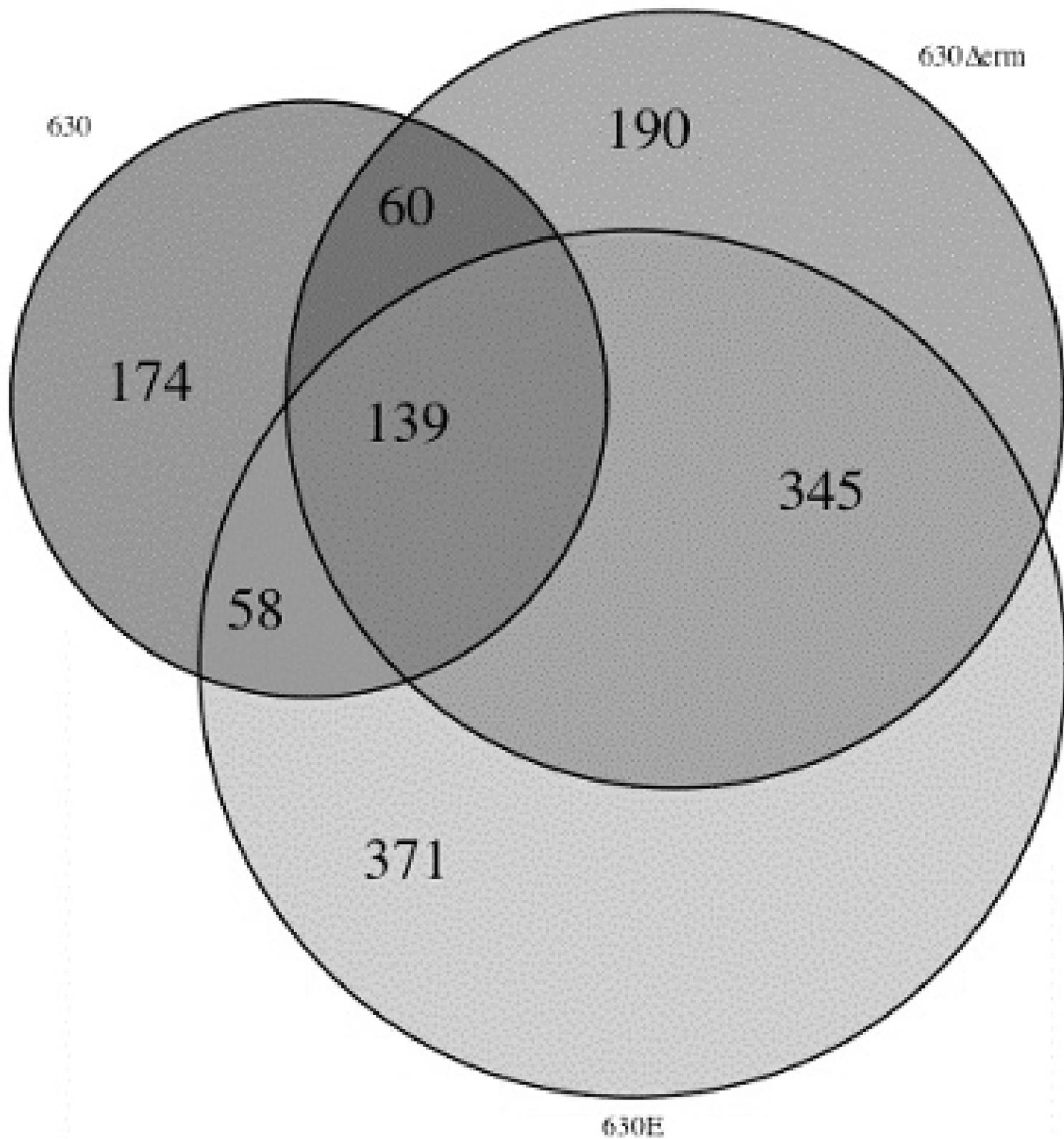


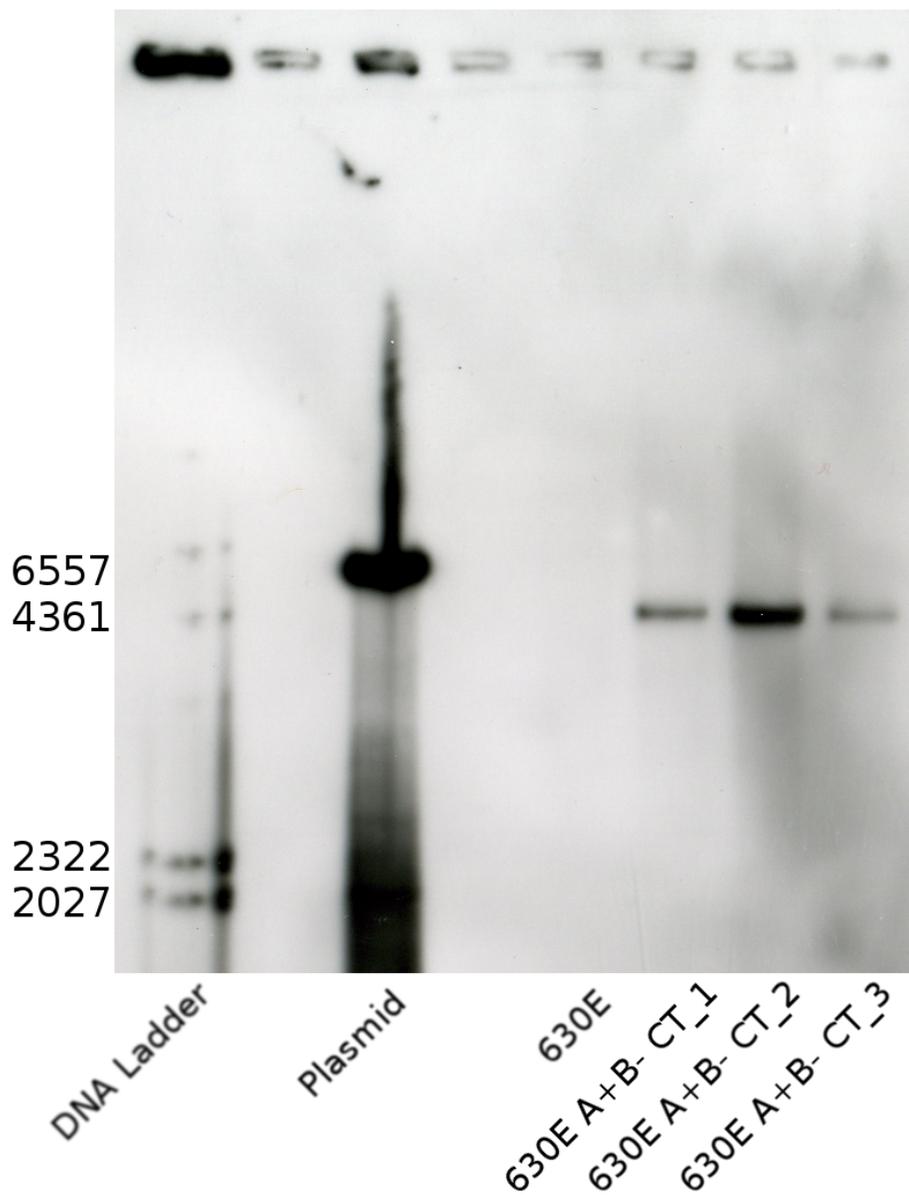
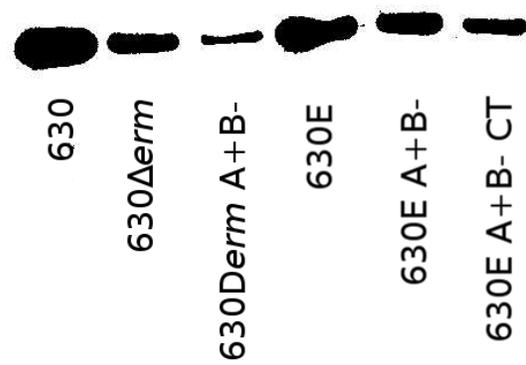
**A****72 h Toxin A and B ELISA****B****Toxin A ELISA****C****Toxin B ELISA**

# Hamster Infection Model







**A****B**

| SNP between strains                              | Name     |
|--|----------|
| <b>630E</b>                                      |          |
| CD630_07610                                      |          |
| CD630_14040                                      |          |
| CD630_20270                                      |          |
| CD630_26670                                      | PTSG-BC  |
| CD630_26270                                      |          |
| CD630_33790                                      |          |
| CD630_12740                                      | topA     |
| CD630_29430                                      |          |
| IG   |          |
| IG   |          |
| CD630_20102                                      | (pseudo) |
| <b>630<math>\Delta</math>erm</b>                 |          |
| CD630_30360                                      |          |
| CD630_19070                                      | eutG     |
| CD630_35650                                      |          |
| IG   |          |
| IG   |          |
| IG   |          |
| CD630_12140                                      | spo0A    |
| CD630_08260                                      |          |
| <b>CD630</b>                                     |          |
| CD630_32450                                      | prdR     |
