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# The *in vitro* and *in vivo* effects of constitutive light expression on the mouse enteropathogen *Citrobacter rodentium*

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Bioluminescent reporter genes, such as those from fireflies and bacteria, let researchers use light production as a non-invasive and non-destructive surrogate measure of microbial numbers in a wide variety of environments. As bioluminescence needs microbial metabolites, tagging microorganisms with luciferases means only live metabolically active cells are detected. Despite the wide use of bioluminescent reporter genes, very little is known about the impact of continuous (also called constitutive) light expression on tagged bacteria. We have previously made a bioluminescent strain of *Citrobacter rodentium*, a bacterium which infects laboratory mice in a similar way to how enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) infect humans. In this study, we investigated whether constitutive light expression makes the bioluminescent *C. rodentium* strain ICC180 less competitive when competed against its non-bioluminescent parent (strain ICC169). To understand more about the metabolic burden of expressing light, we also compared the growth profiles of the two strains under approximately 2000 different conditions. We found that constitutive light expression in ICC180 was near-neutral in almost every non-toxic environment tested. However, we also found that the non-bioluminescent parent strain has a competitive advantage over ICC180 during infection of adult mice, although this was not enough for ICC180 to be completely outcompeted. In conclusion, our data suggests that constitutive light expression is not metabolically costly to *C. rodentium* and supports the view that bioluminescent versions of microbes can be used as a substitute for their non-bioluminescent parents to study bacterial behaviour in a wide variety of environments.



24 **Abstract**

25 Bioluminescent reporter genes, such as those from fireflies and bacteria, let researchers use  
26 light production as a non-invasive and non-destructive surrogate measure of microbial numbers  
27 in a wide variety of environments. As bioluminescence needs microbial metabolites, tagging  
28 microorganisms with luciferases means only live metabolically active cells are detected. Despite  
29 the wide use of bioluminescent reporter genes, very little is known about the impact of  
30 continuous (also called constitutive) light expression on tagged bacteria. We have previously  
31 made a bioluminescent strain of *Citrobacter rodentium*, a bacterium which infects laboratory  
32 mice in a similar way to how enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic  
33 *E. coli* (EHEC) infect humans. In this study, we investigated whether constitutive light  
34 expression makes the bioluminescent *C. rodentium* strain ICC180 less competitive when  
35 competed against its non-bioluminescent parent (strain ICC169). To understand more about the  
36 metabolic burden of expressing light, we also compared the growth profiles of the two strains  
37 under approximately 2000 different conditions. We found that constitutive light expression in  
38 ICC180 was near-neutral in almost every non-toxic environment tested. However, we also found  
39 that the non-bioluminescent parent strain has a competitive advantage over ICC180 during  
40 infection of adult mice, although this was not enough for ICC180 to be completely outcompeted.  
41 In conclusion, our data suggests that constitutive light expression is not metabolically costly to  
42 *C. rodentium* and supports the view that bioluminescent versions of microbes can be used as a  
43 substitute for their non-bioluminescent parents to study bacterial behaviour in a wide variety of  
44 environments.

## 45 Introduction

46 Bioluminescence is the by-product of a chemical reaction which has evolved in a wide variety of  
47 creatures for different purposes. This 'living light' allows fireflies like *Photinus pyralis* to find a  
48 mate<sup>1</sup>, larvae like the New Zealand glow worm *Arachnocampa luminosa* to lure prey<sup>2</sup>, and the  
49 bacterium *Aliivibrio fischeri* (formally *Vibrio fischeri*) to camouflage its nocturnal symbiont, the  
50 Hawaiian bobtail squid, while hunting<sup>3</sup>. Bioluminescence is produced by the oxidation of a  
51 substrate (a luciferin) by an enzyme (a luciferase), which usually requires energy and oxygen.  
52 Cloning of the bioluminescence genes from *P. pyralis*<sup>4</sup>, *V. fischeri*<sup>5</sup> and *Photorhabdus*  
53 *luminescens*<sup>6</sup>, has let researchers use light production as a real-time non-invasive and non-  
54 destructive surrogate measure of microbial numbers in a wide variety of different culture  
55 environments, including within laboratory animals<sup>7</sup>. This has proven particularly useful for  
56 studying microorganisms which take several weeks to grow on selective media, such as the  
57 bacterium *Mycobacterium tuberculosis*<sup>8,9</sup>. As bioluminescence requires microbial metabolites,  
58 such as ATP and reduced flavin mononucleotide (FMNH<sub>2</sub>), tagging microorganisms with  
59 luciferases means only live, metabolically active cells are detected.

60

61 Of the available bioluminescent reporter systems, the most widely used in bacteriology research  
62 is the bacterial luminescence reaction, encoded by the *lux* gene operon. The reaction involves  
63 the oxidation of a long chain aldehyde and FMNH<sub>2</sub>, resulting in the production of oxidised flavin  
64 (FMN), a long chain fatty acid, and the emission of light at 490 nm<sup>10</sup>. The reaction is catalysed  
65 by bacterial luciferase, a 77 kDa enzyme made up of an alpha and a beta subunit encoded by  
66 the *luxA* and *luxB* genes, respectively. The *luxC*, *D* and *E* genes encode the subunits of a multi-  
67 enzyme complex responsible for regenerating the aldehyde substrate from the fatty acid  
68 produced by the reaction. A significant advantage of the bacterial bioluminescence system is  
69 the ability to express the biosynthetic enzymes for substrate synthesis, allowing light to be

70 produced constitutively. One of the underlying motivations for using *lux*-tagged bacteria is the  
71 reduction in the number of animals needed for *in vivo* experiments, a legislative requirement in  
72 many countries. Using a technique known as biophotonic imaging, tagged bacteria can be non-  
73 invasively and non-destructively visualised and quantified on multiple occasions from within the  
74 same group of infected animals, whereas culture based techniques need groups of animals to  
75 be euthanised at each time point of interest<sup>7</sup>. However, very little is known about the impact of  
76 constitutive light expression on tagged bacteria. We hypothesise that light production will  
77 impose a metabolic burden on the tagged bacteria, with the actual fitness costs dependent on  
78 the host bacterial species, the site of insertion of the bioluminescence genes and their  
79 expression levels.

80

81 We have previously made a *lux*-tagged derivative of *Citrobacter rodentium*<sup>11</sup>, a bacterium that  
82 infects laboratory mice using the same virulence mechanisms as the life-threatening pathogens,  
83 enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) use to infect  
84 humans<sup>12,13</sup>. *C. rodentium* ICC180 contains a single chromosomally-located copy of the *lux*  
85 operon from *P. luminescens*, alongside a gene for resistance to the antibiotic kanamycin. We  
86 have previously non-invasively tracked ICC180 during infection of mice<sup>14</sup>, demonstrating that *C.*  
87 *rodentium* rapidly spreads between infected and uninfected animals and that bacteria shed from  
88 infected mice are 1,000 times more infectious than laboratory grown bacteria<sup>15</sup>. While we have  
89 shown that ICC180 can reach similar numbers within the gastro-intestinal tracts of infected mice  
90 when compared to its non-bioluminescent parent strain ICC169<sup>11</sup>, we have never fully  
91 investigated the impact of constitutive light expression on the fitness of ICC180.

92

93 In this study we set out to determine whether constitutive expression of the *lux* operon provides  
94 a competitive disadvantage for *C. rodentium* ICC180 when competed against its non-  
95 bioluminescent parent ICC169 in a range of *in vitro* and *in vivo* environments. We also

96 sequenced the genome and associated plasmids of ICC180 to determine whether there were  
97 any other genetic differences between the two strains, perhaps as a result of the transposon  
98 mutagenesis technique<sup>16</sup> used to generate ICC180. Finally, we compared the growth profiles of  
99 the two strains using the BIOLOG Phenotypic Microarray (PM) system, a rapid 96-well microtitre  
100 plate assay for phenotypically profiling microorganisms based on their growth under  
101 approximately 2000 different metabolic conditions<sup>17</sup>.

102

103 **Materials and methods**

104 **Bacterial strains and culture conditions.** The bacterial strains used in this study were  
105 *Citrobacter rodentium* ICC169 (spontaneous nalidixic acid resistant mutant)<sup>11</sup> and ICC180  
106 (nalidixic acid and kanamycin resistant)<sup>11</sup>. Bacteria were revived and grown from frozen stocks  
107 stored at -80°C in order to prevent adaptation of *C. rodentium* over multiple laboratory  
108 subcultures. Bacteria were grown at 37°C with shaking at 200 revolutions per minute (RPM) in  
109 LB-Lennox media (Fort Richard Laboratories Ltd., Auckland, New Zealand) or in defined  
110 minimal media (modified Davis & Mingioli media<sup>18</sup>), containing ammonium sulphate [1 g l<sup>-1</sup>],  
111 potassium dihydrogen phosphate [4.5 g l<sup>-1</sup>], dipotassium hydrogen phosphate anhydrous [10.5  
112 g l<sup>-1</sup>], sodium citrate dihydrate [5 g l<sup>-1</sup>], magnesium sulfate heptahydrate [24.65 mg l<sup>-1</sup>], thiamine  
113 [0.5 mg l<sup>-1</sup>], supplemented with 1% glucose) at 37°C. Antibiotics (kanamycin [50 ug ml<sup>-1</sup>],  
114 nalidixic acid [50 ug ml<sup>-1</sup>]) were only added to the media if they were required for selection. All  
115 chemicals and antibiotics were obtained from Sigma-Aldrich (Australia).

116 **Genome sequencing and analysis.** Genomic DNA was prepared from bacteria grown  
117 overnight in LB-Lennox broth. Whole genome sequencing was performed using the Illumina  
118 HiSeq platform by BGI (Hong Kong). A total of 3,414,820 paired-end 90 bp reads were  
119 generated for ICC169 and 3,369,194 for ICC180. Data was quality trimmed using  
120 DynamicTrim<sup>19</sup> (minimum Phred score 25) and filtering of reads shorter than 45 bp after quality  
121 trimming was performed using LengthSort<sup>19</sup>; both programmes are part of the SolexaQA  
122 software package<sup>19</sup>. After filtering, 2,444,336 paired reads were retained for ICC169 and  
123 2,383,491 for ICC180. All remaining high quality and properly paired reads were mapped to the  
124 reference strain *C. rodentium* ICC168 (Genbank accession number FN543502.1<sup>20</sup>) using the  
125 default settings in BWA<sup>21</sup>. On average, 95% of all high quality reads mapped uniquely to  
126 ICC168 (94.8% for ICC169 and 95.2% for ICC180) and single nucleotide polymorphisms  
127 (SNPs) and indels that were present only in ICC180 at 100% were identified using Samtools

128 mpileup<sup>22</sup>. SNPs and indels were confirmed by PCR and sequencing. In addition, the reads  
129 were also analysed using BreSeq version 0.24rc6<sup>23</sup>, which identified predicted mutations that  
130 were statistically valid. To locate the insertion site of the *lux* operon and kanamycin resistance  
131 ( $Km^R$ ) gene, we first performed de novo assembly on quality trimmed data for ICC180 data  
132 using EDENA v3.0<sup>24</sup>. All assembled contigs were mapped to the *C. rodentium* reference strain  
133 ICC168 using Geneious<sup>25</sup> and contigs unmapped to ICC168 were BLAST searched against the  
134 *lux* operon and  $Km^R$  gene. We located both the *lux* operon and  $Km^R$  gene on an unmapped  
135 contig 117,921 bp long. To identify the position of this contig, we broke the contig into two  
136 segments based on the location of *lux* operon and  $Km^R$  gene positions on the contig, and  
137 performed additional reference mapping to ICC168 to identify the insertion site. To determine  
138 changes to the plasmids present in *C. rodentium*, reads were also mapped to the sequenced  
139 plasmids pCROD1 (Genbank accession number FN543503.1), pCROD2 (Genbank accession  
140 number FN543504.1), pCROD3 (Genbank accession number FN543505.1), and pCRP3  
141 (Genbank accession number NC\_003114).

142 **Phenotypic microarrays.** Phenotypic microarrays were performed by BIOLOG Inc. (California,  
143 USA) as described previously<sup>17</sup>. Assays were performed in duplicate using plates PM1-20  
144 (Supplementary Table 1). The data was exported and analysed in the software package R as  
145 previously described<sup>26</sup>. Briefly, growth curves were transformed into Signal Values (SVs)<sup>27</sup>  
146 summarising the growth over time while correcting for background signal. PCA showed a clear  
147 separation by genotype, suggesting reproducible differences in metabolism between the two  
148 strains. A histogram of log signal values displayed a clear bimodal distribution, which we  
149 interpreted as representing non-respiring cells ('off', low SV) and respiring cells ('on', high SV),  
150 respectively. Normal distributions were fitted to these two distributions using the R MASS  
151 package, and these models were then used to compute log-odds ratios for each well describing  
152 the probability that each observation originated from the 'on' or 'off' distribution. Wells which

153 were at least 4 times more likely to come from the 'on' distribution than the 'off' in both replicates  
154 were considered to be actively respiring. In order to determine the significance of observed  
155 differences between genotypes, we applied the moderated t-test implemented in the limma  
156 R/Bioconductor package<sup>28</sup>. Wells with a Benjamini-Hochberg corrected P-value of less than  
157 0.05, that is allowing for a false discovery rate of 5%, and which were called as actively respiring  
158 for at least one genotype, were retained for further analysis. The data was also analysed using  
159 the DuctApe software suite<sup>29</sup>. Growth curves were analysed using the dphenome module, with  
160 the background signal subtracted from each well. Based on the results of an elbow test  
161 (Supplementary Fig.1), 7 clusters were chosen for k-means clustering. An Activity Index (AV)  
162 was created based on the clustering, ranging from 0 (minimal activity) to 6 (maximal activity).  
163 AV data was visualised using the plot and ring commands of the dphenome module.

164 **In vitro growth experiments.** Briefly, for individual growth curves, 10 ml of either LB-Lennox or  
165 defined minimal media was inoculated with 20 µl of a culture grown overnight in LB-Lennox  
166 broth. Cultures were grown at 37°C with shaking at 200 RPM and samples were removed at  
167 regular intervals to measure bioluminescence, using a VICTOR X Light Plate reader (Perkin  
168 Elmer), and viable counts, by plating onto LB-Lennox Agar (Fort Richard Laboratories Ltd.,  
169 Auckland, New Zealand). Overnight cultures were plated to retrospectively to determine the  
170 initial inocula. Experiments were performed on seven separate occasions and results used to  
171 calculate Area Under Curve values for each strain. For the competition experiments, 10 µl of a  
172 culture grown overnight in LB-Lennox broth was used to inoculate 1 ml of defined minimal  
173 media, with the mixed culture tubes receiving 5 µl of each strain. Inoculated tubes were  
174 incubated overnight at 37°C with shaking at 200 RPM, followed by serial dilution in sterile  
175 phosphate buffered saline (PBS) for plating onto LB Agar containing either nalidixic acid or  
176 kanamycin. The ratio of colonies that grew on each antibiotic plate was used to determine the  
177 proportion of each strain remaining. Experiments were performed on eight separate occasions

178 and the results used to calculate Area Under Curve (AUC) values and competitive indices (CI).  
179 CI's were calculated as follows:  $CI = [\text{strain of interest output}/\text{competing strain output}]/[\text{strain of}$   
180  $\text{interest input}/\text{competing strain input}]^{30,31}$ .

181

182 **Infection of *Galleria mellonella*.** 5<sup>th</sup> instar *Galleria mellonella* larvae (waxworms) were  
183 obtained from a commercial supplier (Biosuppliers.com, Auckland, New Zealand). Bacteria were  
184 grown overnight in LB-Lennox broth and used to infect waxworms which were pale in colour and  
185 weighed approximately 100-200 mg. Waxworms were injected into one of the last set of prolegs  
186 with 20  $\mu\text{l}$  of approximately  $10^8$  CFU of bacteria using a 1ml fine needle insulin syringe.  
187 Waxworms were injected with either ICC169, ICC180 or a 1:1 mix and incubated at 37°C.  
188 Throughout the course of a 24 h infection, individual waxworms were inspected for phenotypic  
189 changes and scored using a standardised method for assessing waxworm health (the  
190 Caterpillar Health Index [CHI]) which we have developed. Briefly, waxworms were monitored for  
191 movement, cocoon formation, melanisation, and survival. Together, these data form a numerical  
192 scale, with lower CHI scores corresponding with more serious infections and higher scores with  
193 healthier waxworms. Scores were used to calculate AUC values. Bioluminescence (given as  
194 relative light units [RLU]) was measured at regular intervals from waxworms infected with  
195 ICC180. Waxworms were placed into individual wells of a dark OptiPlate-96 well microtitre plate  
196 (Perkin Elmer) and bioluminescence measured for 1 second to provide relative light units  
197 (RLU)/second using the VICTOR X Light Plate reader. Waxworms infected with ICC169 were  
198 used as a control. Following death, or at 24 h, waxworms were homogenised in PBS and plated  
199 onto LB-Lennox Agar containing the appropriate antibiotics. Independent experiments were  
200 performed three times using 10 waxworms per group.

