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1 **All *Yersinia enterocolitica* are pathogenic: Virulence of phylogroup 1 *Y.***
2 ***enterocolitica* in a *Galleria mellonella* infection model.**

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16 **Abstract**

17 *Yersinia enterocolitica* is a zoonotic pathogen and a common cause of
18 gastroenteritis in humans. The species is composed of 6 diverse phylogroups, of
19 which phylogroup 1 strains are considered non pathogenic to mammals due to
20 their lack of the major virulence plasmid pYV and their lack of virulence in a
21 mouse infection model. Here we present data examining the pathogenicity of
22 strains of *Y. enterocolitica* across all six phylogroups in a *Galleria mellonella*
23 model. We show that in this model phylogroup 1 strains exhibit severe
24 pathogenesis with a lethal dose of as low as 10 cfu. We show that this virulence is
25 an active process and that flagella play a major role in the virulence phenotype.
26 Furthermore, we show that the complete lack of virulence in *Galleria* of the
27 mammalian pathogenic phylogroups is not due to carriage of the pYV virulence
28 plasmid. Our data suggest that all *Y. enterocolitica* can be pathogenic, which may
29 be a reflection of the true natural habitat of the species and that we may need to
30 reconsider the eco-evo perspective of this important bacterial species.

31 **Introduction**

32 *Yersinia enterocolitica* is a member of the Enterobacteriaceae, and a common
33 cause of gastroenteritis in humans (Bottone, 1999). The majority of human
34 infections are associated with consumption of, or contamination from, raw and
35 undercooked pork products (Bottone, 1999; Drummond *et al.*, 2012). Carriage of
36 *Y. enterocolitica* is frequently reported in pig tonsil and intestinal tissues
37 (Martinez *et al.*, 2010; McNally *et al.*, 2004; Milnes *et al.*, 2008) as well as faecal
38 samples from cattle and sheep (McNally *et al.*, 2004; Milnes *et al.*, 2008). Human
39 yersiniosis is generally a sporadic infection (Bottone, 1997; Drummond *et al.*,
40 2012), however it is the third most common cause of bacterial gastroenteritis in
41 developed countries, behind *Campylobacter* and *Salmonella* (McNally *et al.*, 2004;
42 van Pelt *et al.*, 2003; Rosner *et al.*, 2010). Large outbreaks have also recently
43 been reported with prolonged epidemic curves (Gierczynski *et al.*, 2009).

44 *Y. enterocolitica* is classically typed using a series of biochemical utilisation tests
45 which separated the species into six distinct biotypes, and further subdivided by
46 classical serotyping (Bottone, 1999; Wauters *et al.*, 1987). More recently whole
47 genome sequences and phylogenetic studies have shown that biotypes are not
48 phylogenetically robust. This has resulted in a proposed a new nomenclature
49 consisting of phylogroup (PG) 1 (biotype 1A), phylogroup 2 (biotype 1B),
50 phylogroup 3 (serotype O:3), phylogroup 4 (serotype O5;27), phylogroup
51 5 (serotype O:9), and phylogroup 6 (biotype 5) (Hall *et al.*, 2015; Reuter *et al.*,
52 2014). PG 1 strains are isolated from a wide range of hosts and habitats and are
53 considered to be non-pathogenic due to the lack of pathology in a mouse
54 infection model (Bottone, 1997) and a lack of the major virulence factors found
55 in *Y. enterocolitica* (Bottone, 1999). PG 2 strains are considered high-pathogenic

56 due to lethality in a mouse infection model, whilst PG 3-6 strains are considered
57 low-pathogenic due to the observed pathology in a mouse infection model
58 (Bottone, 1999). The major genetic difference between PG 1 and PG 2-6 that
59 accounts of the differences in observed pathogenesis are the presence of the
60 virulence plasmid pYV and the adhesion-encoding gene *ail* in PG 2-6 (Reuter *et*
61 *al.*, 2014).