| CD630_02050                                      |          |
| <b>CD630 and 630<math>\Delta</math>erm</b>       |          |
| CD630_26670                                      | PTSG-BC  |
| IG   |          |
| IG   |          |
| <b>CD630, 630<math>\Delta</math>erm and 630E</b> |          |
| CD630_22630                                      | prsA     |
| CD630_30890                                      |          |
| <b>Identical for all strains</b>                 |          |
| CD630_11900                                      |          |
| CD630_13880                                      | (pseudo) |
| <b>Mistake in original sequence</b>              |          |

|             |          |
|-------------|----------|
| CD630_17670 | gapB     |
| CD630_31561 | (pseudo) |

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**Product**

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Putative ATP-dependent RNA helicase  
Putative oligopeptide transporter  
N-carbamoyl-L-amino acid hydrolase  
PTS system, glucose-specific IIBC component  
Conserved hypothetical protein  
Putative conjugative transposon protein  
DNA topoisomerase I  
Putative phage replication protein  
Upstream of flgB  
-  
Fragment of transcriptional regulator

---

Transporter, Major Facilitator Superfamily (MFS)  
Ethanolamine iron-dependent Alcohol dehydrogenase  
Transcriptional regulator, GntR family  
Upstream of Sodium:glutamate/aspartate symporter fam  
Upstream of Lipoprotein signal peptidase  
Upstream Transcriptional regulator, GntR family  
Stage 0 sporulation protein A  
Ferric uptake regulator (perR)