201 **Infection of Mice.** Female 6-7 week old C57BL/6Elite mice were provided by the Vernon  
202 Jansen Unit (University of Auckland) from specific-pathogen free (SPF) stocks. All animals were

203 housed in individually HEPA-filtered cages with sterile bedding and free access to sterilised food  
204 water. Experiments were performed in accordance with the New Zealand Animal Welfare Act  
205 (1999) and institutional guidelines provided by the University of Auckland Animal Ethics  
206 Committee, which reviewed and approved these experiments under applications R1003 and  
207 R1496. Bacteria grown overnight in LB-Lennox broth were spun at 4500 RPM for 5 minutes,  
208 and resuspended in a tenth of the volume of sterile PBS, producing a 10x concentrated  
209 inoculum. Animals were orally inoculated using a gavage needles with 200  $\mu$ l of either ICC169,  
210 ICC180, or a 1:1 mix (containing approximately  $10^8$  CFU of bacteria) and biophotonic imaging  
211 used to determine correct delivery of bacteria to the stomach. The number of viable bacteria  
212 used as an inoculum was determined by retrospective plating onto LB-Lennox Agar containing  
213 either nalidixic acid or kanamycin. Stool samples were recovered aseptically at various time  
214 points after inoculation, and the number of viable bacteria per gram of stool was determined  
215 after homogenisation at  $0.1 \text{ g ml}^{-1}$  in PBS and plating onto LB-Lennox Agar containing the  
216 appropriate antibiotics. The number and ratio of colonies growing on each antibiotic was used to  
217 calculate AUC values and CI's as described above. Independent experiments were performed  
218 twice using 6 animals per group.

219 **In vivo bioluminescence imaging.** Biophotonic imaging was used to noninvasively measure  
220 the bioluminescent signal emitted by *C. rodentium* ICC180 from anaesthetised mice to provide  
221 information regarding the localisation of the bacterium. Prior to being imaged, the abdominal  
222 area of each mouse was shaved, using a Vidal Sasoon handheld facial hair trimmer, to  
223 minimise any potential signal impedance by melanin within pigmented skin and fur.  
224 Bioluminescence (given as photons  $\text{second}^{-1} \text{ cm}^{-2} \text{ steradian} [\text{sr}]^{-1}$ ) was measured after gaseous  
225 anaesthesia with isoflurane using the IVIS<sup>®</sup> Kinetic camera system (Perkin Elmer). A  
226 photograph (reference image) was taken under low illumination before quantification of photons  
227 emitted from ICC180 at a binning of four over 1 minute using the Living Image software (Perkin

228 Elmer). The sample shelf was set to position D (field of view, 12.5 cm). For anatomic  
229 localisation, a pseudocolor image representing light intensity (blue, least intense to red, most  
230 intense) was generated using the Living Image software and superimposed over the gray-scale  
231 reference image. Bioluminescence in specific regions of individual mice also was quantified  
232 using the region of interest tool in the Living Image software program (given as photons second-  
233 <sup>1</sup>) and used to calculate AUC values for each individual animal.

234 **Statistical analyses.** Data was analysed using GraphPad Prism 6. Data was tested for  
235 normality using the D'Agostino-Pearson test; data which failed normality was analysed using a  
236 non-parametric test, while data which passed normality was analysed using a parametric test.  
237 One-tailed tests were used to test the hypothesis that constitutively expressing light gives  
238 ICC180 a differential fitness cost compared to the non-bioluminescent parent strain ICC169.  
239 When comparing multiple experimental groups, Dunn's post hoc multiple comparison test was  
240 applied.

241

242

## 243 Results

244 **Bioluminescent *Citrobacter rodentium* strain ICC180 has three altered chromosomal**  
245 **genes and a large deletion in plasmid pCROD1 in addition to insertion of the *lux* operon**  
246 **and kanamycin resistance gene.**

247 We determined the whole genome draft sequences of *C. rodentium* ICC169 and ICC180 using  
248 Illumina sequence data. Compared with sequenced type strain ICC168 (Genbank accession  
249 number FN543502.1), both strains have a substitution of a guanine (G) to an adenine (A)  
250 residue at 2,475,894 bp, resulting in an amino acid change from serine (Ser) to phenylalanine  
251 (Phe) within *gyrA*, the DNA gyrase subunit, and conferring resistance to nalidixic acid. The  
252 sequencing data indicate that the *lux* operon and kanamycin resistance gene (a 7,759 bp  
253 fragment) has inserted at 5,212,273 bp, disrupting the coding region of a putative site-specific  
254 DNA recombinase (Figure 1). In addition to the presence of the *lux* operon and kanamycin  
255 resistance gene, we found that the genome of ICC180 differs from ICC169 by two single  
256 nucleotide polymorphisms (SNPs), a single base pair insertion (of a G residue at 3,326,092 bp  
257 which results in a frameshift mutation within *ROD\_31611*, a putative membrane transporter) and  
258 a 90 bp deletion in *deoR* (deoxyribose operon repressor) (Table 1). All four plasmids previously  
259 described for *C. rodentium* were present in ICC180, however the largest of these plasmids,  
260 pCROD1, shows evidence of extensive deletion events and is missing 41 out of 60 genes  
261 (Supplementary Table 2).

262

263 **Constitutive light expression does not have a great impact on the metabolism of *C.***  
264 ***rodentium* ICC180.**

265 *C. rodentium* ICC169 and its bioluminescent derivative ICC180 were grown on two separate  
266 occasions using PM plates 1-20. We analysed the data using the DuctApe software suite which  
267 calculates an activity index (AV) for each strain in response to each well. The AV values for

268 ICC169 and ICC80 are given as colour stripes going from red (AV = 0 [not active]) to green (AV  
269 = 6 [active]; 7 total k-means clusters) (Fig. 2).

270

271 Next, the growth curve data were transformed into Signal Values (SVs) as previously  
272 described<sup>26</sup>, summarising the growth of each strain over time for each well. Wells which were  
273 considered to be actively respiring were analysed using the moderated t-test implemented in the  
274 limma R/Bioconductor package<sup>28</sup>. Those wells with a Benjamini-Hochberg corrected P-value of  
275 less than 0.05 are shown in Table 2 (with corresponding growth curves in Supplementary Fig.  
276 2). Our results indicate that the growth of the two strains significantly differed ( $p = <0.05$ ) in  
277 26/1,920 wells. Of these >80% are from the PM11-20 plates, which belong to the chemical  
278 category, suggesting that the expression of bioluminescence is near-neutral in almost every  
279 non-toxic environment. The bioluminescent strain ICC180 is able to use D-glucosamine, cytidine  
280 and Ala-His as nitrogen sources, and inositol hexaphosphate as a phosphate source, and grew  
281 significantly better than ICC169 in the presence of 11 chemicals: the antibiotics kanamycin,  
282 paromomycin, geneticin, spiramycin, rolitetracycline, doxycycline, cefoxitin; the quaternary  
283 ammonium salt dequalinium chloride; coumarin; iodonitrotetrazolium violet; and the  
284 acetaldehyde dehydrogenase inhibitor disulphiram (Table 2). That the expression of a  
285 kanamycin resistance gene also improves growth of ICC180 in the presence of related  
286 aminoglycosides is reassuring. In contrast, the wildtype strain ICC169 was able to use the  
287 nitrogen peptide Lys-Asp and grew significantly better in the presence of 8 chemicals: the metal  
288 chelators, EDTA and EGTA, sodium nitrate, the antibiotics rifampicin and phenethicillin, the  
289 fungicide oxycarboxin, the cyclic polypeptide colistin, the nucleoside analogue cytosine-1-b-D-  
290 arabinofuranoside and (Table 2). The fact that significant differences in growth rate were  
291 observed for so few conditions, provided robust and comprehensive evidence that light  
292 production is near-neutral in *C. rodentium* ICC180.

293

294 **The growth of ICC180 is not impaired during growth in rich laboratory media, when**  
295 **compared to its non-bioluminescent parent strain, but does exhibit an increased lag**  
296 **phase when grown in restricted media.**

297 We grew ICC180 and ICC169 in rich (LB-Lennox) and restricted (minimal A salts with 1%  
298 glucose supplementation) laboratory media. For ICC180, we found that bioluminescence  
299 strongly correlated with the bacterial counts recovered throughout the growth period in both rich  
300 media (Spearman's  $r = 0.9293$  [95% CI = 0.8828 - 0.9578],  $p = <0.0001$ ) and minimal media  
301 (Spearman's  $r = 0.9440$  [95% CI = 0.9001 - 0.9689],  $p = <0.0001$ ) (Fig. 3A & B, 4A & B). We  
302 also found that the growth of each strain was comparable in rich media, with no significant  
303 difference between the bacterial counts recovered over 8 hours (Fig. 3B), as demonstrated by  
304 the calculated AUC values (Fig. 3C).

305

306 In contrast, we found a significant difference between the AUC values calculated from the  
307 bacterial counts recovered from ICC180 and ICC169 growing in restricted media ( $p = 0.0078$ ,  
308 one-tailed Wilcoxon matched-pairs signed rank test) (Fig. 4C), suggesting that the  
309 bioluminescent strain would be at a competitive disadvantage in this medium. We calculated the  
310 slopes of the growth curves and found that there was no difference in the rates of growth of the  
311 two strains during exponential phase. Instead, we found a significant difference between the  
312 slopes calculated during the first 4 hours of growth (1/slope values: ICC169 =  $1.48 \times 10^{-7}$  [SD  
313  $9.98 \times 10^{-8}$ ], ICC180 =  $2.47 \times 10^{-7}$  [SD  $1.10 \times 10^{-7}$ ];  $p = 0.0041$ , one-tailed Paired  $t$  test),  
314 suggesting ICC180 spends longer in lag phase than ICC169 when grown in restricted media.

315

316 **ICC180 is not impaired in the *Galleria mellonella* infection model.**

317 We infected larvae of the Greater Wax Moth *G. mellonella* (waxworms) with ICC169 and  
318 ICC180 in single and 1:1 mixed infections. We monitored the waxworms over a 24-48 hour  
319 period for survival and disease symptoms. The Caterpillar Health Index (CHI) is a numerical

320 scoring system which measures degree of melanisation, silk production, motility, and mortality.  
321 We found that the majority of infected waxworms succumb to *C. rodentium* infection (Fig. 5A),  
322 which is reflected by the concurrent decrease in CHI score (Fig. 5B). This is in contrast to  
323 waxworms injected with PBS, who all survived and consistently scored 9-10 on the CHI scale  
324 throughout the experiments. We also found that the survival and symptoms of waxworms  
325 infected with each strain were comparable, with no significant difference between the survival  
326 curves (Fig. 5A), and calculated AUC values for the CHI scores (Fig. 5C). However, when we  
327 directly compared ICC169 and ICC180 in mixed infections of approximately 1:1, we found a  
328 significant difference in the relative abundance of the bacteria recovered from waxworms at  
329 either time of death or 24 hours, whichever occurred first ( $p = 0.001$ , one-tailed Wilcoxon  
330 matched-pairs signed rank test). Despite a slightly lower infectious dose, higher numbers of  
331 ICC180 were consistently recovered from infected waxworms (Fig. 5D).

332

333 **ICC180 is impaired in mixed but not in single infections in mice when compared to its**  
334 **non-bioluminescent parent strain.**

335 We orally gavaged groups of female 6-8 week old C57Bl/6 mice ( $n=6$ ) with  $\sim 5 \times 10^9$  CFU of  
336 ICC169 and ICC180, either individually or with a 1:1 ratio of each strain. We followed the  
337 infection dynamics by obtaining bacterial counts from stool samples (Fig. 6) and by monitoring  
338 bioluminescence from ICC180 using biophotonic imaging (Fig. 7). We found that the growth of  
339 each strain was comparable during single infections, with no significant difference between the  
340 bacterial counts recovered throughout the infection (Fig. 6A), as demonstrated by the calculated  
341 AUC values (Fig. 6B).

342

343 In contrast, we found a significant difference between the Area Under Curve values calculated  
344 from the bacterial counts recovered from ICC180 and ICC169 during mixed infections ( $p =$   
345  $0.001$ , one-tailed Wilcoxon matched-pairs signed rank test) (Fig. 6D). Our data demonstrates

346 that when in direct competition with ICC169, ICC180 is shed at consistently lower numbers from  
347 infected animals (Fig. 6C). At the peak of infection (days 6-8), this equates to over a 10-fold  
348 difference, with mice shedding a median of  $1.195 \times 10^8$  CFU (SD  $4.544 \times 10^7$ ) for ICC169  
349 compared to  $9.98 \times 10^6$  CFU (SD  $1.544 \times 10^7$ ) for ICC180. This disadvantage is reflected in the  
350 Competitive Indices we calculated from bacterial counts recovered at each time point, which for  
351 ICC180 decreases steadily throughout the course of the infection (Fig. 6E). Despite this  
352 disadvantage, ICC180 is never completely outcompeted and remains detectable in the stools of  
353 infected animals until the clearance of infection (Fig. 6C), and by biophotonic imaging until day  
354 10-13 post-infection (Fig. 7A).

355

## 356 Discussion

357 Bioluminescently-labelled bacteria have gained popularity as a powerful tool for investigating  
358 microbial pathogenicity in vivo, and for preclinical drug and vaccine development<sup>32-35</sup>. Individual  
359 infected and/or treated animals can be followed over time, in contrast to the large numbers of  
360 animals that are euthanised at specific time points of interest for quantifying bacterial loads  
361 using labour-intensive plate count methods. Most widely used is the *lux* operon of the terrestrial  
362 bacterium *P. luminescens*, which encodes for the luciferase enzyme which catalyses the  
363 bioluminescence reaction, and for a multi-enzyme complex responsible for regenerating the  
364 required substrate. As FMNH<sub>2</sub> is also required for light production, it is generally hypothesised  
365 that light production is likely to impose a metabolic burden on tagged bacteria.

366

367 The impact of expression of the *lux* operon has been reported for a number of microbial  
368 species. Sanz and colleagues created strains of *Bacillus anthracis* that emit light during  
369 germination, by introducing plasmids with *lux* operon expression driven by the *sspB* promoter<sup>36</sup>.  
370 The authors noted that the bioluminescent strains were less efficient at germinating, resulting in

371 an increase in the dose required to cause a lethal infection in mice inoculated by either the  
372 subcutaneous or intranasal route. Despite the reduced virulence, bioluminescent *B. anthracis*  
373 was still capable of successfully mounting an infection, and the use of biophotonic imaging  
374 revealed new infection niches which would have been difficult to accurately measure using  
375 traditional plating methods. Similarly, a clinical M75 isolate of *Streptococcus pyogenes* with the  
376 *lux* operon chromosomally inserted at the *spy0535* gene was found to have significantly  
377 attenuated maximal growth in vitro, as well as reduced survival in an intranasal mouse model<sup>37</sup>.  
378 The bioluminescent *Listeria monocytogenes* Xen32 strain was shown to have reduced mortality  
379 after oral inoculation of BALB/cJ mice, however subsequent investigation revealed that the  
380 chromosomally-located *lux* operon had inserted into the *flaA* gene, disrupting the ability of  
381 Xen32 to produce flagella. This suggests that the virulence attenuation observed is likely due to  
382 the location of the *lux* operon rather than the metabolic cost of light production<sup>38</sup>.

383

384 In this study, we have compared a bioluminescent-derivative of the mouse enteropathogen *C.*  
385 *rodentium*, strain ICC180, with its non-bioluminescent parent strain ICC169, using the BIOLOG  
386 Phenotypic Microarray (PM) system, which tests microbial growth under approximately 2000  
387 different metabolic conditions. Rather surprisingly, our results demonstrated that the expression  
388 of bioluminescence in ICC180 is near-neutral in almost every non-toxic environment tested,  
389 suggesting that light production is not metabolically costly to *C. rodentium*. This supports the  
390 “free lunch hypothesis” proposed by Falls and colleagues, namely that cells have an excess of  
391 metabolic power available to them<sup>39</sup>. Interestingly, ICC180 grew significantly better than its non-  
392 bioluminescent parent strain in the presence of a number of different chemicals, including  
393 several antibiotics, supporting previous findings that bacteria have many pleiotropic ways to  
394 resist toxins<sup>40</sup>. In the case of the artificial electron acceptor iodinitrotetrazolium violet, we

395 hypothesise that light production may be altering the redox balance of the cell, thus making the  
396 dye less toxic.

397

398 We also compared the ability of ICC180 and ICC169 to directly compete with one another  
399 during infection of their natural host, laboratory mice, as well as larvae of the Greater Wax Moth  
400 *G. mellonella* (waxworms). Wax worms are becoming an increasingly popular surrogate host for  
401 infectious diseases studies due to legislative requirements in many countries to replace the use  
402 of animals in scientific research. Wax worms have a well-developed innate immune system  
403 involving a cellular immune response in the form of haemocytes, and a humoral immune  
404 response in the form of antimicrobial peptides in the hemolymph<sup>41</sup>. Detection of bacterial cell  
405 wall components leads to activation of the prophenoloxidase cascade, which is similar to the  
406 complement system in mammals<sup>42</sup>, and subsequent endocytosis of bacteria by haemocytes.  
407 The haemocytes function in a similar way to mammalian neutrophils, and kill bacteria via  
408 NADPH oxidase and production of reactive oxygen species<sup>43</sup>. Again, we observed no fitness  
409 costs to constitutive light production by ICC180. Interestingly, we recovered significantly more  
410 ICC180 from wax worms infected with both ICC180 and ICC169. Similar to the response to  
411 iodinitrotetrazolium violet, an altered redox balance caused by light production could make  
412 reactive oxygen species generated by the wax worm immune response, less toxic.

413

414 In contrast, our data shows that the non-bioluminescent parent strain ICC169 has a clear  
415 competitive advantage over ICC180 during infection of adult C57Bl/6 mice, with the  
416 bioluminescent strain shed from infected animals at consistently lower numbers. Surprisingly  
417 though, this competitive advantage is not sufficient for the parent strain to outcompete and  
418 displace its bioluminescent derivative, which remains present in the gastrointestinal tract until  
419 clearance of both strains by the immune system. This suggests that there are sufficient niches

420 within the gastrointestinal tract for the two strains to coexist. This also leads us to conclude that,  
421 while ICC180 does have a fitness disadvantage, it is negligible.

422

423 It is important to note that in addition to light production, ICC180 differs from its non-  
424 bioluminescent parent strain ICC169 by lacking a putative site-specific DNA recombinase,  
425 disrupted by insertion of the *lux* operon. *C. rodentium* ICC180 was constructed by random  
426 transposon mutagenesis of ICC169 with a mini-Tn5 vector containing an unpromoted *lux* operon  
427 and kanamycin-resistance gene. As an aside, previous characterisation of the site of insertion of  
428 the *lux* operon suggested that the transposon had inserted within a homologue of the *xyIE* gene.  
429 However, whole genome sequencing has revealed that this was incorrect and the *lux* operon  
430 has inserted at 5,212,273 bp, disrupting the coding region of the putative site-specific DNA  
431 recombinase. Whole genome sequencing also revealed that ICC180 differs from ICC169 by 2  
432 non-synonymous SNPs, a single base pair insertion and a 90 bp deletion. It is unclear if these  
433 changes occurred during the process of transposon mutagenesis, and are merely 'hitch-hikers',  
434 or after laboratory passage. The single base pair insertion revealed by sequencing is of a G  
435 residue at 3,326,092 bp which results in a frameshift mutation within a putative membrane  
436 transporter, while the 90 bp deletion is within the deoxyribose operon repressor gene *deoR*. The  
437 DeoR protein represses the *deoCABD* operon, which is involved in the catabolism of  
438 deoxyribonucleotides. One SNP is the substitution of an aspartic acid (D) for a glycine (G) at  
439 residue 471 of Cts1V, a Type 6 secretion system protein involved in ATP binding. The other  
440 SNP is the substitution of a glutamic acid (E) for a glycine (G) at residue 89 of the formate  
441 acetyltransferase 2 gene *pflD*, which is involved in carbon utilisation under anaerobic  
442 conditions. Modelling suggests that once mutated, residue 89 will be unable to make several  
443 key contacts, suggesting the function of PflD will be affected. As we have not introduced these  
444 genetic differences into the non-bioluminescent parent strain, we cannot be certain that the

445 fitness costs we observed are not a result of any single or combination of these differences,  
446 rather than expression of the *lux* operon. In addition, at 54 kb the largest *C. rodentium* plasmid  
447 pCROD1 is dramatically altered in ICC180, missing 41 out of 60 of genes. This is in contrast to  
448 previous results which indicated that pCROD1 is entirely absent in ICC180<sup>44</sup>. We do not  
449 anticipate that the loss of a large part of this plasmid will have any significant impact however,  
450 as it has been shown that pCROD1 is frequently lost in *C. rodentium*, and that strains lacking  
451 pCROD1 do not show any attenuation of virulence in a C57BL/6 mouse model<sup>44</sup>.

452

453 In conclusion, our data suggests that constitutive light expression is surprisingly neutral in *C.*  
454 *rodentium*, and while it may confer a fitness disadvantage, it is negligible. This supports the view  
455 that bioluminescent versions of microbes can be used as a substitute for their non-  
456 bioluminescent parents, at least in theory. In reality, the actual fitness costs will likely depend on  
457 the host bacterial species, whether the *lux* operon is located on a multi-copy plasmid or  
458 integrated into the chromosome (and if chromosomal, the site of insertion of the operon), and  
459 the levels of expression of the *lux* genes.

460

461

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- 594  
595

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601

602

603

604

605 **Tables**

606

Position	Base change	Amino acid change	Gene	Function
2,936,285	T→C	D471G (GAC→GGC)	<i>cts1V</i>	T6SS protein Cts1V
3,999,002	T→C	E89G (GAG→GGG)	<i>pflD</i>	Formate acetyltransferase 2
3,326,092	CAG→ CAGG	Frameshift	<i>ROD_31611</i>	Major Facilitator Superfamily transporter

607

608 **Table 1. SNPs and indels that differ between the bioluminescent *C. rodentium* derivative**609 **ICC180 and its parent strain ICC169.** Sequencing revealed three points of difference between

610 ICC180 and ICC169. Two SNPs are present, each cytosine substitutions, and one guanine

611 insertion inducing a frameshift mutation. Sequencing data was analysed using BreSeq<sup>23</sup>.

612

613

614

615

616

PM Class	Substrate	Adjusted p value	Improved growth by ICC169	Improved growth by ICC180	Comment
Nitrogen	D-glucosamine	0.0159		✓	
	Cytidine	0.0280		✓	
	Ala-His	0.0316		✓	
Phosphate	Inositol hexaphosphate	0.0280		✓	
Nitrogen peptides	Lys-Asp	0.0306	✓		
Chemicals	Kanamycin	0.0076		✓	Conferred by KanR gene
	Paromomycin	0.0048		✓	Aminoglycoside -- the kanamycin cassette will be mediating resistance
	Geneticin	0.0048		✓	Aminoglycoside -- the kanamycin cassette will be mediating resistance
	Dequalinium chloride	0.0116		✓	Quaternary ammonium salt
	Spiramycin	0.0088		✓	Macrolide -- acts at ribosomal 50S, c.f. aminoglycosides at 30S
	Rolitetracycline	0.0316		✓	Tetracycline; prevents tRNA binding at 30S A-site
	Doxycycline	0.0210		✓	Tetracycline; prevents tRNA binding at 30S A-site
	Coumarin	0.0333		✓	Fragrant organic compound found in many plants
	Iodonitro tetrazolium violet (INT)	0.0087		✓	Electron acceptor, reduced by succinate dehydrogenase (and by superoxide radicals)
	EDTA	0.0048	✓		Metal chelator

	EGTA	0.0210	✓		Metal chelator
	Rifampicin	0.0048	✓		RNA polymerase inhibitor
	Colistin	0.0048	✓		Cyclic polypeptide; disrupts outer membrane
	Oxycarboxin	0.0121	✓		Fungicide
	Phenethicillin	0.0048	✓		Beta-lactam
	Cytosine-1-b-D-arabinofuranoside	0.0123	✓		Nucleoside analogue (anti-cancer/-viral)
	Sodium Nitrate	0.0306	✓		
	Cefoxitin	0.0316		✓	Beta-lactam
	Disulphiram	0.0349		✓	Inhibits acetaldehyde dehydrogenase

617

618 **Table 2. Phenotypic microarray (PM) wells in which the growth of bioluminescent *C.***  
619 ***rodentium* derivative ICC180 significantly differs from its non-bioluminescent parent**  
620 **strain ICC169.**

621

622 **Figure legends**

623

624 **Figure 1. Whole genome sequencing shows that the *lux* operon and kanamycin**  
625 **resistance gene have inserted at position 5,212,273 in the chromosome of *C. rodentium***  
626 **ICC180, disrupting a putative site-specific DNA recombinase.**

627

628 **Figure 2. The growth of *C. rodentium* ICC180 compared to its non-bioluminescent parent**  
629 **strain ICC169 as assessed by phenotypic microarray (PM).** Wildtype *C. rodentium* ICC169  
630 and its bioluminescent derivative ICC180 were grown on two separate occasions using PM  
631 plates 1-20. Activity rings from the PM data are shown where the grey inner circles indicate the  
632 strains' order and the external circle indicates the PM categories (see Key). The activity index  
633 (AV) was calculated for each strain in response to each well and the values for ICC169 are  
634 shown as colour stripes going from red (AV = 0 [not active]) to green (AV = 6 [active]; 7 total k-  
635 means clusters.

636

637 **Figure 3. *C. rodentium* ICC180 is not impaired during growth in rich laboratory media**  
638 **when compared to its non-bioluminescent parent strain ICC169.** Wildtype *C. rodentium*  
639 ICC169 (shown as purple circles) and its bioluminescent derivative ICC180 (shown as blue  
640 triangles) were grown in LB-Lennox broth and monitored for changes in bioluminescence (given  
641 as relative light units [RLU] ml<sup>-1</sup>) (A) and bacterial counts (given as colony forming units [CFU]  
642 ml<sup>-1</sup>) (B). Bacterial count data was used to calculate Area Under Curve values for each strain  
643 (C). Data (medians with ranges where appropriate) is presented from experiments performed on  
644 eight separate occasions.

645

646 **Figure 4. *C. rodentium* ICC180 is mildly impaired during growth in a defined minimal**  
647 **laboratory media when compared to its non-bioluminescent parent strain ICC169.**  
648 Wildtype *C. rodentium* ICC169 (shown as purple circles) and its bioluminescent derivative  
649 ICC180 (shown as blue triangles) were grown in minimal A salts supplemented with 1% glucose  
650 and monitored for changes in bioluminescence (given as relative light units [RLU] ml<sup>-1</sup>) (A) and  
651 bacterial counts (given as colony forming units [CFU] ml<sup>-1</sup>) (B). Bacterial count data was used to  
652 calculate Area Under Curve values for each strain, which were found to be significantly different  
653 ( $p=0.0078$ ; Wilcoxon Matched pairs-signed rank test) (C). Data (medians with ranges where  
654 appropriate) is presented from experiments performed on eight separate occasions.

655

656 **Figure 5. Bioluminescent *C. rodentium* ICC180 is not impaired in the *Galleria mellonella***  
657 **infection model.** Groups of larvae ( $n = 10$ ) of the Greater Wax Moth *Galleria mellonella* were  
658 infected with ICC169 and ICC180 in single and 1:1 mixed infections and monitored for survival  
659 (%) (A) and for disease symptoms using the Caterpillar Health Index (CHI), a numerical scoring  
660 system which measures degree of melanisation, silk production, motility, and mortality (given as  
661 median CHI values) (B). Survival curves (A) and calculated Area Under Curve data of CHI  
662 scores reveals no difference between waxworm response to infection from either strain (C).  
663 Waxworms infected with a 1:1 mix of ICC169 and ICC180 were homogenised at 24-hours, or at  
664 time of death if earlier. Actual infecting doses for each strain were determined by retrospective  
665 plating, and are indicated by \*. The bacterial burden of ICC180 and ICC169 in individual  
666 caterpillars (indicated by the dotted line), was calculated after plating onto differential media and  
667 found to be significantly different ( $p=0.001$ ; one-tailed Wilcoxon matched pairs-signed rank test)  
668 (D). Data (medians with ranges where appropriate) is presented from experiments performed on  
669 3 separate occasions, except (A) and (D), where the results of a representative experiment are  
670 shown.

671

672 **Figure 6. *C. rodentium* ICC180 is impaired during mixed, but not in single, infections in**  
673 **mice when compared to its non-bioluminescent parent strain ICC169.** Groups of female 6-  
674 8 week old C57Bl/6 mice (n=6) were orally-gavaged with  $\sim 5 \times 10^9$  CFU of wildtype *C. rodentium*  
675 ICC169 (shown as purple circles) and its bioluminescent derivative ICC180 (shown as blue  
676 triangles) in single infections (A, B) or 1:1 mixed infections (C, D) and monitored for changes in  
677 bacterial counts (given as colony forming units [CFU]  $g^{-1}$  stool) (A, B). Bacterial count data was  
678 used to calculate Area Under Curve values for each strain in single (B) and mixed (D) infections,  
679 and were found to be significantly different only for the mixed infections ( $p=0.001$ ; one-tailed  
680 Wilcoxon Matched pairs-signed rank test). This is reflected in the competitive indices (CI)  
681 calculated from the bacterial counts recovered during mixed infections, with ICC180 showing a  
682 growing competitive disadvantage from day 2 post-infection (E). Data (medians with ranges  
683 where appropriate) is presented from experiments performed on two separate occasions.

684

685 **Figure 7. Despite having a fitness disadvantage in mixed infections of mice, ICC180 is**  
686 **still visible by biophotonic imaging.** Groups of female 6-8 week old C57Bl/6 mice (n=6) were  
687 orally-gavaged with  $\sim 5 \times 10^9$  CFU of wildtype *C. rodentium* ICC169 and its bioluminescent  
688 derivative ICC180 in single infections or 1:1 mixed infections. Bioluminescence (given as  
689 photons  $second^{-1} cm^{-2} sr^{-1}$ ) from ICC180 was measured after gaseous anesthesia with isoflurane  
690 using the IVIS<sup>®</sup> Kinetic camera system (Perkin Elmer). A photograph (reference image) was  
691 taken under low illumination before quantification of photons emitted from ICC180 at a binning  
692 of four over 1 minute using the Living Image software (Perkin Elmer). The sample shelf was set  
693 to position D (field of view, 12.5 cm). The images show peak bioluminescence with variations in  
694 colour representing light intensity at a given location and superimposed over the grey-scale  
695 reference image (A). Red represents the most intense light emission, whereas blue corresponds  
696 to the weakest signal. The color bar indicates relative signal intensity (as  
697 photons/second/cm<sup>2</sup>/steradian [Sr]). Bioluminescence from the abdominal region of individual

698 mice also was quantified using the region of interest tool in the Living Image software program  
699 (given as photons second<sup>-1</sup>) and used to calculate Area Under Curve values for each individual  
700 animal (B). Dotted line represents background. Experiments were performed on two separate  
701 occasions. Three representative animals are shown.

702 **Supplementary Fig. 1. Elbow tests of phenotypic microarray array data to determine the**  
703 **number of clusters appropriate for k-means clustering. Data was analysed using the**  
704 **DuctApe software suite.**

705

706 **Supplementary Fig. 2. The growth of *C. rodentium* ICC180 compared to its non-**  
707 **bioluminescent parent strain ICC169 as assessed by phenotypic microarray (PM).**  
708 Wildtype *C. rodentium* ICC169 (shown as purple lines) and its bioluminescent derivative ICC180  
709 (shown as blue lines) were grown on two separate occasions using PM plates 1-20 (categorised  
710 by colour [see Key]). Differences between the growth of ICC169 and ICC180 in each individual  
711 well were analysed using the moderated t-test provided by limma<sup>28</sup>. Wells in which the  
712 differences had an adjusted p-value of less than 0.5 (stringent cut-off) are shown.

713

714 **Supplementary Fig. 3. Infection of larvae of the Greater Wax Moth *Galleria mellonella* with**  
715 **bioluminescent *C. rodentium* ICC180 can be visualised by luminometry.** Groups of larvae  
716 (n = 10) of the Greater Wax Moth *Galleria mellonella* were infected with ~10<sup>8</sup> CFU of *C.*  
717 *rodentium* ICC169 or ICC180 and monitored for bioluminescence using a plate luminometer.  
718 Data (medians with ranges) is presented from experiments performed on 3 separate occasions  
719 and is given as relative light units [RLU] waxworm<sup>-1</sup>.