---

Transcriptional regulator, sigma-54-dependent  
Transcription antiterminator, PTS operon regulator

---

PTS system, glucose-specific IIBC component  
Upstream of phage regulator

---

Peptidyl-prolyl cis-trans isomerase, PpiC-type  
PTS system, glucose-like IIBC component

---

Putative acyl-CoA N-acyltransferase  
Fragment of transcriptional regulator

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)  
Fragment of conserved hypothetical protein

| <b>Metabolic Pathway (KEEG, Microscope)</b> | <b>Position</b> | <b>630 reference</b> |
|---|-----------------|----------------------|
| RNA degradation                             | 933,139         | G                    |
| -   | 1,626,977       | A                    |
| Purine metabolism                           | 2,339,506       | G                    |
| Phosphotransferase system (PTS)             | 3,079,815       | A                    |
| -   | 3,034,953       | C                    |
| -   | 3,951,559       | C                    |
| -   | 1,480,649       | C                    |
| -   | 3,422,569       | T                    |
| -   | 309,208         | -                    |
| -   | 3,528,736       | G                    |
| -   | 2,320,410       | A                    |
| -   | 3,526,888       | T                    |
| Ethanolamine utilization                    | 2,209,236       | G                    |
| Sporulation                                 | 4,166,495       | G                    |
| -   | 2,937,176       | C                    |
| -   | 3,005,866       | T                    |
| -   | 3,591,103       | G                    |
| Sporulation                                 | 1,413,057       | INS (18)*            |
| superoxide radicals degradation             | 1,000,995       | A                    |
| Proline degradation                         | 3,797,112       | C                    |
| Phosphotransferase system (PTS)             | 268,934         | G                    |
| Phosphotransferase system (PTS)             | 3,080,703       | C                    |
| -   | 2,203,033       | A                    |
| -   | 4,007,463       | -                    |
| -   | 2,623,495       | G                    |
| Phosphotransferase system (PTS)             | 3,590,230       | T                    |
| -   | 1,391,850       | T                    |
| -   | 1,607,453       | INS (1)              |

Glycolysis / Gluconeogenesis

2,044,514

C

-

3,686,535

INS (1)

---

| Complete genome     |                       | DNA-Seq |                |                 |
|---------------------|-----------------------|---------|----------------|-----------------|
| 630 GenomeA         | 630 GenomeA           | 630wt   | 630Δerm (M)    | 630E (R)        |
| <i>Riedel et al</i> | <i>Van Eijk et al</i> |         |                |                 |
| -                   | -                     | -       | -              | T               |
| -                   | -                     | -       | -              | G               |
| -                   | -                     | -       | -              | A               |
| -                   | -                     | -       | -              | C               |
| A                   | A                     | A       | A              | -               |
| -                   | -                     | -       | -              | A               |
| -                   | -                     | -       | -              | T               |
| -                   | -                     | -       | -              | C               |
| -                   | -                     | -       | -              | INV (150 bases) |
| -                   | -                     | -       | -              | T               |
| -                   | -                     | -       | -              | C               |
| -                   | -                     | -       | A              | -               |
| -                   | A                     | -       | A              | -               |
| -                   | -                     | -       | -              | A               |
| -                   | A                     | -       | A              | -               |
| -                   | G                     | -       | G              | -               |
| -                   | A                     | -       | A              | -               |
| -                   | AATGTAGGAAATAT        | -       | AATGTAGGAAATAT | -               |
| -                   | G                     | -       | G              | -               |
| -                   | -                     | T       | -              | -               |
| -                   | -                     | T       | -              | -               |
| -                   | T                     | T       | T              | C               |
| -                   | T                     | T       | T              | -               |
| -                   | -                     | C       | C              | -               |
| -                   | -                     | -       | -              | T               |
| -                   | -                     | G       | -              | -               |
| -                   | C                     | C       | C              | C               |
| -                   | T                     | T       | T              | T               |