720

721

## 722 Supplementary Table 1. BIOLOG Phenotypic Microarray assays.

PM1 – Carbon Sources	A1, Negative Control; A2, L-Arabinose; A3, N-Acetyl-D Glucosamine; A4, D-Saccharic Acid; A5, Succinic Acid; A6, D-Galactose; A7, L-Aspartic Acid; A8, L-Proline; A9, D-Alanine; A10, D-Trehalose; A11, D-Mannose; A12, Dulcitol; B1, D-Serine; B2, D-Sorbitol; B3, Glycerol; B4, L-Fucose; B5, D-Glucuronic Acid; B6, D-Gluconic Acid; B7, D,L- $\alpha$ -Glycerol Phosphate; B8, D-Xylose; B9, L-Lactic Acid; B10, Formic Acid; B11, D-Mannitol; B12, L-Glutamic Acid; C1, D-Glucose-6-Phosphate; C2, D-Galactonic Acid- $\gamma$ -Lactone; C3, D,L-Malic Acid; C4, D-Ribose; C5, Tween 20; C6, L-Rhamnose; C7, D-Fructose; C8, Acetic Acid; C9, $\alpha$ -D-Glucose; C10, Maltose; C11, D-Melibiose; C12, Thymidine; D1, L-Asparagine; D2, D-Aspartic Acid; D3, D-Glucosaminic Acid; D4, 1,2- Propanediol; D5, Tween 40; D6, $\alpha$ -Keto-Glutaric Acid; D7, $\alpha$ -Keto-Butyric Acid; D8, $\alpha$ -Methyl-D Galactoside; D9, $\alpha$ -D-Lactose; D10, Lactulose; D11, Sucrose; D12, Uridine; E1, L-Glutamine; E2, m-Tartaric Acid; E3, D-Glucose-1-Phosphate; E4, D-Fructose-6-Phosphate; E5, Tween 80; E6, $\alpha$ -Hydroxy Glutaric Acid- $\alpha$ -Lactone; E7, $\alpha$ -Hydroxy Butyric Acid; E8, $\alpha$ -Methyl-DGlucoside; E9, Adonitol; E10, Maltotriose; E11, 2-Deoxy Adenosine; E12, Adenosine; F1, Glycyl-L-Aspartic Acid; F2, Citric Acid; F3, m-Inositol; F4, D-Threonine; F5, Fumaric Acid; F6, Bromo Succinic Acid; F7, Propionic Acid; F8, Mucic Acid; F9, Glycolic Acid; F10, Glyoxylic Acid; F11, D-Cellobiose; F12, Inosine; G1, Glycyl-L-Glutamic Acid; G2, Tricarballic Acid; G3, L-Serine; G4, L-Threonine; G5, L-Alanine; G6, L-Alanyl-Glycine; G7, Acetoacetic Acid; G8, N-Acetyl- $\beta$ -D-Mannosamine; G9, Mono Methyl Succinate; G10, Methyl Pyruvate; G11, D-Malic Acid; G12, L-Malic Acid; H1, Glycyl-L-Proline; H2, p-Hydroxy Phenyl Acetic Acid; H3, m-Hydroxy Phenyl Acetic Acid; H4, Tyramine; H5, D-Psicose; H6, L-Lyxose; H7, Glucuronamide; H8, Pyruvic Acid; H9, L-Galactonic Acid- $\gamma$ -Lactone; H10, D-Galacturonic Acid; H11, Phenylethylamine; H12, 2-Amino Ethanol.
PM2 – Carbon Sources	A1, Negative Control; A2, Chondroitin Sulfate C; A3, $\alpha$ -Cyclodextrin; A4, $\beta$ -Cyclodextrin; A5, $\gamma$ -Cyclodextrin; A6, Dextrin; A7, Gelatin; A8, Glycogen; A9, Inulin; A10, Laminarin; A11, Mannan; A12, Pectin; B1, N-Acetyl-D Galactosamine; B2, N-Acetyl Neuraminic Acid; B3, $\beta$ -D-Allose; B4, Amygdalin; B5, D-Arabinose; B6, D-Arabitol; B7, L-Arabitol; B8, Arbutin; B9, 2-Deoxy-D Ribose; B10, i-Erythritol; B11, D-Fucose; B12,3-0- $\beta$ -D-Galactopyranosyl-D Arabinose; C1, Gentiobiose; C2, L-Glucose; C3, Lactitol; C4, D-Melezitose; C5, Maltitol; C6, $\alpha$ -Methyl-D Glucoside; C7, $\beta$ -Methyl-D Galactoside; C8, 3-Methyl Glucose; C9, $\beta$ -Methyl-D Glucuronic Acid; C10, $\alpha$ -Methyl-D Mannoside; C11, $\beta$ -Methyl-D Xyloside; C12, Palatinose; D1, D-Raffinose; D2, Salicin; D3, Sedoheptulosan; D4, L-Sorbose; D5, Stachyose; D6, D-Tagatose; D7, Turanose; D8, Xylitol; D9, N-Acetyl-D Glucosaminitol; D10, $\gamma$ -Amino Butyric Acid; D11, $\delta$ -Amino Valeric Acid; D12, Butyric Acid; E1, Capric Acid; E2, Caproic Acid; E3, Citraconic Acid; E4, Citramalic Acid; E5, D-Glucosamine; E6, 2-Hydroxy Benzoic Acid; E7, 4-Hydroxy Benzoic Acid; E8, $\beta$ -Hydroxy Butyric Acid; E9, $\delta$ -Hydroxy Butyric Acid; E10, $\alpha$ -Keto-Valeric Acid; E11, Itaconic Acid; E12, 5-Keto-D Gluconic Acid; F1, D-Lactic Acid Methyl Ester; F2, Malonic Acid; F3, Melibionc Acid; F4, Oxalic Acid; F5, Oxalomalic Acid; F6, Quinic Acid; F7, D-Ribono-1,4-Lactone; F8, Sebacic Acid; F9, Sorbic Acid; F10, Succinamic Acid; F11, D-Tartaric Acid; F12, L-Tartaric Acid; G1, Acetamide; G2, L-Alaninamide; G3, N-Acetyl-L Glutamic Acid; G4, L-Arginine; G5, Glycine; G6, L-Histidine; G7, L-Homoserine; G8, Hydroxy-L Proline; G9, L-Isoleucine; G10, L-Leucine; G11, L-Lysine; G12, L-Methionine; H1, L-Ornithine; H2, L-Phenylalanine; H3, L-Pyroglutamic Acid; H4, L-Valine; H5, D,L-Carnitine; H6, Sec-Butylamine; H7, D,L-Octopamine; H8, Putrescine; H9, Dihydroxy Acetone; H10, 2,3-Butanediol; H11, 2,3-Butanone; H12, 3-Hydroxy 2-Butanone.
PM3 – Nitrogen Sources	A1, Negative Control; A2, Ammonia; A3, Nitrite; A4, Nitrate; A5, Urea; A6, Biuret; A7, L-Alanine; A8, L-Arginine; A9, L-Asparagine; A10, L-Aspartic Acid; A11, L-Cysteine; A12, L-

	<p>Glutamic Acid; B1, L-Glutamine; B2, Glycine; B3, L-Histidine; B4, L-Isoleucine; B5, L-Leucine; B6, L-Lysine; B7, L-Methionine; B8, L-Phenylalanine; B9, L-Proline; B10, L-Serine; B11, L-Threonine; B12, L-Tryptophan; C1, L-Tyrosine; C2, L-Valine; C3, D-Alanine; C4, D-Asparagine; C5, D-Aspartic Acid; C6, D-Glutamic Acid; C7, D-Lysine; C8, D-Serine; C9, D-Valine; C10, L-Citrulline; C11, L-Homoserine; C12, L-Ornithine; D1, N-Acetyl-L Glutamic Acid; D2, N-Phthaloyl-L Glutamic Acid; D3, L-Pyroglutamic Acid; D4, Hydroxylamine; D5, Methylamine; D6, N-Amylamine; D7, N-Butylamine; D8, Ethylamine; D9, Ethanolamine; D10, Ethylenediamine; D11, Putrescine; D12, Agmatine; E1, Histamine; E2, <math>\beta</math>-Phenylethylamine; E3, Tyramine; E4, Acetamide; E5, Formamide; E6, Glucuronamide; E7, D,L-Lactamide; E8, D-Glucosamine; E9, D-Galactosamine; E10, D-Mannosamine; E11, N-Acetyl-D Glucosamine; E12, N-Acetyl-D Galactosamine; F1, N-Acetyl-D Mannosamine; F2, Adenine; F3, Adenosine; F4, Cytidine; F5, Cytosine; F6, Guanine; F7, Guanosine; F8, Thymine; F9, Thymidine; F10, Uracil; F11, Uridine; F12, Inosine; G1, Xanthine; G2, Xanthosine; G3, Uric Acid; G4, Alloxan; G5, Allantoin; G6, Parabanic Acid; G7, D,L-<math>\alpha</math>-Amino-N Butyric Acid; G8, <math>\beta</math>-Amino-N Butyric Acid; G9, <math>\epsilon</math>-Amino-N Caproic Acid; G10, D,L-<math>\alpha</math>-Amino Caprylic Acid; G11, <math>\delta</math>-Amino-N Valeric Acid; G12, <math>\alpha</math>-Amino-N Valeric Acid; H1, Ala-Asp; H2, Ala-Gln; H3, Ala-Glu; H4, Ala-Gly; H5, Ala-His; H6, Ala-Leu; H7, Ala-Thr; H8, Gly-Asn; H9, Gly-Gln; H10, Gly-Glu; H11, Gly-Met; H12, Met-Ala.</p>
PM4 – Phosphorus and Sulfur Sources	<p>A1, Negative Control; A2, Phosphate; A3, Pyrophosphate; A4, Trimetaphosphate; A5, Tripolyphosphate; A6, Triethyl Phosphate; A7, Hypophosphite; A8, Adenosine-2'-monophosphate; A9, Adenosine-3'-monophosphate; A10, Adenosine-5'-monophosphate; A11, Adenosine-2',3'-cyclic monophosphate; A12, Adenosine-3',5'-cyclic monophosphate; B1, Thiophosphate; B2, Dithiophosphate; B3, D,L-<math>\alpha</math>-Glycerol Phosphate; B4, <math>\beta</math>-Glycerol Phosphate; B5, Carbamyl Phosphate; B6, D-2-Phospho Glyceric Acid; B7, D-3-Phospho Glyceric Acid; B8, Guanosine-2'-monophosphate; B9, Guanosine-3'-monophosphate; B10, Guanosine-5'-monophosphate; B11, Guanosine-2',3'-cyclic monophosphate; B12, Guanosine-3',5'-cyclic monophosphate; C1, Phosphoenol Pyruvate; C2, Phospho Glycolic Acid; C3, D-Glucose-1-Phosphate; C4, D-Glucose-6-Phosphate; C5, 2-Deoxy-D Glucose 6-Phosphate; C6, D-Glucosamine-6-Phosphate; C7, 6-Phospho Gluconic Acid; C8, Cytidine-2'-monophosphate; C9, Cytidine-3'-monophosphate; C10, Cytidine-5'-monophosphate; C11, Cytidine-2',3'-cyclic monophosphate; C12, Cytidine-3',5'-cyclic monophosphate; D1, D-Mannose-1-Phosphate; D2, D-Mannose-6-Phosphate; D3, Cysteamine-S Phosphate; D4, Phospho-L Arginine; D5, O-Phospho-D Serine; D6, O-Phospho-L Serine; D7, O-Phospho-L Threonine; D8, Uridine-2'-monophosphate; D9, Uridine-3'-monophosphate; D10, Uridine-5'-monophosphate; D11, Uridine-2',3'-cyclic monophosphate; D12, Uridine-3',5'-cyclic monophosphate; E1, O-Phospho-D Tyrosine; E2, O-Phospho-L Tyrosine; E3, Phosphocreatine; E4, Phosphoryl Choline; E5, O-Phosphoryl Ethanolamine; E6, Phosphono Acetic Acid; E7, 2-Aminoethyl Phosphonic Acid; E8, Methylene Diphosphonic Acid; E9, Thymidine-3'-monophosphate; E10, Thymidine-5'-monophosphate; E11, Inositol Hexaphosphate; E12, Thymidine 3',5'-cyclic monophosphate; F1, Negative Control; F2, Sulfate; F3, Thiosulfate; F4, Tetrathionate; F5, Thiophosphate; F6, Dithiophosphate; F7, L-Cysteine; F8, D-Cysteine; F9, L-Cysteinyl Glycine; F10, L-Cysteic Acid; F11, Cysteamine; F12, L-Cysteine Sulfinic Acid; G1, N-Acetyl-L Cysteine; G2, S-Methyl-L Cysteine; G3, Cystathionine; G4, Lanthionine; G5, Glutathione; G6, D,L-Ethionine; G7, L-Methionine; G8, D-Methionine; G9, Glycyl-L Methionine; G10, N-Acetyl-D,L Methionine; G11, L- Methionine Sulfoxide; G12, L-Methionine Sulfone; H1, L-Djenkolic Acid; H2, Thiourea; H3, 1-Thio-<math>\beta</math>-D Glucose; H4, D,L-Lipoamide; H5, Taurocholic Acid; H6, Taurine; H7, Hypotaurine; H8, p-Amino Benzene Sulfonic Acid; H9, Butane Sulfonic Acid; H10, 2-Hydroxyethane Sulfonic Acid; H11, Methane Sulfonic Acid;</p>

	H12, Tetramethylene Sulfone.
PM5 – Nutrient Supplements	A1, Negative Control; A2, Positive Control; A3, L-Alanine; A4, L-Arginine; A5, L-Asparagine; A6, L-Aspartic Acid; A7, L-Cysteine, A8, L-Glutamic Acid; A9, Adenosine-3',5'-cyclic monophosphate; A10, Adenine; A11, Adenosine; A12, 2'-Deoxy Adenosine; B1, L-Glutamine; B2, Glycine; B3, L-Histidine; B4, L-Isoleucine; B5, L-Leucine; B6, L-Lysine; B7, L-Methionine; B8, L-Phenylalanine; B9, Guanosine-3',5'-cyclic monophosphate; B10, Guanine; B11, Guanosine; B12, 2'-Deoxy Guanosine; C1, L-Proline; C2, L-Serine; C3, L-Threonine; C4, L-Tryptophan; C5, L-Tyrosine; C6, L-Valine; C7, L-isoleucine + L-Valine; C8, trans-4-Hydroxy L-Proline; C9, (5) 4-Aminolmidazole-4(5)-Carboxamide; C10, Hypoxanthine; C11, Inosine; C12, 2'-Deoxy Inosine; D1, L-Ornithine; D2, L-Citrulline; D3, Chorismic Acid; D4, (-)Shikimic Acid; D5, L-Homoserine Lactone; D6, D-Alanine; D7, D-Aspartic Acid; D8, D-Glutamic Acid; D9, D,L- $\alpha,\epsilon$ -Diaminopimelic Acid; D10, Cytosine; D11, Cytidine; D12, 2'-Deoxy Cytidine; E1, Putrescine; E2, Spermidine; E3, Spermine; E4, Pyridoxine; E5, Pyridoxal; E6, Pyridoxamine; E7, $\beta$ -Alanine; E8, D-Pantothenic Acid; E9, Orotic Acid; E10, Uracil; E11, Uridine; E12, 2'-Deoxy Uridine; F1, Quinolinic Acid; F2, Nicotinic Acid; F3, Nicotinamide; F4, $\beta$ -Nicotinamide Adenine Dinucleotide; F5, $\delta$ -Amino Levulinic Acid; F6, Hematin F7, Deferoxamine; Mesylate; F8, D-(+)-Glucose; F9, N-Acetyl D-Glucosamine; F10, Thymine; F11, Glutathione (reduced form); F12, Thymidine; G1, Oxaloacetic Acid; G2, D-Biotin; G3, CyanoCobalamine; G4, p-Amino Benzoic Acid; G5, Folic Acid; G6, Inosine +Thiamine; G7, Thiamine; G8, Thiamine Pyrophosphate; G9, Riboflavin; G10, Pyrrolo-Quinoline Quinone; G11, Menadione; G12, m-Inositol; H1, Butyric Acid; H2, D,L- $\alpha$ -Hydroxy Butyric Acid; H3, $\alpha$ -Keto Butyric Acid; H4, Caprylic Acid; H5, D,L- $\alpha$ -Lipoic Acid (oxidized form); H6, D,L-Mevalonic Acid; H7, D,L-Carnitine; H8, Choline; H9, Tween 20; H10, Tween 40; H11, Tween 60; H12, Tween 80.
PM6 – Peptide Nitrogen sources	A1, Negative Control; A2, Positive Control: L-Glutamine; A3, Ala-Ala; A4, Ala-Arg; A5, Ala-Asn; A6, Ala-Glu; A7, Ala-Gly; A8, Ala-His; A9, Ala-Leu; A10, Ala-Lys; A11, Ala-Phe; A12, Ala-Pro; B1, Ala-Ser; B2, Ala-Thr; B3, Ala-Trp; B4, Ala-Tyr; B5, Arg-Ala; B6, Arg-Arg; B7, Arg-Asp; B8, Arg-Gln; B9, ; rg-Glu; B10, Arg-Ile; B11, Arg-Leu; B12, Arg-Lys; C1, Arg-Met; C2, Arg-Phe; C3, Arg-Ser; C4, Arg-; rp; C5, Arg-Tyr; C6, Arg-Val; C7, Asn-Glu; C8, Asn-Val; C9, Asp-Asp; C10, Asp-Glu; C11, Asp-Leu; C12, Asp-Lys; D1, Asp-Phe; D2, Asp-Trp; D3, Asp-Val; D4, Cys-Gly; D5, Gln-Gln ;D6, Gln-Gly; D7, Glu-Asp; D8, Glu-Glu; D9, Glu-Gly; D10, Glu-Ser; D11, Glu-Trp; D12, Glu-Tyr; E1, Glu-Val; E2, Gly-Ala; E3, Gly-Arg; E4, Gly-Cys; E5, Gly-Gly; E6, Gly-His; E7, Gly-Leu; E8, Gly-Lys; E9, Gly-Met; E10, Gly-Phe; E11, Gly-Pro; E12, Gly-Ser; F1, Gly-Thr; F2, Gly-Trp; F3, Gly-Tyr; F4, Gly-Val; F5, His-Asp; F6, His-Gly; F7, His-Leu; F8, His-Lys; F9, His-Met; F10, His-Pro; F11 ,His-Ser; F12, His-Trp; G1, His-Tyr; G2, His-Val; G3, Ile-Ala; G4, Ile-Arg; G5, Ile-Gln; G6, Ile-Gly; G7, Ile-His; G8, Ile-Ile; G9, Ile-Met; G10, Ile-Phe; G11, Ile-Pro; G12, Ile-Ser; H1, Ile-Trp; H2, Ile-Tyr; H3, Ile-Val; H4, Leu-Ala; H5, Leu-Arg; H6, Leu-Asp; H7, Leu-Glu; H8, Leu-Gly; H9, Leu-Ile; H10, Leu-Leu; H11, Leu-Met; H12, Leu-Phe.
PM7 – Peptide Nitrogen sources	A1, Negative Control; A2, Positive Control: L-Glutamine; A3, Leu-Ser; A4, Leu-Trp; A5, Leu-Val; A6, Lys-Ala; A7, Lys-Arg; A8, Lys-Glu; A9, Lys-Ile; A10, Lys-Leu; A11, Lys-Lys; A12, Lys-Phe; B1, Lys-Pro; B2, Lys-Ser; B3, Lys-Thr; B4, Lys-Trp; B5 ,Lys-Tyr; B6, Lys-Val; B7, Met-Arg; B8, Met-Asp; B9, Met-Gln; B10, Met-Glu; B11, Met-Gly; B12, Met-His; C1, Met-Ile; C2, Met-Leu; C3, Met-Lys; C4, Met-Met; C5, Met-Phe; C6, Met-Pro; C7, Met-Trp; C8, Met-Val; C9, Phe-Ala; C10, Phe-Gly; C11, Phe-Ile; C12, Phe-Phe; D1, Phe-Pro; D2, Phe-Ser; D3, Phe-Trp; D4, Pro-Ala; D5, Pro-Asp; D6, Pro-Gln; D7, Pro-Gly; D8, Pro-Hyp; D9, Pro-Leu; D10, Pro-Phe; D11, Pro-Pro; D12, Pro-Tyr; E1, Ser-Ala; E2, Ser-Gly; E3, Ser-His; E4, Ser-Leu; E5, Ser-Met; E6, Ser-Phe; E7, Ser-Pro; E8, Ser-Ser; E9, Ser-Tyr; E10, Ser-Val; E11, Thr-Ala; E12, Thr-Arg; F1, Thr-Glu; F2, Thr-Gly; F3, Thr-Leu; F4, Thr-Met; F5, Thr-Pro; F6, Trp-Ala; F7, Trp-Arg; F8, Trp-Asp; F9, Trp-Glu; F10, Trp-Gly;

	F11, Trp-Leu; F12, Trp-Lys; G1, Trp-Phe; G2, Trp-Ser; G3, Trp-Trp; G4, Trp-Tyr; G5, Tyr-Ala; G6, Tyr-Gln; G7, Tyr-Glu; G8, Tyr-Gly G9, Tyr-His; G10, Tyr-Leu; G11, Tyr-Lys; G12, Tyr-Phe; H1, Tyr-Trp; H2, Tyr-Tyr; H3, Val-Arg; H4, Val-Asn; H5, Val-Asp; H6, Val-Gly; H7, Val-His; H8, Val-Ile; H9, Val-Leu; H10, Val-Tyr; H11, Val-Val; H12, Y-Glu-Gly.
PM8 – Peptide Nitrogen sources	A1, Negative Control; A2, Positive Control: L-Glutamine; A3, Ala-Asp; A4, Ala-Gln; A5, Ala-Ile; A6, Ala-Met; A7, Ala-Val; A8, Asp-Ala; A9, Asp-Gln; A10, Asp-Gly; A11, Glu-Ala; A12, Gly-Asn; B1, Gly-Asp; B2, Gly-Ile; B3, His-Ala; B4, His-Glu; B5, His-His; B6, Ile-Asn; B7, Ile-Leu; B8, Leu-Asn; B9, Leu-His; B10, Leu-Pro; B11, Leu-Tyr; B12, Lys-Asp; C1, Lys-Gly; C2, Lys-Met; C3, Met-Thr; C4, Met-Tyr; C5, Phe-Asp; C6, Phe-Glu; C7, Gln-Glu; C8, Phe-Met; C9, Phe-Tyr; C10, Phe-Val; C11, Pro-Arg; C12, Pro-Asn; D1, Pro-Glu; D2, Pro-Ile; D3, Pro-Lys; D4, Pro-Ser; D5, Pro-Trp; D6, Pro-Val; D7, Ser-Asn; D8, Ser-Asp; D9, Ser-Gln; D10, Ser-Glu; D11, Thr-Asp; D12, Thr-Gln; E1, Thr-Phe; E2, Thr-Ser; E3, Trp-Val; E4, Tyr-Ile; E5, Tyr-Val; E6, Val-Ala; E7, Val-Gln; E8, Val-Glu; E9, Val-Lys; E10, Val-Met; E11, Val-Phe; E12, Val-Pro; F1, Val-Ser; F2, $\beta$ -Ala-Ala; F3, $\beta$ -Ala-Gly; F4, $\beta$ -Ala-His; F5, Met- $\beta$ -Ala; F6, $\beta$ -Ala-Phe; F7, D-Ala-D-Ala; F8, D-Ala-Gly; F9, D-Ala-Leu; F10, D-Leu-D-Leu; F11, D-Leu-Gly; F12, D-Leu-Tyr; G1, Y-Glu-Gly; G2, Y-D-Glu-Gly; G3, Gly-D-Ala; G4, Gly-D-Asp; G5, Gly-D-Ser; G6, Gly-D-Thr; G7, Gly-D-Val; G8, Leu- $\beta$ -Ala; G9, Leu-D-Leu; G10, Phe- $\beta$ -Ala; G11, Ala-Ala-Ala; G12, D-Ala-Gly-Gly; H1, Gly-Gly-Ala; H2, Gly-Gly-D-Leu; H3, Gly-Gly-Gly; H4, Gly-Gly-Ile; H5, Gly-Gly-Leu; H6, Gly-Gly-Phe; H7, Val-Tyr-Val; H8, Gly-Phe-Phe; H9, Leu-Gly-Gly; H10, Leu-Leu-Leu; H11, Phe-Gly-Gly; H12, Tyr-Gly-Gly.
PM9 – Osmolytes	A1, NaCl 1%; A2, NaCl 2%; A3, NaCl 3%; A4, NaCl 4%; A5, NaCl 5%; A6, NaCl 5.5%; A7, NaCl 6%; A8, NaCl 6.5%; A9, NaCl 7%; A10, NaCl 8%; A11, NaCl 9%; A12, NaCl 10%; B1, NaCl 6%; B2, NaCl 6% +Betaine; B3, NaCl 6% +N-N Dimethyl Glycine; B4, NaCl 6% + Sarcosine; B5, NaCl 6% + Dimethyl sulphonyl propionate; B6, NaCl 6% + MOPS; B7, NaCl 6% + Ectoine; B8, NaCl 6% + Choline; B9, NaCl 6% + Phosphoryl Choline; B10, NaCl 6% + Creatine; B11, NaCl 6% + Creatinine; B12, NaCl 6% + L-Carnitine; C1, NaCl 6% + KCl; C2, NaCl 6% + L-Proline; C3, NaCl 6% + N-Acetyl L-Glutamine; C4, NaCl 6% + $\beta$ -Glutamic Acid; C5, NaCl 6% + $\gamma$ -Amino -N Butyric Acid; C6, NaCl 6% + Glutathione; C7, NaCl 6% + Glycerol; C8, NaCl 6% +Trehalose; C9, NaCl 6% + TrimethylamineN-oxide; C10, NaCl 6% + Trimethylamine; C11, NaCl 6% + Octopine; C12, NaCl 6% + Trigonelline; D1, Potassium chloride 3%; D2, Potassium chloride 4%; D3, Potassium chloride 5%; D4, Potassium chloride 6%; D5, Sodium sulphate 2%; D6, Sodium sulphate 3%; D7, Sodium sulphate 4%; D8, Sodium sulphate 5%; D9, Ethylene glycol 5%; D10, Ethylene glycol 10%; D11, Ethylene glycol 15%; D12, Ethylene glycol 20%; E1, Sodium formate 1%; E2, Sodium formate 2%; E3, Sodium formate 3%; E4, Sodium formate 4%; E5, Sodium formate 5%; E6, Sodium formate 6%; E7, Urea 2%; E8, Urea 3%; E9, Urea 4%; E10, Urea 5%; E11, Urea 6%; E12, Urea 7%; F1, Sodium Lactate 1%; F2, Sodium Lactate 2%; F3, Sodium Lactate 3%; F4, Sodium Lactate 4%; F5, Sodium Lactate 5%; F6, Sodium Lactate 6%; F7, Sodium Lactate 7%; F8, Sodium Lactate 8%; F9, Sodium Lactate 9%; F10, Sodium Lactate 10%; F11, Sodium Lactate 11%; F12, Sodium Lactate 12%; G1, Sodium Phosphate pH 7 20mM; G2, Sodium Phosphate pH 7 50mM; G3, Sodium Phosphate pH 7 100mM; G4, Sodium Phosphate pH 7 200mM; G5, Sodium Benzoate pH 5.2 20mM; G6, Sodium Benzoate pH 5.2 50mM; G7, Sodium Benzoate pH5.2 100mM; G8, Sodium Benzoate pH 5.2 200mM; G9, Ammonium sulfate pH8 10mM; G10, Ammonium sulfate pH 8 20mM; G11, Ammonium sulfate pH 8 50mM; G12, Ammonium sulfate pH8 100mM; H1, Sodium Nitrate 10mM; H2, Sodium Nitrate 20mM; H3, Sodium Nitrate 40mM; H4, Sodium Nitrate 60mM; H5, Sodium Nitrate 80mM; H6, Sodium Nitrate 100mM; H7, Sodium Nitrite 10mM; H8, Sodium Nitrite 20mM; H9, Sodium Nitrite 40mM; H10, Sodium Nitrite 60mM; H11, Sodium Nitrite 80mM; H12, Sodium Nitrite 100mM.

PM10 – pH	<p>A1, pH 3.5; A2, pH 4; A3, pH 4.5; A4, pH 5; A5, pH 5.5; A6, pH 6; A7, pH 7; A8, pH 8; A9, pH 8.5; A10, pH 9; A11, pH 9.5; A12, pH 10; B1, pH 4.5; B2, pH 4.5 + L-Alanine; B3, pH 4.5 + L-Arginine; B4, pH 4.5 + L-Asparagine; B5, pH 4.5 + L-Aspartic Acid; B6, pH 4.5 + L-Glutamic Acid; B7, pH 4.5 + L-Glutamine; B8, pH 4.5 + Glycine; B9, pH 4.5 + L-Histidine; B10, pH 4.5 + L-Isoleucine; B11, pH 4.5 + L-Leucine; B12, pH 4.5 + L-Lysine; C1, pH 4.5 + L-Methionine; C2, pH 4.5 + L-Phenylalanine; C3, pH 4.5 + L-Proline; C4, pH 4.5 + L-Serine; C5, pH 4.5 + L-Threonine; C6, pH 4.5 + L-Tryptophan; C7, pH 4.5 + L-Citrulline; C8, pH 4.5 + L-Valine; C9, pH 4.5 + HydroxyL-Proline; C10, pH 4.5 + L-Ornithine; C11, pH 4.5 + L-Homoarginine; C12, pH 4.5 + L-Homoserine; D-1, pH 4.5 + Anthranilic Acid; D2, pH 4.5 + L-Norleucine; D3, pH 4.5 + L-Norvaline; D4, pH 4.5 + <math>\alpha</math>-Amino-N Butyric Acid; D5, pH 4.5 + p-Amino Benzoic Acid; D6, pH 4.5 + L-Cysteic Acid; D7, pH 4.5 + D-Lysine; D8, pH 4.5 + 5-Hydroxy Lysine; D9, pH 4.5 + 5-Hydroxy Tryptophan; D10, pH 4.5 + D,L-Diamino pimelic Acid; D11, pH 4.5 + Trimethylamine N-oxide; D12, pH 4.5 + Urea; E1, pH 9.5; E2, pH 9.5 + L-Alanine; E3, pH 9.5 + L-Arginine; E4, pH 9.5 + L-Asparagine; E5, pH 9.5 + L-Aspartic Acid; E6, pH 9.5 + L-Glutamic Acid; E7, pH 9.5 + L-Glutamine; E8, pH 9.5 + Glycine; E9, pH 9.5 + L-Histidine; E10, pH 9.5 + L-Isoleucine; E11, pH 9.5 + L-Leucine; E12, pH 9.5 + L-Lysine; F1, pH 9.5 + L-Methionine; F2, pH 9.5 + L-Phenylalanine; F3, pH 9.5 + L-Proline; F4, pH 9.5 + L-Serine; F5, pH 9.5 + L-Threonine; F6, pH 9.5 + L-Tryptophan; F7, pH 9.5 + L-Tyrosine; F8, pH 9.5 + L-Valine; F9, pH 9.5 + Hydroxy L-Proline; F10, pH 9.5 + L-Ornithine; F11, pH 9.5 + L-Homoarginine; F12, pH 9.5 + L-Homoserine; G1, pH 9.5 + Anthranilic acid; G2, pH 9.5 + L-Norleucine; G3, pH 9.5 + L-Norvaline; G4, pH 9.5 + Agmatine; G5, pH 9.5 + Cadaverine; G6, pH 9.5 + Putrescine; G7, pH 9.5 + Histamine; G8, pH 9.5 + Phenylethylamine; G9, pH 9.5 + Tyramine; G10, pH 9.5 + Creatine; G11, pH 9.5 + Trimethylamine N-oxide; G12, pH 9.5 + Urea; H1, X-Caprylate; H2, X-<math>\alpha</math>-DGlucoside; H3, X-<math>\beta</math>-DGlucoside; H4, X-<math>\alpha</math>-DGalactoside; H5, X-<math>\beta</math>-DGalactoside; H6, X-<math>\alpha</math>-DGlucuronide; H7, X-<math>\beta</math>-DGlucuronide; H8, X-<math>\beta</math>-DGlucosaminide; H9, X-<math>\beta</math>-DGalactosaminide; H10, X-<math>\alpha</math>-DMannoside; H11, X-PO4; H12, X-SO4.</p>
PM11C – chemical	<p>A1, Amikacin (1); A2, Amikacin (2); A3, Amikacin (3); A4, Amikacin (4); A5, Chlortetracycline (1); A6, Chlortetracycline (2); A7, Chlortetracycline (3); A8, Chlortetracycline (4); A9, Lincomycin (1); A10, Lincomycin (2); A11, Lincomycin (3); A12, Lincomycin (4); B1, Amoxicillin (1); B2, Amoxicillin (2); B3, Amoxicillin (3); B4, Amoxicillin (4); B5, Cloxacillin (1); B6, Cloxacillin (2); B7, Cloxacillin (3); B8, Cloxacillin (4); B9, Lomefloxacin (1); B10, Lomefloxacin (2); B11, Lomefloxacin (3); B12, Lomefloxacin (4); C1, Bleomycin (1); C2, Bleomycin (2); C3, Bleomycin (3); C4, Bleomycin (4); C5, Colistin (1); C6, Colistin (2); C7, Colistin (3); C8, Colistin (4); C9, Minocycline (1); C10, Minocycline (2); C11, Minocycline (3); C12, Minocycline (4); D1, Capreomycin (1); D2, Capreomycin (2); D3, Capreomycin (3); D4, Capreomycin (4); D5, Demeclocycline (1); D6, Demeclocycline (2); D7, Demeclocycline (3); D8, Demeclocycline (4); D9, Nafcillin (1); D10, Nafcillin (2); D11, Nafcillin (3); D12, Nafcillin (4); E1, Cefazolin (1); E2, Cefazolin (2); E3, Cefazolin (3); E4, Cefazolin (4); E5, Enoxacin (1); E6, Enoxacin (2); E7, Enoxacin (3); E8, Enoxacin (4); E9, Nalidixic acid (1); E10, Nalidixic acid (2); E11, Nalidixic acid (3); E12, Nalidixic acid (4); F1, Chloramphenicol (1); F2, Chloramphenicol (2); F3, Chloramphenicol (3); F4, Chloramphenicol (4); F5, Erythromycin (1); F6, Erythromycin (2); F7, Erythromycin (3); F8, Erythromycin (4); F9, Neomycin (1); F10, Neomycin (2); F11, Neomycin (3); F12, Neomycin (4); G1, Ceftriaxone (1); G2, Ceftriaxone (2); G3, Ceftriaxone (3); G4, Ceftriaxone (4); G5, Gentamicin (1); G6, Gentamicin (2); G7, Gentamicin (3); G8, Gentamicin (4); G9, Potassium tellurite (1); G10, Potassium tellurite (2); G11, Potassium tellurite (3); G12, Potassium tellurite (4); H1, Cephalothin (1); H2, Cephalothin (2); H3, Cephalothin (3); H4, Cephalothin (4); H5, Kanamycin (1); H6, Kanamycin (2); H7, Kanamycin (3); H8, Kanamycin (4); H9, Ofloxacin (1); H10, Ofloxacin</p>