-

same base as reference



DNA-Seq more than 70%

DNA-Seq less than 70%

| RNA-Seq Mapping (coverage) |                     |                   | RNA-Seq* 630wt |          |
|----------------------------|---------------------|-------------------|----------------|----------|
| 630wt                      | 630Δerm (M)         | 630E (R)          | 14h / 6h       | 24h / 6h |
| G (42) / T (7)             | G (27) / T (2)      | T (3) / G (1)     | 1.00           | 1.00     |
| A (58) G (46)              | A (40) G (23)       | G (3)             | 1.00           | 1.00     |
| G (31) / A (2)             | G (27) / A (6)      | A (15) / G (5)    | 1.00           | 1.00     |
| A (88) / C (49)            | A (80)              | C (1)             | 1.00           | 1.00     |
| A (50) / C (25)            | A (60) / C (40)     | C (46) / A (6)    | 1.00           | 1.00     |
| C (3)                      |                     | C (1)             | 4.43           | 1.00     |
| T (205) / C (136)          | T (93) / C (24)     | T (49) / C (1)    | 1.00           | 1.00     |
| C (9) / T (2)              | T (1)               | C (2)             | 1.00           | 3.48     |
|                            |                     |                   | 1.00           | 1.00     |
|                            |                     |                   | 1.00           | 1.00     |
| T (12)                     |                     |                   | 1.00           | 1.00     |
| G (3)                      | G (2)               |                   | 1.00           | 5.91     |
| A (47) / G (38)            | G (17) / A (7)      | G (5) / A (1)     | 1.00           | 1.00     |
|                            |                     |                   | 8.34           | 3.00     |
|                            |                     |                   | 1.00           | 1.00     |
|                            |                     |                   | 4.19           | 1.73     |
|                            |                     |                   | 1.00           | 1.00     |
| G (250) / A (21)           | G (>1500) / A (300) | G (268) / A (250) | 7.12           | 1.00     |
|                            |                     |                   |                |          |
| C (3) / T (0)              | T (3) / C (1)       |                   | 1.00           | 1.00     |
| T (1)                      |                     |                   | 0.11           | 0.02     |
|                            |                     |                   |                |          |
| T (7)                      | T (5)               |                   | 1.00           | 1.00     |
|                            |                     |                   | 1.00           | 1.00     |
|                            |                     |                   | 1.00           | 1.00     |
|                            |                     |                   |                |          |
| G (2)                      |                     | G (1)             | 1.00           | 1.00     |
| T (140) / G (10)           | T (45) / G (7)      | T (24)            | 1.00           | 1.00     |
|                            |                     |                   |                |          |
| C (34)                     | C (11)              | C (5)             | 1.00           | 1.00     |
|                            |                     |                   | 1.00           | 1.00     |
|                            |                     |                   |                |          |

|        |       |        |      |      |
|--------|-------|--------|------|------|
| G (43) | G (8) | G (32) | 0.26 | 1.00 |
|        |       |        | 1.00 | 1.00 |

RNA-Seq mapping = DNA-Seq
Difference ex  
RNA-Seq mapping  $\neq$  DNA-Seq
INV 150 bp invers  
*\*RNA-Seq FC  $\geq 3$ , IG: gene expression after the mutation*
INS (18)\*
Identical in 6:

| RNA-Seq* 630Δerm (M) |          | RNA-Seq* 630E (R) |          | Comments                      |
|----------------------|----------|-------------------|----------|-------------------------------|
| 14h / 6h             | 24h / 6h | 14h / 6h          | 24h / 6h |                               |
| 1.00                 | 1.00     | 0.12              | 1.00     | OK                            |
| 1.00                 | 1.00     | 1.00              | 0.27     | OK                            |
| 1.00                 | 1.00     | 1.00              | 3.13     | OK                            |
| 0.05                 | 0.03     | 0.32              | 0.01     | OK                            |
| 1.00                 | 1.00     | 1.00              | 1.00     | OK                            |
| 4.5                  | 1.00     | 5.28              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 4.47     | 1.00              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 1.00     | 1.00              | 5.10     | OK with CD630wt               |
| 1.00                 | 1.00     | 1.00              | 1.00     | No expression                 |
| 1.00                 | 1.00     | 1.00              | 1.00     | CD630_20101 ermC              |
| 1.00                 | 1.00     | 1.00              | 1.00     | Low expression                |
| 1.00                 | 1.00     | 1.00              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 1.00     | 1.00              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 7.35                 | 5.68     | 5.03              | 3.39     | No expression                 |
| 1.00                 | 1.00     | 1.00              | 1.00     | No expression                 |
| 1.00                 | 3.00     | 1.00              | 1.00     | Gene after : CD630_30900 TreR |
| 1.00                 | 1.00     | 0.17              | 0.23     | No expression                 |
| 10.45                | 1.00     | 5.42              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 1.00     | 1.00              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 0.04                 | 0.08     | 0.01              | 0.01     | Low expression                |
| 0.05                 | 0.03     | 0.32              | 0.01     | OK                            |
| 1.00                 | 1.00     | 1.00              | 1.00     | No expression                 |
| 1.00                 | 1.00     | 1.00              | 1.00     | No expression                 |
| 1.00                 | 1.00     | 1.00              | 1.00     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 13.07    | 1.00              | 7.38     | RNA-Seq ≠ DNA-Seq             |
| 1.00                 | 1.00     | 1.00              | 1.00     | OK                            |
| 1.00                 | 1.00     | 1.00              | 1.00     | No expression                 |

|      |      |      |      |               |
|------|------|------|------|---------------|
| 1.00 | 1.00 | 1.00 | 3.54 | OK            |
| 1.00 | 1.00 | 1.00 | 1.00 | No expression |

pression in agreement with SNP  
ion promoter region of flgB  
30 GenomeA and 630E (R) strains