	(2); H11, Ofloxacin (3); H12, Ofloxacin (4).
PM12B – chemical	A1, Penicillin G (1); A2, Penicillin G (2); A3, Penicillin G (3); A4, Penicillin G (4); A5, Tetracycline (1); A6, Tetracycline (2); A7, Tetracycline (3); A8, Tetracycline (4); A9, Carbenicillin (1); A10, Carbenicillin (2); A11, Carbenicillin (3); A12, Carbenicillin (4); B1, Oxacillin (1); B2, Oxacillin (2); B3, Oxacillin (3); B4, Oxacillin (4); B5, Penimepicycline (1); B6, Penimepicycline (2); B7, Penimepicycline (3); B8, Penimepicycline (4); B9, Polymyxin B (1); B10, Polymyxin B (2); B11, Polymyxin B (3); B12, Polymyxin B (4); C1, Paromomycin (1); C2, Paromomycin (2); C3, Paromomycin (3); C4, Paromomycin (4); C5, Vancomycin (1); C6, Vancomycin (2); C7, Vancomycin (3); C8, Vancomycin (4); C9, D,L-Serinehydroxamate (1); C10, D,L-Serine hydroxamate (2); C11, D,L-Serine hydroxamate (3); C12, D,L-Serine hydroxamate (4); D1, Sisomicin (1); D2, Sisomicin (2); D3, Sisomicin (3); D4, Sisomicin (4); D5, Sulfamethazine (1); D6, Sulfamethazine (2); D7, Sulfamethazine (3); D8, Sulfamethazine (4); D9, Novobiocin (1); D10, Novobiocin (2); D11, Novobiocin (3); D12, Novobiocin (4); E1, 2,4-Diamino-6,7-diisopropylpteridine (1); E2, 2,4-Diamino-6,7-diisopropylpteridine (2); E3, 2,4-Diamino-6,7-diisopropylpteridine (3); E4, 2,4-Diamino-6,7-diisopropylpteridine (4); E5, Sulfadiazine (1); E6, Sulfadiazine (2); E7, Sulfadiazine (3); E8, Sulfadiazine (4); E9, Benzethoniumchloride (1); E10, Benzethoniumchloride (2); E11, Benzethoniumchloride (3); E12, Benzethoniumchloride (4); F1, Tobramycin (1); F2, Tobramycin (2); F3, Tobramycin (3); F4, Tobramycin (4); F5, Sulfathiazole (1); F6, Sulfathiazole (2); F7, Sulfathiazole (3); F8, Sulfathiazole (4); F9, 5-Fluoroorotic acid (1); F10, 5-Fluoroorotic acid (2); F11, 5-Fluoroorotic acid (3); F12, 5-Fluoroorotic acid (4); G1, Spectinomycin (1); G2, Spectinomycin (2); G3, Spectinomycin (3); G4, Spectinomycin (4); G5, Sulfamethoxazole (1); G6, Sulfamethoxazole (2); G7, Sulfamethoxazole (3); G8, Sulfamethoxazole (4); G9, L-Aspartic- $\beta$ -hydroxamate (1); G10, L-Aspartic- $\beta$ -hydroxamate (2); G11, L-Aspartic- $\beta$ -hydroxamate (3); G12, L-Aspartic- $\beta$ -hydroxamate (4); H1, Spiramycin (1); H2, Spiramycin (2); H3, Spiramycin (3); H4, Spiramycin (4); H5, Rifampicin (1); H6, Rifampicin (2); H7, Rifampicin (3); H8, Rifampicin (4); H9, Dodecyltrimethyl ammonium bromide (1); H10, Dodecyltrimethyl ammonium bromide (2); H11, Dodecyltrimethyl ammonium bromide (3); H12, Dodecyltrimethyl ammonium bromide (4).
PM13B – chemical	A1, Ampicillin (1); A2, Ampicillin (2); A3, Ampicillin (3); A4, Ampicillin (4); A5, Dequalinium chloride (1); A6, Dequalinium chloride (2); A7, Dequalinium chloride (3); A8, Dequalinium chloride (4); A9, Nickel chloride (1); A10, Nickel chloride (2); A11, Nickel chloride (3); A12, Nickel chloride (4); B1, Azlocillin (1); B2, Azlocillin (2); B3, Azlocillin (3); B4, Azlocillin (4); B5, 2, 2'-Dipyridyl (1); B6, 2, 2'-Dipyridyl (2); B7, 2, 2'-Dipyridyl (3); B8, 2, 2'-Dipyridyl (4); B9, Oxolinic acid (1); B10, Oxolinic acid (2); B11, Oxolinic acid (3); B12, Oxolinic acid (4); C1, 6-Mercaptopurine (1); C2, 6-Mercaptopurine (2); C3, -Mercaptopurine (3); C4, 6-Mercaptopurine (4); C5, Doxycycline (1); C6, Doxycycline (2); C7, Doxycycline (3); C8, Doxycycline (4); C9, Potassium chromate (1); C10, Potassium chromate (2); C11, Potassium chromate (3); C12, Potassium chromate (4); D1, Cefuroxime (1); D2, Cefuroxime (2); D3, Cefuroxime (3); D4, Cefuroxime (4); D5, 5-Fluorouracil (1); D6, 5-Fluorouracil (2); D7, 5-Fluorouracil (3); D8, 5-Fluorouracil (4); D9, Rolitetracycline (1); D10, Rolitetracycline (2); D11, Rolitetracycline (3); D12, Rolitetracycline (4); E1, Cytosine-1- $\beta$ D-arabinofuranoside (1); E2, Cytosine-1- $\beta$ D-arabinofuranoside (2); E3, Cytosine-1- $\beta$ D-arabinofuranoside (3); E4, Cytosine-1- $\beta$ D-arabinofuranoside (4); E5, Geneticin (G418) (1); E6, Geneticin (G418) (2); E7, Geneticin (G418) (3); E8, Geneticin (G418) (4); E9, Ruthenium red (1); E10, Ruthenium red (2); E11, Ruthenium red (3); E12, Ruthenium red (4); F1, Cesium chloride (1); F2, Cesium chloride (2); F3, Cesium chloride (3); F4, Cesium chloride (4); F5, Glycine (1); F6, Glycine (2); F7, Glycine (3); F8, Glycine (4); F9, Thallium (I) acetate (1); F10, Thallium (I) acetate (2); F11, Thallium (I) acetate (3); F12, Thallium (I) acetate (4); G1, Cobalt

	chloride (1); G2, Cobalt chloride (2); G3, Cobalt chloride (3); G4, Cobalt chloride (4); G5, Manganese chloride (1); G6, Manganese chloride (2); G7, Manganese chloride (3); G8, Manganese chloride (4); G9, Trifluoperazine (1); G10, Trifluoperazine (2); G11, Trifluoperazine (3); G12, Trifluoperazine (4); H1, Cupric chloride (1); H2, Cupric chloride (2); H3, Cupric chloride (3); H4, Cupric chloride (4); H5, Moxalactam (1); H6, Moxalactam (2); H7, Moxalactam (3); H8, Moxalactam (4); H9, Tylosin (1); H10, Tylosin (2); H11, Tylosin (3); H12, Tylosin (4).
PM14A – chemical	A1, Acriflavine (1); A2, Acriflavine (2); A3, Acriflavine (3); A4, Acriflavine (4); A5, Furaltadone (1); A6, Furaltadone (2); A7, Furaltadone (3); A8, Furaltadone (4); A9, Sanguinarine (1); A10, Sanguinarine (2); A11, Sanguinarine (3); A12, Sanguinarine (4); B1, 9-Aminoacridine (1); B2, 9-Aminoacridine (2); B3, 9-Aminoacridine (3); B4, 9-Aminoacridine (4); B5, Fusaric acid (1); B6, Fusaric acid (2); B7, Fusaric acid (3); B8, Fusaric acid (4); B9, Sodium arsenate (1); B10, Sodium arsenate (2); B11, Sodium arsenate (3); B12, Sodium arsenate (4); C1, Boric Acid (1); C2, Boric Acid (2); C3, Boric Acid (3); C4, Boric Acid (4); C5, 1-Hydroxypyridine-2-thione (1); C6, 1-Hydroxypyridine-2-thione (2); C7, 1-Hydroxypyridine-2-thione (3); C8, 1-Hydroxypyridine-2-thione (4); C9, Sodium cyanate (1); C10, Sodium cyanate (2); C11, Sodium cyanate (3); C12, Sodium cyanate (4); D1, Cadmium chloride (1); D2, Cadmium chloride (2); D3, Cadmium chloride (3); D4, Cadmium chloride (4); D5, Iodoacetate (1); D6, Iodoacetate (2); D7, Iodoacetate (3); D8, Iodoacetate (4); D9, Sodium dichromate (1); D10, Sodium dichromate (2); D11, Sodium dichromate (3); D12, Sodium dichromate (4); E1, Cefoxitin (1); E2, Cefoxitin (2); E3, Cefoxitin (3); E4, Cefoxitin (4); E5, Nitrofurantoin (1); E6, Nitrofurantoin (2); E7, Nitrofurantoin (3); E8, Nitrofurantoin (4); E9, Sodium metaborate (1); E10, Sodium metaborate (2); E11, Sodium metaborate (3); E12, Sodium metaborate (4); F1, Chloramphenicol (1); F2, Chloramphenicol (2); F3, Chloramphenicol (3); F4, Chloramphenicol (4); F5, Piperacillin (1); F6, Piperacillin (2); F7, Piperacillin (3); F8, Piperacillin (4); F9, Sodium metavanadate (1); F10, Sodium metavanadate (2); F11, Sodium metavanadate (3); F12, Sodium metavanadate (4); G1, Chelerythrine (1); G2, Chelerythrine (2); G3, Chelerythrine (3); G4, Chelerythrine (4); G5, Carbenicillin (1); G6, Carbenicillin (2); G7, Carbenicillin (3); G8, Carbenicillin (4); G9, Sodium nitrite (1); G10, Sodium nitrite (2); G11, Sodium nitrite (3); G12, Sodium nitrite (4); H1, EGTA (1); H2, EGTA (2); H3, EGTA (3); H4, EGTA (4); H5, Promethazine (1); H6, Promethazine (2); H7, Promethazine (3); H8, Promethazine (4); H9, Sodium orthovanadate (1); H10, Sodium orthovanadate (2); H11, Sodium orthovanadate (3); H12, Sodium orthovanadate (4).
PM15B – chemical	A1, Procaine (1); A2, Procaine (2); A3, Procaine (3); A4, Procaine (4); A5, Guanidine hydrochloride (1); A6, Guanidine hydrochloride (2); A7, Guanidine hydrochloride (3); A8, Guanidine hydrochloride (4); A9, Cefmetazole (1); A10, Cefmetazole (2); A11, Cefmetazole (3); A12, Cefmetazole (4); B1, D-Cycloserine (1); B2, D-Cycloserine (2); B3, D-Cycloserine (3); B4, D-Cycloserine (4); B5, EDTA (1); B6, EDTA (2); B7, EDTA (3); B8, EDTA (4); B9, 5,7-Dichloro-8-hydroxyquinaldine (1); B10, 5,7-Dichloro-8-hydroxyquinaldine (2); B11, 5,7-Dichloro-8-hydroxyquinaldine (3); B12, 5,7-Dichloro-8-hydroxyquinaldine (4); C1, 5,7-Dichloro-8-hydroxyquinoline (1); C2, 5,7-Dichloro-8-hydroxyquinoline (2); C3, 5,7-Dichloro-8-hydroxyquinoline (3); C4, 5,7-Dichloro-8-hydroxyquinoline (4); C5, Fusidic acid (1); C6, Fusidic acid (2); C7, Fusidic acid (3); C8, Fusidic acid (4); C9, 1,10-Phenanthroline (1); C10, 1,10-Phenanthroline (2); C11, 1,10-Phenanthroline (3); C12, 1,10-Phenanthroline (4); D1, Phleomycin (1); D2, Phleomycin (2); D3, Phleomycin (3); D4, Phleomycin (4); D5, Domiphen bromide (1); D6, Domiphen bromide (2); D7, Domiphen bromide (3); D8, Domiphen bromide (4); D9, Nordihydroguaia retic acid (1); D10, Nordihydroguaia retic acid (2); D11, Nordihydroguaia retic acid (3); D12, Nordihydroguaia retic acid (4); E1, Alexidine (1); E2, Alexidine (2); E3, Alexidine (3);

	E4, Alexidine (4); E5, 5-Nitro-2-furaldehyde semicarbazone (1); E6, 5-Nitro-2-furaldehyde semicarbazone (2); E7, 5-Nitro-2-furaldehyde semicarbazone (3); E8, 5-Nitro-2-furaldehyde semicarbazone (4); E9, Methyl viologen (1); E10, Methyl viologen (2); E11, Methyl viologen (3); E12, Methyl viologen (4); F1, 3, 4-Dimethoxybenzyl alcohol (1); F2, 3, 4-Dimethoxybenzyl alcohol (2); F3, 3, 4-Dimethoxybenzyl alcohol (3); F4, 3, 4-Dimethoxybenzyl alcohol (4); F5, Oleandomycin (1); F6, Oleandomycin (2); F7, Oleandomycin (3); F8, Oleandomycin (4); F9, Puromycin (1); F10, Puromycin (2); F11, Puromycin (3); F12, Puromycin (4); G1, CCCP (1); G2, CCCP (2); G3, CCCP (3); G4, CCCP (4); G5, Sodium azide (1); G6, Sodium azide (2); G7, Sodium azide (3); G8, Sodium azide (4); G9, Menadione (1); G10, Menadione (2); G11, Menadione (3); G12, Menadione (4); H1, 2-Nitroimidazole (1); H2, 2-Nitroimidazole (2); H3, 2-Nitroimidazole (3); H4, 2-Nitroimidazole (4); H5, Hydroxyurea (1); H6, Hydroxyurea (2); H7, Hydroxyurea (3); H8, Hydroxyurea (4); H9, Zinc chloride (1); H10, Zinc chloride (2); H11, Zinc chloride (3); H12, Zinc chloride (4).
PM16A – chemical	A1, Cefotaxime (1); A2, Cefotaxime (2); A3, Cefotaxime (3); A4, Cefotaxime (4); A5, Phosphomycin (1); A6, Phosphomycin (2); A7, Phosphomycin (3); A8, Phosphomycin (4); A9, 5-Chloro-7-iodo-8-hydroxyquinoline (1); A10, 5-Chloro-7-iodo-8-hydroxyquinoline (2); A11, 5-Chloro-7-iodo-8-hydroxyquinoline (3); A12, 5-Chloro-7-iodo-8-hydroxyquinoline (4); B1, Norfloxacin (1); B2, Norfloxacin (2); B3, Norfloxacin (3); B4, Norfloxacin (4); B5, Sulfanilamide (1); B6, Sulfanilamide (2); B7, Sulfanilamide (3); B8, Sulfanilamide (4); B9, Trimethoprim (1); B10, Trimethoprim (2); B11, Trimethoprim (3); B12, Trimethoprim (4); C1, Dichlofluanid (1); C2, Dichlofluanid (2); C3, Dichlofluanid (3); C4, Dichlofluanid (4); C5, Protamine sulfate (1); C6, Protamine sulfate (2); C7, Protamine sulfate (3); C8, Protamine sulfate (4); C9, Cetylpyridinium chloride (1); C10, Cetylpyridinium chloride (2); C11, Cetylpyridinium chloride (3); C12, Cetylpyridinium chloride (4); D1, 1-Chloro -2,4-dinitrobenzene (1); D2, 1-Chloro -2,4-dinitrobenzene (2); D3, 1-Chloro -2,4-dinitrobenzene (3); D4, 1-Chloro -2,4-dinitrobenzene (4); D5, Diamide (1); D6, Diamide (2); D7, Diamide (3); D8, Diamide (4); D9, Cinoxacin (1); D10, Cinoxacin (2); D11, Cinoxacin (3); D12, Cinoxacin (4); E1, Streptomycin (1); E2, Streptomycin (2); E3, Streptomycin (3); E4, Streptomycin (4); E5, 5-Azacytidine (1); E6, 5-Azacytidine (2); E7, 5-Azacytidine (3); E8, 5-Azacytidine (4); E9, Rifamycin SV (1); E10, Rifamycin SV (2); E11, Rifamycin SV (3); E12, Rifamycin SV (4); F1, Potassium tellurite (1); F2, Potassium tellurite (2); F3, Potassium tellurite (3); F4, Potassium tellurite (4); F5, Sodium selenite (1); F6, Sodium selenite (2); F7, Sodium selenite (3); F8, Sodium selenite (4); F9, Aluminum sulfate (1); F10, Aluminum sulfate (2); F11, Aluminum sulfate (3); F12, Aluminum sulfate (4); G1, Chromium chloride (1); G2, Chromium chloride (2); G3, Chromium chloride (3); G4, Chromium chloride (4); G5, Ferric chloride (1); G6, Ferric chloride (2); G7, Ferric chloride (3); G8, Ferric chloride (4); G9, L-Glutamic-ghydroxamate (1); G10, L-Glutamic-ghydroxamate (2); G11, L-Glutamic-ghydroxamate (3); G12, L-Glutamic-ghydroxamate (4); H1, Glycine hydroxamate (1); H2, Glycine hydroxamate (2); H3, Glycine hydroxamate (3); H4, Glycine hydroxamate (4); H5, Chloroxylenol (1); H6, Chloroxylenol (2); H7, Chloroxylenol (3); H8, Chloroxylenol (4); H9, Sorbic acid (1); H10, Sorbic acid (2); H11, Sorbic acid (3); H12, Sorbic acid (4).
PM17A – chemical	A1, D-Serine (1); A2, D-Serine (2); A3, D-Serine (3); A4, D-Serine (4); A5, $\beta$ -ChloroL-alanine hydrochloride (1); A6, $\beta$ -ChloroL-alanine hydrochloride (2); A7, $\beta$ -ChloroL-alanine hydrochloride (3); A8, $\beta$ -ChloroL-alanine hydrochloride (4); A9, Thiosalicylic acid (1); A10, Thiosalicylic acid (2); A11, Thiosalicylic acid (3); A12, Thiosalicylic acid (4); B1, Sodium salicylate (1); B2, Sodium salicylate (2); B3, Sodium salicylate (3); B4, Sodium salicylate (4); B5, Hygromycin B (1); B6, Hygromycin B (2); B7, Hygromycin B (3); B8, Hygromycin B (4); B9, Ethionamide (1); B10, Ethionamide (2); B11, Ethionamide (3); B12, Ethionamide (4); C1, 4-Aminopyridine (1); C2, 4-Aminopyridine (2); C3, 4-Aminopyridine

	<p>(3); C4, 4-Aminopyridine (4); C5, Sulfachloropyridazine (1); C6, Sulfachloropyridazine (2); C7, Sulfachloropyridazine (3); C8, Sulfachloropyridazine (4); C9, Sulfamonomethoxine (1); C10, Sulfamonomethoxine (2); C11, Sulfamonomethoxine (3); C12, Sulfamonomethoxine (4); D1, Oxycarboxin (1); D2, Oxycarboxin (2); D3, Oxycarboxin (3); D4, Oxycarboxin (4); D5, 3-Amino-1,2,4-triazole (1); D6, 3-Amino-1,2,4-triazole (2); D7, 3-Amino-1,2,4-triazole (3); D8, 3-Amino-1,2,4-triazole (4); D9, Chlorpromazine (1); D10, Chlorpromazine (2); D11, Chlorpromazine (3); D12, Chlorpromazine (4); E1, Niaproof (1); E2, Niaproof (2); E3, Niaproof (3); E4, Niaproof (4); E5, Compound 48/80 (1); E6, Compound 48/80 (2); E7, Compound 48/80 (3); E8, Compound 48/80 (4); E9, Sodium tungstate (1); E10, Sodium tungstate (2); E11, Sodium tungstate (3); E12, Sodium tungstate (4); F1, Lithium chloride (1); F2, Lithium chloride (2); F3, Lithium chloride (3); F4, Lithium chloride (4); F5, DL-Methionine hydroxamate (1); F6, DL-Methionine hydroxamate (2); F7, DL-Methionine hydroxamate (3); F8, DL-Methionine hydroxamate (4); F9, Tannic acid (1); F10, Tannic acid (2); F11, Tannic acid (3); F12, Tannic acid (4); G1, Chlorambucil (1); G2, Chlorambucil (2); G3, Chlorambucil (3); G4, Chlorambucil (4); G5, Cefamandole nafate (1); G6, Cefamandole nafate (2); G7, Cefamandole nafate (3); G8, Cefamandole nafate (4); G9, Cefoperazone (1); G10, Cefoperazone (2); G11, Cefoperazone (3); G12, Cefoperazone (4); H1, Cefsulodin (1); H2, Cefsulodin (2); H3, Cefsulodin (3); H4, Cefsulodin (4); H5, Caffeine (1); H6, Caffeine (2); H7, Caffeine (3); H8, Caffeine (4); H9, Phenylarsine oxide (1); H10, Phenylarsine oxide (2); H11, Phenylarsine oxide (3); H12, Phenylarsine oxide (4).</p>
PM18C – chemical	<p>A1, Ketoprofen (1); A2, Ketoprofen (2); A3, Ketoprofen (3); A4, Ketoprofen (4); A5, Sodium pyrophosphate decahydrate (1); A6, Sodium pyrophosphate decahydrate (2); A7, Sodium pyrophosphate decahydrate (3); A8, Sodium pyrophosphate decahydrate (4); A9, Thiamphenicol (1); A10, Thiamphenicol (2); A11, Thiamphenicol (3); A12, Thiamphenicol (4); B1, Trifluorothymidine (1); B2, Trifluorothymidine (2); B3, Trifluorothymidine (3); B4, Trifluorothymidine (4); B5, Pipemidic Acid (1); B6, Pipemidic Acid (2); B7, Pipemidic Acid (3); B8, Pipemidic Acid (4); B9, Azathioprine (1); B10, Azathioprine (2); B11, Azathioprine (3); B12, Azathioprine (4); C1, Poly-L-lysine (1); C2, Poly-L-lysine (2); C3, Poly-L-lysine (3); C4, Poly-L-lysine (4); C5, Sulfisoxazole (1); C6, Sulfisoxazole (2); C7, Sulfisoxazole (3); C8, Sulfisoxazole (4); C9, Pentachlorophenol (1); C10, Pentachlorophenol (2); C11, Pentachlorophenol (3); C12, Pentachlorophenol (4); D1, Sodium m-arsenite (1); D2, Sodium m-arsenite (2); D3, Sodium m-arsenite (3); D4, Sodium m-arsenite (4); D5, Sodium bromate (1); D6, Sodium bromate (2); D7, Sodium bromate (3); D8, Sodium bromate (4); D9, Lidocaine (1); D10, Lidocaine (2); D11, Lidocaine (3); D12, Lidocaine (4); E1, Sodium metasilicate (1); E2, Sodium metasilicate (2); E3, Sodium metasilicate (3); E4, Sodium metasilicate (4); E5, Sodium m-periodate (1); E6, Sodium m-periodate (2); E7, Sodium m-periodate (3); E8, Sodium m-periodate (4); E9, Antimony (III) chloride (1); E10, Antimony (III) chloride (2); E11, Antimony (III) chloride (3); E12, Antimony (III) chloride (4); F1, Semicarbazide (1); F2, Semicarbazide (2); F3, Semicarbazide (3); F4, Semicarbazide (4); F5, Tinidazole (1); F6, Tinidazole (2); F7, Tinidazole (3); F8, Tinidazole (4); F9, Aztreonam (1); F10, Aztreonam (2); F11, Aztreonam (3); F12, Aztreonam (4); G1, Triclosan (1); G2, Triclosan (2); G3, Triclosan (3); G4, Triclosan (4); G5, 3,5-Diamino-1,2,4-triazole (Guanazole) (1); G6, 3,5-Diamino-1,2,4-triazole (Guanazole) (2); G7, 3,5-Diamino-1,2,4-triazole (Guanazole) (3); G8, 3,5-Diamino-1,2,4-triazole (Guanazole) (4); G9, Myricetin (1); G10, Myricetin (2); G11, Myricetin (3); G12, Myricetin (4); H1, 5-fluoro-5'-deoxyuridine (1); H2, 5-fluoro-5'-deoxyuridine (2); H3, 5-fluoro-5'-deoxyuridine (3); H4, 5-fluoro-5'-deoxyuridine (4); H5, 2-Phenylphenol (1); H6, 2-Phenylphenol (2); H7, 2-Phenylphenol (3); H8, 2-Phenylphenol (4); H9, Plumbagin (1); H10, Plumbagin (2); H11, Plumbagin (3); H12, Plumbagin (4).</p>
PM19 – chemical	<p>A1, Josamycin (1); A2, Josamycin (2); A3, Josamycin (3); A4, Josamycin (4); A5, Gallic</p>

	<p>acid (1); A6, Gallic acid (2); A7, Gallic acid (3); A8, Gallic acid (4); A9, Coumarin (1); A10, Coumarin (2); A11, Coumarin (3); A12, Coumarin (4); B1, Methyltrioctylammonium chloride (1); B2, Methyltrioctylammonium chloride (2); B3, Methyltrioctylammonium chloride (3); B4, Methyltrioctylammonium chloride (4); B5, Harmane (1); B6, Harmane (2); B7, Harmane (3); B8, Harmane (4); B9, 2,4-Dinitrophenol (1); B10, 2,4-Dinitrophenol (2); B11, 2,4-Dinitrophenol (3); B12, 2,4-Dinitrophenol (4); C1, Chlorhexidine (1); C2, Chlorhexidine (2); C3, Chlorhexidine (3); C4, Chlorhexidine (4); C5, Umbelliferone (1); C6, Umbelliferone (2); C7, Umbelliferone (3); C8, Umbelliferone (4); C9, Cinnamic acid (1); C10, Cinnamic acid (2); C11, Cinnamic acid (3); C12, Cinnamic acid (4); D1, Disulphiram (1); D2, Disulphiram (2); D3, Disulphiram (3); D4, Disulphiram (4); D5, Iodonitro Tetrazolium Violet (1); D6, Iodonitro Tetrazolium Violet (2); D7, Iodonitro Tetrazolium Violet (3); D8, Iodonitro Tetrazolium Violet (4); D9, Phenyl-methylsulfonylfluoride (PMSF) (1); D10, Phenyl- methylsulfonylfluoride (PMSF) (2); D11, Phenyl- methylsulfonylfluoride (PMSF) (3); D12, Phenyl- methylsulfonylfluoride (PMSF) (4); E1, FCCP (1); E2, FCCP (2); E3, FCCP (3); E4, FCCP (4); E5, D,L-Thioctic Acid (1); E6, D,L-Thioctic Acid (2); E7, D,L-Thioctic Acid (3); E8, D,L-Thioctic Acid (4); E9, Lawsone (1); E10, Lawsone (2); E11, Lawsone (3); E12, Lawsone (4); F1, Phenethicillin (1); F2, Phenethicillin (2); F3, Phenethicillin (3); F4, Phenethicillin (4); F5, Blastocidin S (1); F6, Blastocidin S (2); F7, Blastocidin S (3); F8, Blastocidin S (4); F9, Sodium caprylate (1); F10, Sodium caprylate (2); F11, Sodium caprylate (3); F12, Sodium caprylate (4); G1, Lauryl sulfobetaine (1); G2, Lauryl sulfobetaine (2); G3, Lauryl sulfobetaine (3); G4, Lauryl sulfobetaine (4); G5, Dihydrostreptomycin (1); G6, Dihydrostreptomycin (2); G7, Dihydrostreptomycin (3); G8, Dihydrostreptomycin (4); G9, Hydroxylamine (1); G10, Hydroxylamine (2); G11, Hydroxylamine (3); G12, Hydroxylamine (4); H1, Hexamine cobalt (III) chloride (1); H2, Hexamine cobalt (III) chloride (2); H3, Hexamine cobalt (III) chloride (3); H4, Hexamine cobalt (III) chloride (4); H5, Thioglycerol (1); H6, Thioglycerol (2); H7, Thioglycerol (3); H8, Thioglycerol (4); H9, Polymyxin B (1); H10, Polymyxin B (2); H11, Polymyxin B (3); H12, Polymyxin B (4).</p>
PM20B – chemical	<p>A1, Amitriptyline (1); A2, Amitriptyline (2); A3, Amitriptyline (3) A4, Amitriptyline (4); A5, Apramycin (1); A6, Apramycin(2); A7, Apramycin (3); A8, Apramycin (4); A9, Benserazide (1); A10, Benserazide (2); A11, Benserazide (3); A12, Benserazide (4); B1, Orphenadrine (1); B2, Orphenadrine (2); B3, Orphenadrine (3); B4, Orphenadrine (4); B5, D,L-Propranolol (1); B6, D,L-Propranolol (2); B7, D,L-Propranolol (3), B8, D,L-Propranolol (4); B9, Tetrazolium Violet (1); B10, Tetrazolium Violet (2); B11, Tetrazolium Violet (3); B12, Tetrazolium Violet (4); C1, Thioridazine (1); C2, Thioridazine (2); C3, Thioridazine (3); C4, Thioridazine (4); C5, Atropine (1); C6, Atropine (2); C7, Atropine (3); C8, Atropine (4); C9, Ornidazole (1); C10, Ornidazole (2); C11, Ornidazole (3); C12, Ornidazole (4); D1, Proflavine (1); D2, Proflavine (2); D3, Proflavine (3); D4, Proflavine (4); D5, Ciprofloxacin (1); D6, Ciprofloxacin (2); D7, Ciprofloxacin (3); D8, Ciprofloxacin (4); D9, 18-Crown-6 Ether (1); D10, 18-Crown-6 Ether (2); D11, 18-Crown-6 Ether (3); D12, 18-Crown-6 Ether (4); E1, Crystal violet (1); E2, Crystal violet (2); E3, Crystal violet (3); E4, Crystal violet (4); E5, Dodine (1); E6, Dodine (2); E7, Dodine (3); E8, Dodine (4); E9, Hexachlorophene (1); E10, Hexachlorophene (2); E11, Hexachlorophene (3); E12, Hexachlorophene (4); F1, 4-Hydroxycoumarin (1); F2, 4-Hydroxycoumarin (2); F3, 4-Hydroxycoumarin (3); F4, 4-Hydroxycoumarin (4); F5, Oxytetracycline (1); F6, Oxytetracycline (2); F7, Oxytetracycline (3); F8, Oxytetracycline (4); F9, Pridinol (1); F10, Pridinol (2); F11, Pridinol (3); F12, Pridinol (4); G1, Captan (1); G2, Captan (2); G3, Captan (3); G4, Captan (4); G5, 3,5-Dinitrobenzene (1); G6, 3,5-Dinitrobenzene (2); G7, 3,5-Dinitrobenzene (3); G8, 3,5-Dinitrobenzene (4); G9, 8-Hydroxyquinoline (1); G10, 8-Hydroxyquinoline (2); G11, 8-Hydroxyquinoline (3); G12, 8-Hydroxyquinoline (4); H1, Patulin (1); H2, Patulin (2); H3, Patulin (3); H4, Patulin (4); H5, Tolyfluanid (1); H6,</p>

	Tolyfluanid (2); H7, Tolyfluanid (3); H8, Tolyfluanid (4); H9, Troleandomycin (1); H10, Troleandomycin (2); H11, Troleandomycin (3); H12, Troleandomycin (4).
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724 **Supplementary Table 2. Genes missing from pCROD1 of *C. rodentium* ICC180**

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Gene	Location	Function
ROD_RS25055	240..494	Replication regulatory protein repA2
ROD_RS25060	797..1654	Replication protein
ROD_RS25065	2593..3246	Hypothetical protein
ROD_RS25070	3339..3596	Antitoxin
ROD_RS25075	3598..3930	Hypothetical protein
ROD_RS25080	4318..4614	Transposase
ROD_RS25085	5726..6955	Autotransporter strand-loop-strand
ROD_RS25090	6939..11720	Autotransporter
ROD_RS25095	12358..12558	Hypothetical protein
ROD_RS25100	12814..13043	Transposase
ROD_RS25105	14045..14563	Fimbrial protein
ROD_RS25110	14636..17053	Fimbrial protein
ROD_RS25115	17046..17738	Fimbrial protein
ROD_RS25120	18251..18820	Hypothetical protein
ROD_RS25125	18945..19904	Hypothetical protein
ROD_RS25130	20068..20844	EAL domain-containing protein
ROD_RS25135	22328..26254	Autotransporter
ROD_RS25140	26743..26993	Toxin HigB-2
ROD_RS25145	27079..27339	Transcriptional regulator
ROD_RS25150	27957..30536	Usher protein
ROD_RS25155	30578..31048	Hypothetical protein
ROD_RS25160	33519..33941	Twitching motility protein PilT

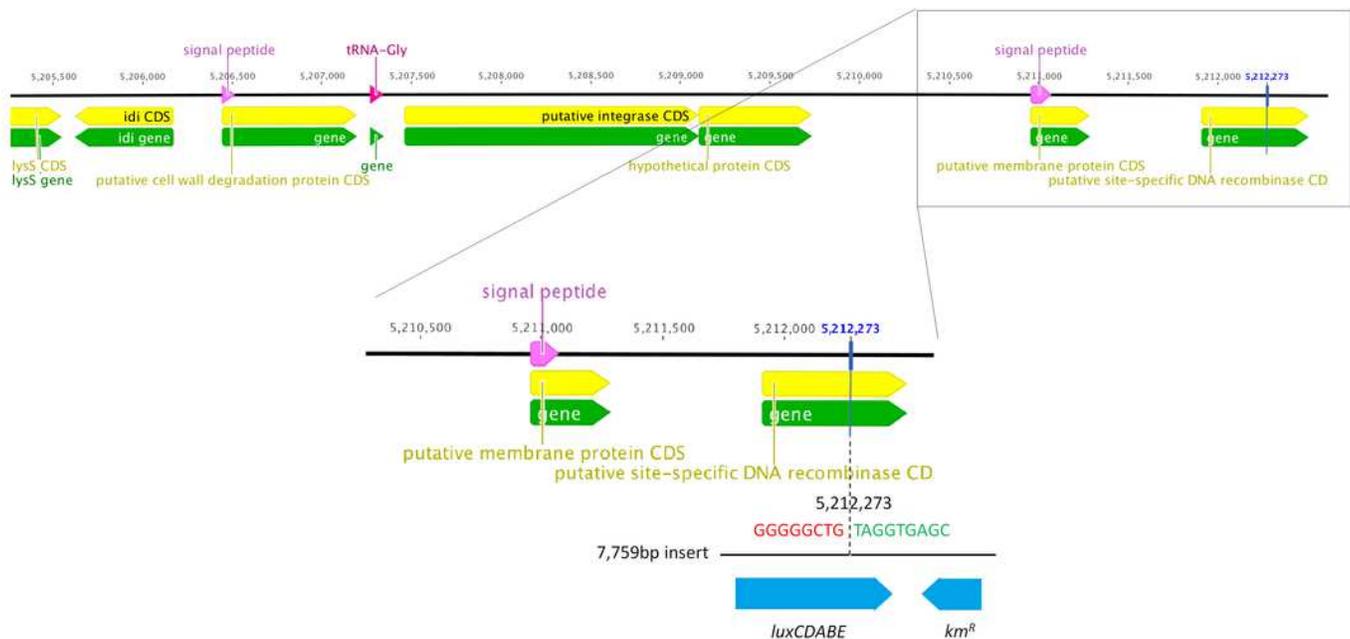
<i>ROD_RS25165</i>	33938..34168	Virulence factor
<i>ROD_RS25170</i>	34837..35055	Hypothetical protein
<i>ROD_RS25175</i>	35057..35362	Hypothetical protein
<i>ROD_RS25180</i>	35364..35690	Hypothetical protein
<i>ROD_RS25185</i>	35680..36471	Resolvase
<i>ROD_RS25190</i>	36627..40730	Autotransporter
<i>ROD_RS25730</i>	41808..43358	Hypothetical protein
<i>ROD_RS25205</i>	43907..44329	Entry exclusion protein 2
<i>ROD_RS25210</i>	44567..45523	Hypothetical protein
<i>ROD_RS25215</i>	45875..46504	Serine recombinase
<i>ROD_RS25220</i>	46779..47306	Putative resolvase
<i>ROD_RS25225</i>	47600..48241	Chromosome partitioning protein ParA
<i>ROD_RS25230</i>	48333..48665	Molecular chaperone GroEL
<i>ROD_RS25235</i>	49280..50002	DNA repair protein
<i>ROD_RS25240</i>	50082..51653	Transposase
<i>ROD_RS25250</i>	52020..52697	Transposase
<i>ROD_RS25255</i>	52721..52750	Endonuclease
<i>ROD_RS25260</i>	53458..54144	Hypothetical protein
<i>ROD_RS25265</i>	54141..54449	Hypothetical protein

726

727

## 1

Figure 1. Whole genome sequencing shows that the *lux* operon and kanamycin resistance gene have inserted at position 5,212,273 in the chromosome of *C. rodentium* ICC180, disrupting a putative site-specific DNA recombinase.