\_\_\_\_\_

| Class                   | Gene ID           | Name | Product   | ES0 14h / GH | ES0 24h / GH |
|-------------------------|-------------------|------|---|--------------|--------------|
| Cell Factor             | CD610_0001        |      | Putative peptidase, M20 family  | 0.23         |              |
| Cell Factor             | CD610_06490       |      | Putative peptidase, S9 family   | 1            | 5.88         |
| Cell Factor             | CD610_06500       |      | Putative peptidase, S9 family   | 1            | 6.92         |
| Cell Factor             | CD610_07100       |      | Putative iron-sulfur protein  | 1            | 3.32         |
| Cell Factor             | CD610_07110       |      | Putative radical SAM superfamily protein  | 1            | 0.33         |
| Cell Factor             | CD610_07130       |      | Putative radical SAM superfamily protein  | 1            | 0.28         |
| Cell Factor             | CD610_07130       |      | Putative biotin/ipoate-protein ligase   | 1            | 0.31         |
| Cell Factor             | CD610_12770       |      | Putative acyltransferase  | 1            | 3.08         |
| Cell Factor             | CD610_15600       |      | Conserved hypothetical protein  | 1            | 4.12         |
| Cell Factor             | CD610_15610       |      | Putative dehydrogenase  | 1            | 4.13         |
| Cell Factor             | CD610_16230       |      | Conserved hypothetical protein  | 1            | 1.71         |
| Cell Factor             | CD610_16786.gpp   |      | Pyruvate carboxylase  | 1            | 4.96         |
| Cell Factor             | CD610_17630       |      | Putative hydrolase, NUDIX family  | 1            | 5.13         |
| Cell Factor             | CD610_15460       |      | Putative ATP-binding protein  | 1            | 1.85         |
| Cell Factor             | CD610_21552.80a   |      | Biotin synthase   | 1            | 3.97         |
| Cell Factor             | CD610_21552.80a   |      | Thiamine biosynthesis protein ThiH  | 1            | 6.94         |
| Cell Factor             | CD610_2131.cyt1   |      | Carbonic anhydrase  | 1            | 0.29         |
| Cell Factor             | CD610_20550       |      | Putative NAD(P)-binding oxidoreductase  | 1            | 0.25         |
| Cell Factor             | CD610_30390       |      | Putative pyridine nucleotide-dehydrogenase, class I   | 1            | 3.86         |
| Cell Wall               | CD610_02410       |      | Phosphate threonine kinase  | 1            | 0.03         |
| Cell Wall               | CD610_02420       |      | Phosphotransferase N-acetylglucosamine catalysis  | 1            | 0.04         |
| Cell Wall               | CD610_02430       |      | Methyltransferase   | 1            | 0.04         |
| Cell Wall               | CD610_02440       |      | Putative GDP-glycerol-3-phosphatase/glycerophosphatase  | 1            | 0.06         |
| Cell Wall               | CD610_03060       |      | Conserved hypothetical protein  | 1            | 4.41         |
| Cell Wall               | CD610_07860       |      | Putative membrane protein   | 1            | 4.13         |
| Cell Wall               | CD610_08330       |      | Putative membrane protein   | 1            | 1.7          |
| Cell Wall               | CD610_10290       |      | Putative cell wall anchored protein   | 1            | 8.05         |
| Cell Wall               | CD610_1033.80a    |      | UDP-N-acetylglucosamine 2-epimerase (UDP-G2NAc-2-epimerase)                                       | 1            | 4.23         |
| Cell Wall               | CD610_1036.caps1  |      | Putative N-acetylglucosamoyl-L-alanine amidase, autolysin   | 1            | 0.33         |
| Cell Wall               | CD610_15850       |      | Putative N-acetylglucosamine kinase NAGK-like   | 1            | 4.51         |
| Cell Wall               | CD610_16450       |      | Putative membrane protein   | 1            | 0.25         |
| Cell Wall               | CD610_16460       |      | Putative peptidase, M19 family  | 1            | 0.23         |
| Cell Wall               | CD610_24580       |      | Putative membrane protein   | 1            | 0.21         |
| Fermentation            | CD610_1054.dct2   |      | Butyryl-CoA dehydrogenase   | 1            | 0.27         |
| Fermentation            | CD610_1055.effB   |      | Electron transfer flavoprotein subunit beta   | 1            | 0.25         |
| Fermentation            | CD610_1055.effB   |      | Electron transfer flavoprotein subunit alpha  | 1            | 0.28         |
| Fermentation            | CD610_1057.cit2   |      | 3-hydroxybutyryl-CoA dehydrogenase (Citronase)  | 1            | 0.31         |
| Fermentation            | CD610_2954.cme    |      | Aldehyde-acylating enzyme   | 1            | 0.26         |
| Membrane Transport      | CD610_03610       |      | ABC-type transport system, multidrug family ATP-binding protein                                   | 0.31         | 0.25         |
| Membrane Transport      | CD610_03620       |      | Putative radical SAM family protein   | 0.33         | 0.26         |
| Membrane Transport      | CD610_04930       |      | PTS system, mannose-specific IIC component  | 1            | 0.32         |
| Membrane Transport      | CD610_04940       |      | ABC-type transport system, tetracycline/multidrug family ATP-binding protein                      | 1            | 0.28         |
| Membrane Transport      | CD610_0563.effB   |      | ABC-type transport system, IIC component  | 1            | 0.32         |
| Membrane Transport      | CD610_0854.pqc    |      | ABC-type transport system, oligopeptide-family permease   | 1            | 0.32         |