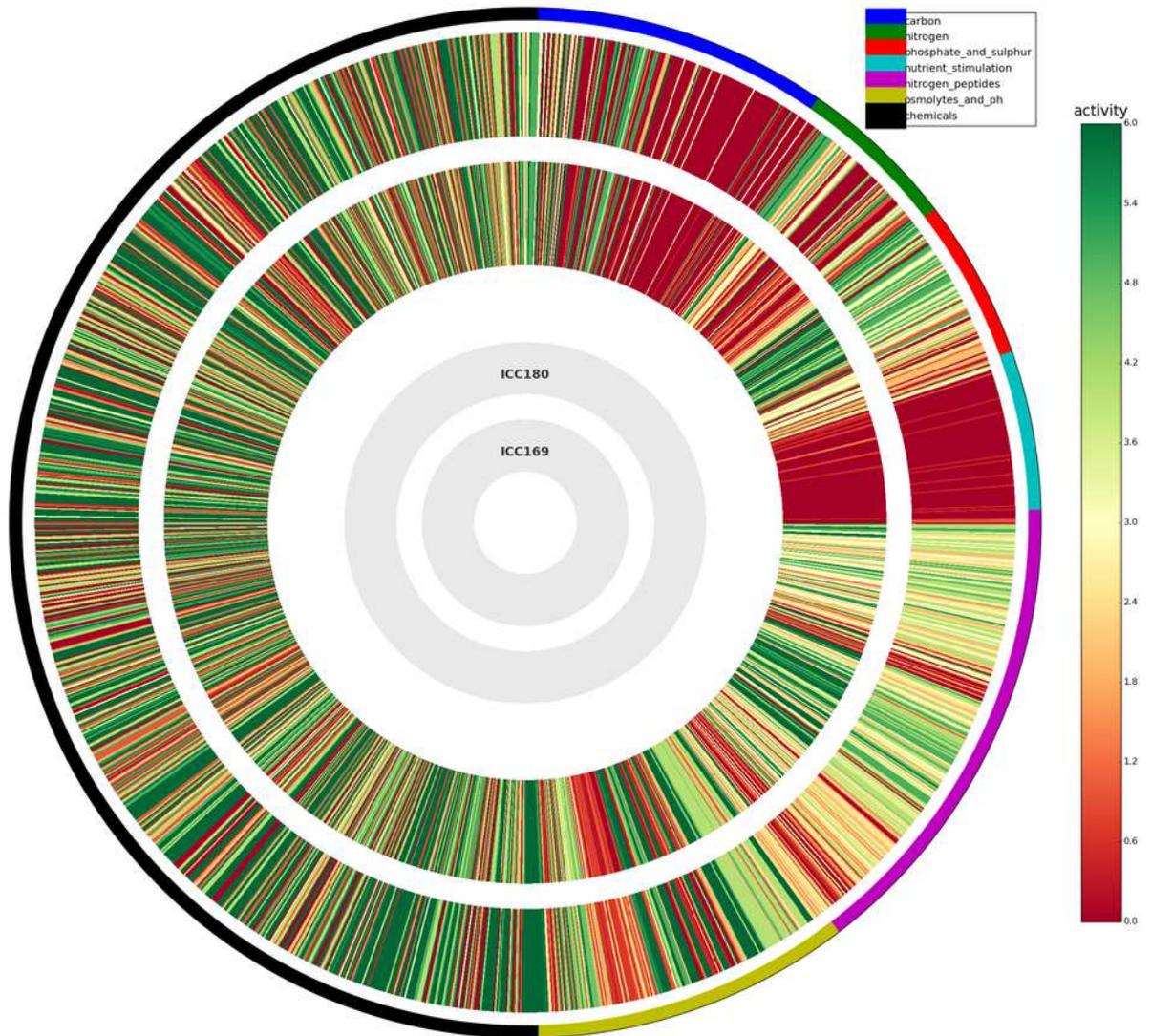


## 2

Figure 2. The growth of *Citrobacter rodentium* ICC180 compared to its non-bioluminescent parent strain ICC169 as assessed by phenotypic microarray (PM).

Wildtype *C. rodentium* ICC169 and its bioluminescent derivative ICC180 were grown on two separate occasions using PM plates 1-20. Activity rings from the PM data are shown where the grey inner circles indicate the strains' order and the external circle indicates the PM categories (see Key). The activity index (AV) was calculated for each strain in response to each well and the values for ICC169 are shown as colour stripes going from red (AV = 0 [not active]) to green (AV = 6 [active]); 7 total k-means clusters.

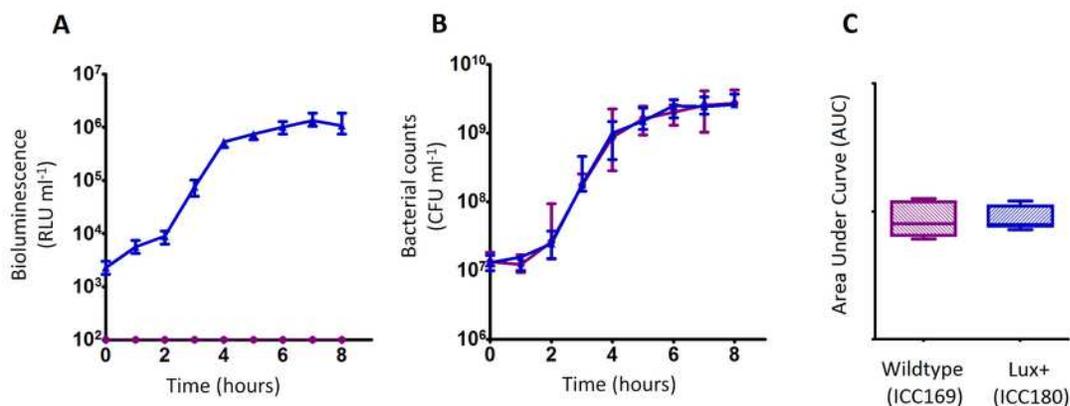
Activity ring



## 3

Figure 3. *C. rodentium* ICC180 is not impaired during growth in rich laboratory media when compared to its non-bioluminescent parent strain ICC169.

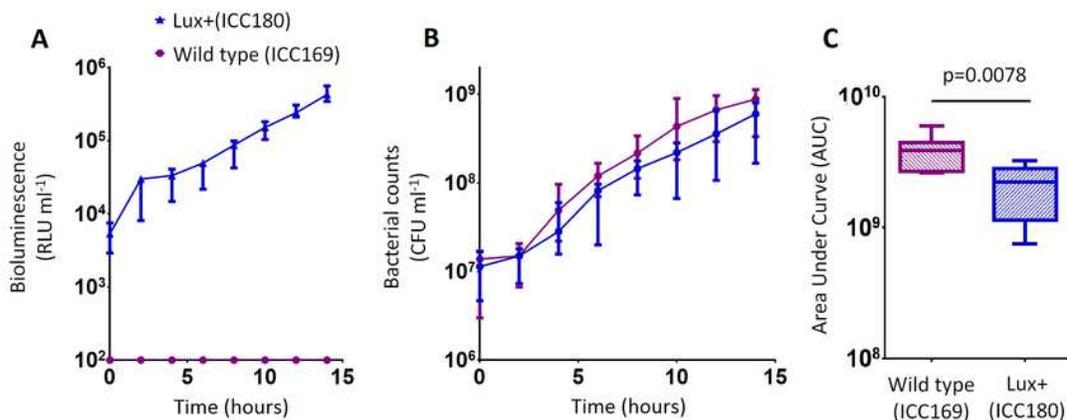
Wildtype *C. rodentium* ICC169 (shown as purple circles) and its bioluminescent derivative ICC180 (shown as blue triangles) were grown in LB-Lennox broth and monitored for changes in bioluminescence (given as relative light units [RLU] ml<sup>-1</sup>) (A) and bacterial counts (given as colony forming units [CFU] ml<sup>-1</sup>) (B). Bacterial count data was used to calculate Area Under Curve values for each strain (C). Data (medians with ranges where appropriate) is presented from experiments performed on eight separate occasions.



## 4

Figure 4. *C. rodentium* ICC180 is mildly impaired during growth in a defined minimal laboratory media when compared to its non-bioluminescent parent strain ICC169.

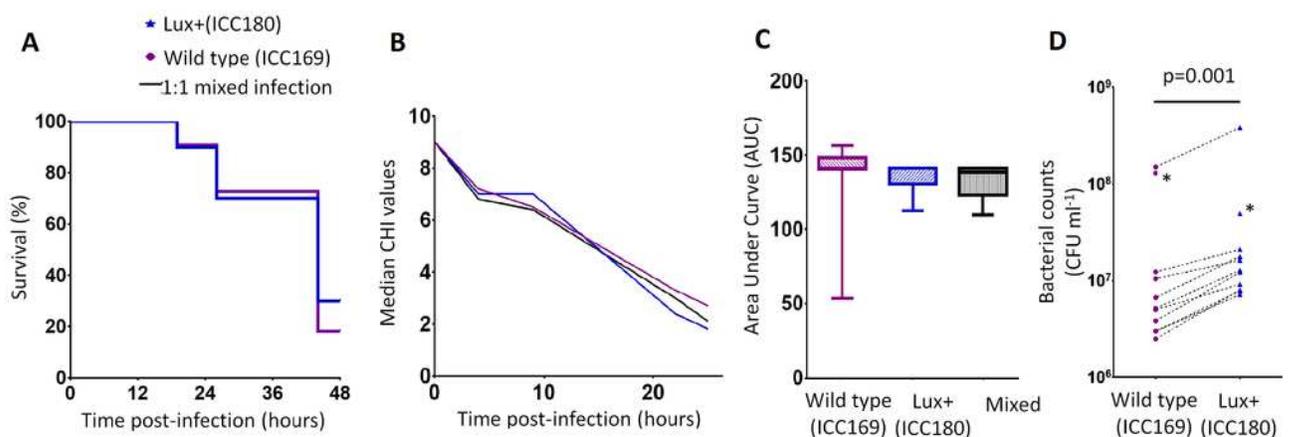
Wildtype *C. rodentium* ICC169 (shown as purple circles) and its bioluminescent derivative ICC180 (shown as blue triangles) were grown in minimal A salts supplemented with 1% glucose and monitored for changes in bioluminescence (given as relative light units [RLU] ml<sup>-1</sup>) (A) and bacterial counts (given as colony forming units [CFU] ml<sup>-1</sup>) (B). Bacterial count data was used to calculate Area Under Curve values for each strain, which were found to be significantly different ( $p=0.0078$ ; Wilcoxon Matched pairs-signed rank test) (C). Data (medians with ranges where appropriate) is presented from experiments performed on eight separate occasions.



## 5

Figure 5. Bioluminescent *C. rodentium* ICC180 is not impaired in the *Galleria mellonella* infection model.

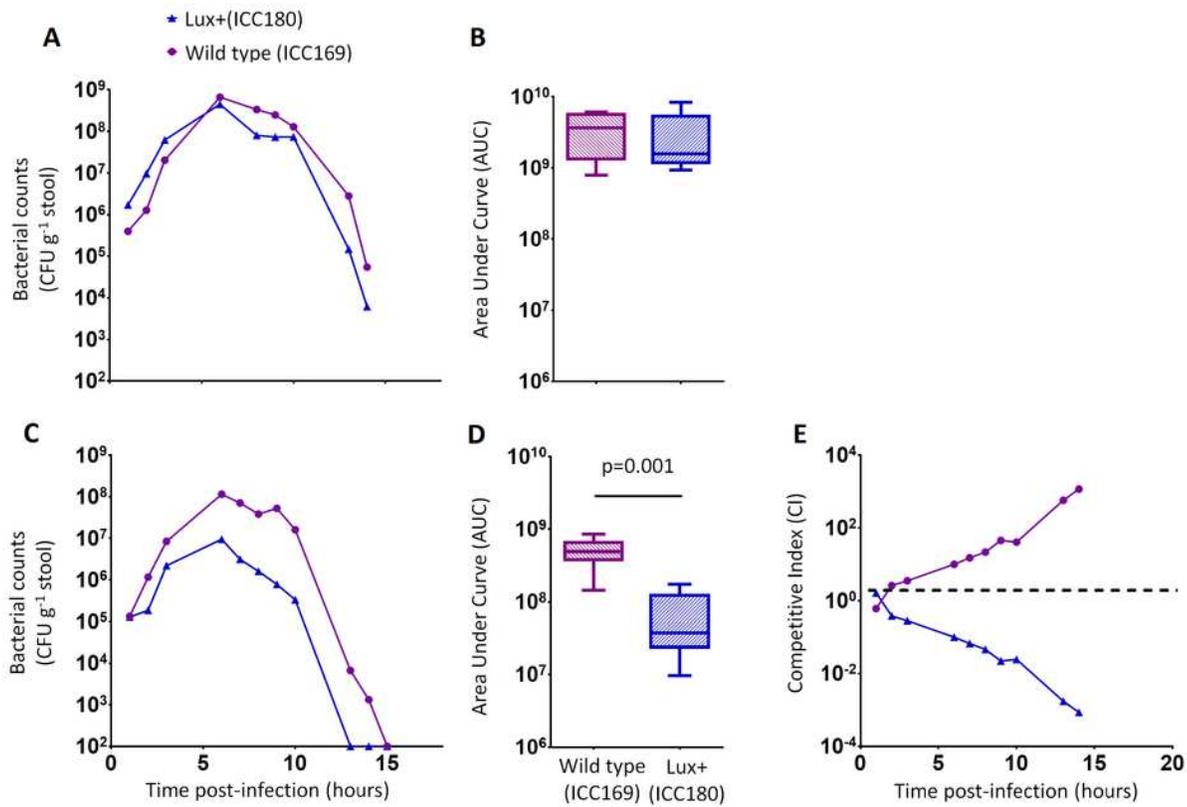
Groups of larvae (n = 10) of the Greater Wax Moth *Galleria mellonella* were infected with ICC169 and ICC180 in single and 1:1 mixed infections and monitored for survival (%) (A) and for disease symptoms using the Caterpillar Health Index (CHI), a numerical scoring system which measures degree of melanisation, silk production, motility, and mortality (given as median CHI values) (B). Survival curves (A) and calculated Area Under Curve data of CHI scores reveals no difference between waxworm response to infection from either strain (C). Waxworms infected with a 1:1 mix of ICC169 and ICC180 were homogenised at 24-hours, or at time of death if earlier. Actual infecting doses for each strain were determined by retrospective plating, and are indicated by \*. The bacterial burden of ICC180 and ICC169 in individual caterpillars (indicated by the dotted line), was calculated after plating onto differential media and found to be significantly different ( $p=0.001$ ; one-tailed Wilcoxon matched pairs-signed rank test) (D). Data (medians with ranges where appropriate) is presented from experiments performed on 3 separate occasions, except (A) and (D), where the results of a representative experiment are shown.



## 6

Figure 6. *C. rodentium* ICC180 is impaired during mixed, but not in single, infections in mice when compared to its non-bioluminescent parent strain ICC169.

Groups of female 6-8 week old C57Bl/6 mice (n=6) were orally-gavaged with  $\sim 5 \times 10^9$  CFU of wildtype *C. rodentium* ICC169 (shown as purple circles) and its bioluminescent derivative ICC180 (shown as blue triangles) in single infections (A, B) or 1:1 mixed infections (C, D) and monitored for changes in bacterial counts (given as colony forming units [CFU] g<sup>-1</sup> stool) (A, B). Bacterial count data was used to calculate Area Under Curve values for each strain in single (B) and mixed (D) infections, and were found to be significantly different only for the mixed infections (p=0.001; one-tailed Wilcoxon Matched pairs-signed rank test). This is reflected in the competitive indices (CI) calculated from the bacterial counts recovered during mixed infections, with ICC180 showing a growing competitive disadvantage from day 2 post-infection (E). Data (medians with ranges where appropriate) is presented from experiments performed on two separate occasions.



## 7

Figure 7. Despite having a fitness disadvantage in mixed infections of mice, ICC180 is still visible by biophotonic imaging.

Groups of female 6-8 week old C57Bl/6 mice (n=6) were orally-gavaged with  $\sim 5 \times 10^9$  CFU of wildtype *C. rodentium* ICC169 and its bioluminescent derivative ICC180 in single infections or 1:1 mixed infections. Bioluminescence (given as photons second<sup>-1</sup> cm<sup>-2</sup> sr<sup>-1</sup>) from ICC180 was measured after gaseous anesthesia with isoflurane using the IVIS<sup>®</sup> Kinetic camera system (Perkin Elmer). A photograph (reference image) was taken under low illumination before quantification of photons emitted from ICC180 at a binning of four over 1 minute using the Living Image software (Perkin Elmer). The sample shelf was set to position D (field of view, 12.5 cm). The images show peak bioluminescence with variations in colour representing light intensity at a given location and superimposed over the grey-scale reference image (A). Red represents the most intense light emission, whereas blue corresponds to the weakest signal. The color bar indicates relative signal intensity (as photons/second/cm<sup>2</sup>/steradian [Sr]). Bioluminescence from the abdominal region of individual mice also was quantified using the region of interest tool in the Living Image software program (given as photons second<sup>-1</sup>) and used to calculate Area Under Curve values for each individual animal (B). Dotted line represents background. Experiments were performed on two separate occasions. Three representative animals are shown.