| Membrane Transport      | CD610_0950.pqa    |      | ABC-type transport system, oligopeptide-family extracellular solute-binding protein               | 1            | 0.27         |
| Membrane Transport      | CD610_10270       |      | ABC-type transport system, multidrug family permease  | 1            | 0.77         |
| Membrane Transport      | CD610_12840       |      | ABC-type transport system, multidrug family ATP-binding protein                                   | 1            | 4.87         |
| Membrane Transport      | CD610_1584.couA   |      | ABC-type transport system, akanaevoraxin-family extracellular solute-binding protein              | 1            | 4.33         |
| Membrane Transport      | CD610_15230       |      | Putative transporter  | 1            | 3.3          |
| Membrane Transport      | CD610_17600       |      | Putative permease   | 1            | 0.16         |
| Membrane Transport      | CD610_21020       |      | Putative Hcp1/Hcp1 antipporter  | 1            | 0.04         |
| Membrane Transport      | CD610_21070       |      | Xanthine/uracil/thiamine/acarboxate permease family protein                                       | 1            | 3.62         |
| Membrane Transport      | CD610_24880       |      | PTS system, fructose-like IIC component   | 1            | 0.25         |
| Membrane Transport      | CD610_24890       |      | Transcription antiterminator, PTS operon regulator  | 1            | 4.99         |
| Membrane Transport      | CD610_2870.kglT1  |      | 3-keto-3-deoxyglucuronate permease I  | 1            | 0.28         |
| Membrane Transport      | CD610_2883.couA   |      | PTS system, cellobiose-specific IIC component   | 1            | 0.3          |
| Membrane Transport      | CD610_2884.couA   |      | PTS system, cellobiose-specific IIC component   | 1            | 0.29         |
| Membrane Transport      | CD610_30320       |      | Putative alpha-glucosylase  | 0.3          | 1            |
| Membrane Transport      | CD610_30330       |      | PTS system, mannose-specific IIC component  | 0.29         | 0.3          |
| Membrane Transport      | CD610_30340       |      | PTS system, mannose-specific IIC component  | 0.3          | 0.29         |
| Membrane Transport      | CD610_30350       |      | PTS system, mannose-specific IIC component  | 1            | 0.31         |
| Membrane Transport      | CD610_30360       |      | Transcription antiterminator, PTS operon regulator  | 0.31         | 1            |
| Membrane Transport      | CD610_3076.pgf    |      | Tageline 4-phosphatase  | 1            | 0.24         |
| Membrane Transport      | CD610_34410       |      | Putative pyridoxal phosphate-dependent transferase (PLP-dependent transferase)                    | 1            | 3.83         |
| Membrane Transport      | CD610_34420       |      | Putative peptidase, M24 family  | 1            | 3.97         |
| Membrane Transport      | CD610_34430       |      | PTS system, lactulose/cellobiose-family IIC component   | 1            | 0.22         |
| Membrane Transport      | CD610_34450       |      | PTS system, lactulose/cellobiose-family IIC component   | 1            | 3.67         |
| Membrane Transport      | CD610_35250       |      | ABC-type transport system, iron-family extracellular solute-binding protein                       | 1            | 0.08         |
| Membrane Transport      | CD610_35260       |      | ABC-type transport system, iron-family permease   | 1            | 0.06         |
| Membrane Transport      | CD610_35270       |      | ABC-type transport system, iron-family ATP-binding protein  | 1            | 0.07         |
| Metabolism Amino Acid   | CD610_03560       |      | Putative carbonylase  | 1            | 0.01         |
| Metabolism Amino Acid   | CD610_14810       |      | Putative tyrosine carbonylase   | 1            | 0.31         |
| Metabolism Amino Acid   | CD610_15473.cyt   |      | ATP phosphoribosyltransferase regulatory subunit  | 1            | 0.23         |
| Metabolism Amino Acid   | CD610_15483.hcg   |      | ATP phosphoribosyltransferase (ATP-PRFase) (ATP-PRF)  | 1            | 0.09         |
| Metabolism Amino Acid   | CD610_15483.hcg   |      | Histidinol phosphate aminotransferase (imidazole acetyl-phosphate transaminase)                   | 1            | 0.17         |
| Metabolism Amino Acid   | CD610_15505.hb    |      | Imidazole acetyl-phosphate dehydrogenase  | 1            | 0.18         |
| Metabolism Amino Acid   | CD610_15513.hch   |      | Imidazole glycerol phosphate synthase subunit HsfH  | 1            | 0.15         |
| Metabolism Amino Acid   | CD610_15523.hca   |      | 1-5-phosphoribosyl-5-S-phosphoribosylaminomethyladenine/imidazole 4-carbamoyl ion                 | 1            | 0.12         |
| Metabolism Amino Acid   | CD610_15533.hsf   |      | Imidazole glycerol phosphate synthase subunit HsfF  | 1            | 0.12         |
| Metabolism Amino Acid   | CD610_15541       |      | Histidine biosynthesis bifunctional protein HsdR [Includes: Phosphoribosyl-AMP cyclitolase Ph     | 1            | 0.2          |
| Metabolism Amino Acid   | CD610_15542.hcm2  |      | Homoserine dehydrogenase  | 1            | 0.73         |
| Metabolism Amino Acid   | CD610_17640       |      | Putative phenylalanyl-tRNA synthetase beta chain  | 1            | 0.28         |
| Metabolism Amino Acid   | CD610_20533.cya   |      | Diaminopimellic decarboxylase   | 1            | 0.27         |
| Metabolism Amino Acid   | CD610_20547.cyc   |      | Aspartakase   | 1            | 0.25         |
| Metabolism Amino Acid   | CD610_21183.cyc   |      | Threonine synthase  | 1            | 3.35         |
| Metabolism Amino Acid   | CD610_22520       |      | Glutamate 2,3-aminomutase   | 1            | 3.08         |
| Metabolism Amino Acid   | CD610_29020       |      | Putative pyridoxal phosphate-dependent transferase  | 1            | 0.17         |
| Metabolism Amino Acid   | CD610_30990       |      | Putative aminohydrolase, M202 family  | 0.04         | 0.31         |
| Metabolism Amino Acid   | CD610_31000       |      | Putative CA-4-carboxylate anaerobic carrier, Douc family  | 0.33         | 0.03         |
| Metabolism Amino Acid   | CD610_23870       |      | Alpha-hydroxy acid oxidoreductase, FMN-dependent  | 1            | 0.17         |
| Metabolism Carbon       | CD610_2410.pgdK   |      | Pyruvate phosphate dikinase (ATP-pyruvate, phosphate phosphotransferase)                          | 0.31         | 1            |
| Metabolism Carbon       | CD610_2410.pgdK   |      | Tartrate semialdehyde reductase   | 1            | 0.24         |
| Metabolism Carbon       | CD610_30140       |      | Conserved hypothetical protein  | 1            | 0.21         |
| Metabolism Cofactor     | CD610_21690       |      | Putative iron-sulfur binding protein  | 0.32         | 0.21         |
| Metabolism Lipids       | CD610_18773.couA  |      | Ethanolamine iron-dependent alcohol dehydrogenase   | 1            | 5.61         |
| Metabolism Lipids       | CD610_19085.cu5   |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 3.56         |
| Metabolism Lipids       | CD610_19095.couF  |      | Ethanolamine utilization protein, OTFase family   | 1            | 3.1          |
| Metabolism Lipids       | CD610_19123.couA  |      | Ethanolamine reductase factor for ammonia lyase-cuBC  | 1            | 3.88         |
| Metabolism Lipids       | CD610_19133.couB  |      | Ethanolamine ammonia lyase large subunit  | 1            | 12.31        |
| Metabolism Lipids       | CD610_19133.couB  |      | Ethanolamine ammonia lyase small subunit  | 1            | 28.68        |
| Metabolism Lipids       | CD610_19154.cu1   |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 17.38        |
| Metabolism Lipids       | CD610_19164.couF  |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 21.53        |
| Metabolism Lipids       | CD610_19173.couE  |      | Ethanolamine succinate dehydrogenase  | 1            | 0.16         |
| Metabolism Lipids       | CD610_19183.couM  |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 14.47        |
| Metabolism Lipids       | CD610_19193.couM  |      | Ethanolamine succinate dehydrogenase  | 1            | 16.74        |
| Metabolism Lipids       | CD610_19205.couD  |      | Putative phosphotransacetylase  | 1            | 22.6         |
| Metabolism Lipids       | CD610_19210       |      | Putative ethanolamine utilization protein   | 1            | 15.57        |
| Metabolism Lipids       | CD610_19223.couM  |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 0.32         |
| Metabolism Lipids       | CD610_19230       |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 12.09        |
| Metabolism Lipids       | CD610_19244.couM  |      | Ethanolamine carbonylase structural protein, BMC family   | 1            | 0.72         |
| Metabolism Lipids       | CD610_19255.couE2 |      | Ethanolamine utilization protein, Cupin barrels family  | 1            | 11.59        |
| Metabolism Nucleic Acid | CD610_06260       |      | Putative replicative helicase DnaB-like   | 1            | 7.06         |
| Metabolism Nucleic Acid | CD610_12720       |      | Putative magnesium chelatase  | 1            | 0.33         |
| Metabolism Nucleic Acid | CD610_17760       |      | Putative replication associated recombination protein A Rank                                      | 1            | 3.08         |
| Metabolism Nucleic Acid | CD610_20735.couA  |      | Xanthine dehydrogenase, molybdenum binding subunit  | 1            | 0.31         |
| Metabolism Nucleic Acid | CD610_24750       |      | Putative membrane protein   | 1            | 3.09         |
| Metabolism Nucleic Acid | CD610_25210       |      | Putative hydrolase  | 1            | 0.31         |
| Metabolism Nucleic Acid | CD610_25242.couD  |      | Nucleotide-metabolic adenylyltransferase  | 1            | 0.25         |
| Metabolism Nucleic Acid | CD610_30420       |      | Putative selenocysteine synthase  | 1            | 0.24         |
| Metabolism Nucleic Acid | CD610_30430       |      | Oligonucleotase   | 1            | 0.31         |
| Mobile Element          | CD610_03730       |      | Putative membrane protein Tn916-like, Ctn1-Ctn17  | 3.14         | 1            |
| Mobile Element          | CD610_06260       |      | Putative conjugative transposon protein Tn1549-like, Ctn2-Ctn21                                   | 1            | 0.28         |
| Mobile Element          | CD610_09101       |      | Putative conjugative transposon protein Tn1597, Ctn3-Ctn8   | 1            | 0.22         |
| Mobile Element          | CD610_06250       |      | Putative phage protein  | 1            | 8.03         |
| Mobile Element          | CD610_09030       |      | Putative phage protein  | 1            | 3.1          |
| Mobile Element          | CD610_09380       |      | Putative phage protein  | 1            | 3.16         |
| Mobile Element          | CD610_15630       |      | Putative phage XduK-like protein  | 1            | 3.16         |
| Mobile Element          | CD610_15640       |      | Putative phage XduK-like protein  | 1            | 3.88         |
| Mobile Element          | CD610_15650       |      | Putative phage XduK-like protein  | 1            | 4.87         |
| Mobile Element          | CD610_20090       |      | Putative phage protein  | 1            | 3.16         |
| Mobile Element          | CD610_29120       |      | Conserved hypothetical protein  | 1            | 3.76         |
| Mobile Element          | CD610_29130       |      | Putative phage protein  | 1            | 3.1          |
| Mobile Element          | CD610_29140       |      | Putative phage protein  | 1            | 3.39         |
| Mobile Element          | CD610_29190       |      | Putative phage protein  | 1            | 4.13         |
| Mobile Element          | CD610_31880       |      | Putative cell wall hydrolase Tn916-like, Ctn1-Ctn17   | 1            | 3.48         |
| Mobility                | CD610_02283.N     |      | Flagellar motor switch protein FlhK   | 1            | 0.24         |
| Mobility                | CD610_03133.flw   |      | Flagellar assembly factor Flw   | 1            | 0.03         |
| Mobility                | CD610_17940       |      | Conserved hypothetical protein  | 1            | 3.11         |
| OPFONS (Automatic)      | CD610_17600       |      | Putative iron-sulfur protein  | 1            | 0.27         |
| OPFONS (Automatic)      | CD610_18690       |      | Putative endonuclease ribonuclease Tn1549-like, Ctn2-Ctn20  | 1            | 0.33         |
| OPFONS (Automatic)      | CD610_23970       |      | Putative exonuclease  | 1            | 3.11         |
| OPFONS (Automatic)      | CD610_35038.80A   |      | BirA bifunctional protein [includes: biotin operon repressor and both--[acyl]-CoA-carboxylase(s)] | 1            | 3.31         |
| OPFONS (Automatic)      | CD610_35750       |      | Putative sodium/calcium symporter   | 1            | 0.16         |
| Regulators              | CD610_02920       |      | Transcriptional regulator, HTH-type   | 1            | 3.99         |
| Regulators              | CD610_06170       |      | Putative membrane protein   | 1            | 0.18         |
| Regulators              | CD610_06270       |      | Putative transcriptional regulator, activator Mor   | 1            | 5.63         |
| Regulators              | CD610_12460       |      | Two-component response regulator  | 1            | 3.35         |
| Regulators              | CD610_12700       |      | Two-component sensor histidine kinase   | 1            | 3.35         |
| Regulators              | CD610_17250       |      | Transcriptional regulator, ArcA family  | 1            | 3.49         |
| Regulators              | CD610_18410       |      | Two-component sensor histidine kinase   | 1            | 3.24         |
| Regulators              | CD610_23850       |      | Putative diguanylate cyclase signaling protein  | 1            | 4.63         |
| Regulators              | CD610_33613.couB  |      | ABC-type transport system, phosphate-specific ATP-binding   | 1            | 4.23         |
| Respiration Anaerobic   | CD610_3476.gap2   |      | ATP synthase translocator protein Atp2  | 1            | 4.24         |
| Sporulation             | CD610_12133.couV8 |      | Stage IV sporulation protein B, peptidase S55 family  | 1            | 3.68         |
| Stress                  | CD610_03140       |      | Putative beta-lactamase-like protein  | 1            | 0.21         |
| Stress                  | CD610_11740       |      | Putative beta-lactamase-inhibitor protein II  | 1            | 3.54         |
| Stress                  | CD610_10255.cou5  |      | Two-component sensor histidine kinase   | 1            | 3.61         |
| UNKNOWN                 | CD610_11560       |      | Conserved hypothetical protein  | 1            | 0.02         |
| UNKNOWN                 | CD610_15830       |      | Conserved hypothetical protein  | 1            | 4.18         |
| UNKNOWN                 | CD610_15831       |      | Conserved hypothetical protein  | 1            | 0.21         |
| UNKNOWN                 | CD610_17170       |      | Conserved hypothetical protein, DUF917 family   | 1            | 0.18         |
| UNKNOWN                 | CD610_17170       |      | Conserved hypothetical protein  | 1            | 0.2          |
| UNKNOWN                 | CD610_20620       |      | Conserved hypothetical protein  | 1            | 3.12         |
| UNKNOWN                 | CD610_21570       |      | Conserved hypothetical protein  | 1            | 5.84         |
| UNKNOWN                 | CD610_22590       |      | Conserved hypothetical protein with BMC-like jelly roll fold                                      | 1            | 0.28         |
| UNKNOWN                 | CD610_23860       |      | Conserved hypothetical protein  | 1            | 3.59         |
| UNKNOWN                 | CD610_24300       |      | Conserved hypothetical protein  | 1            | 0.28         |
| UNKNOWN                 | CD610_28790       |      | Conserved hypothetical protein  | 1            | 0.29         |
| UNKNOWN                 | CD610_30220       |      | Conserved hypothetical protein  | 1            | 3.28         |
| UNKNOWN                 | CD610_30430       |      | Conserved hypothetical protein  | 1            | 0.04         |
| UNKNOWN                 | CD610_31320       |      | Conserved hypothetical protein  | 1            | 3.04         |
| UNKNOWN                 | CD610_31340       |      | Conserved hypothetical protein  | 1            | 0.65         |
| UNKNOWN                 | CD610_33512       |      | Conserved hypothetical protein  | 1            | 3.3          |
| Virulence Factors       | CD610_06642.couC  |      | Negative regulator of toxin gene expression   | 1            | 0.16         |