62 Despite lacking the key virulence factors involved in mammalian pathogenesis,
63 there is still some debate as to the true pathogenic potential of PG 1 *Y.*
64 *enterocolitica*. The suitability of the mouse infection model has been questioned
65 as a suitable proxy for human pathogenesis, with different mouse models giving
66 different levels of observed pathology (Schippers *et al.*, 2008). Epidemiological
67 studies have isolated PG 1 strains from humans with gastroenteritis (Mallik &
68 Viridi, 2010; McNally *et al.*, 2004), and experimental studies have shown that PG
69 1 isolates exhibit the ability to invade cultured epithelial cells (Grant *et al.*, 1999;
70 McNally *et al.*, 2006; Tennant *et al.*, 2003). PG 1 isolates have also been shown to
71 survive inside cultured macrophages for longer time-frames than pathogenic PG
72 2-5 isolates and to trigger a pro-inflammatory response upon macrophages
73 uptake (McNally *et al.*, 2006). It is known that many PG 1 isolates carry genes
74 that have been proposed to be *Yersinia* virulence factors (Kumar & Viridi, 2012;
75 Singh & Viridi, 2004; Tennant *et al.*, 2005) and population genomic studies have
76 shown that many genes purported to play a role in *Y. enterocolitica* pathogenesis
77 of other PGs are found in PG 1 isolates, and additionally a putative type III
78 secretion system is found exclusively in PG 1 (Reuter *et al.*, 2014). To date the
79 only factor that has been shown to be involved in a virulence associated trait in

80 PG 1 is the requirement of flagella to survive inside macrophages (McNally *et al.*,
81 2007b).

82 Here we present data examining the ability of PG 1 *Y. enterocolitica* to infect the
83 wax-moth insect larvae *Galleria mellonella*, a commonly used alternative
84 infection model for enteropathogens (Gaspar *et al.*, 2009; Senior *et al.*, 2011).

85 Our data shows that PG 1 isolates exhibit severe virulence in infected *Galleria*,
86 with a LD₅₀ of just 10 cfu, and that virulence is enhanced at 25°C compared to
87 37°C. We also show that the severe virulence of PG 1 isolates is in direct contrast
88 to mammalian pathogenic PG 2-5 isolates that exhibit almost no virulent
89 phenotype in *Galleria*. We also show that mutations in potential virulence genes
90 previously identified in PG 1 strains show no effect on the *Galleria* virulence
91 phenotype, but that the loss of flagella function previously shown to be
92 necessary for survival in macrophages also attenuates pathogenesis in *Galleria*.
93 Therefore the term non-pathogenic should not be applied to PG 1 *Y.*
94 *enterocolitica* given the high levels of entemopathogenesis observed here, and
95 that a more comprehensive understanding of *Y. enterocolitica* ecology is required
96 to fully dissect the lifecycle of this highly diverse bacterial species.

97 **Materials and methods**

98 **Bacterial strains and plasmids**

99 A full list of bacterial isolates (Table 1) and plasmids (Table 2) used in this study
100 is provided. *Y. enterocolitica* isolates were collected from human, pig, cattle, and
101 sheep faecal samples (McNally *et al.*, 2004). The isolates investigated in depth in
102 the study represent comprehensively characterised type strains of each
103 phylogroup (McNally *et al.*, 2006) and for which a reference genome sequence
104 has been produced (Reuter *et al.*, 2014). All strains were routinely cultured from

105 glycerol stocks stored at -80°C using LB agar at 25°C. For all experiments 10
106 colonies from an agar plate were incubated in 5 ml LB broth at 25°C with shaking
107 at 200rpm for 18 hours. Strains YE8081, YE1203 and YE14902 had pYV minus
108 derivative constructed by 2 x 18 hour serial passages on LB agar at 37°C
109 followed by incubation on CRMOX agar plates at 37°C and selection of large non-
110 pigmented colonies (Farmer *et al.*, 1992). Absence of pYV was confirmed by
111 Kado and Liu gel electrophoresis (Kado & Liu, 1981) and by PCR using primers
112 Yscp1 and Yscp2 to detect the YscP gene present on pYV.

113 ***Galleria* infection assay:** *Galleria mellonella* were infected as previously
114 described (Fuchs *et al.*, 2008). *Galleria* larvae were infected with a series of
115 bacterial suspensions containing 10¹, 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸ & 10⁹ cfu of
116 each *Y. enterocolitica* strain. Each dose was injected sub-cutaneously in 10 µl-
117 aliquots into a group of 10 active *G. mellonella* larvae using a Hamilton syringe.
118 After injection, each group was placed on a separate 90 mm sterile Petri Dish
119 containing a 90mm diameter Whatman filter paper. The injected *Galleria* groups
120 were then incubated in the dark at 25°C or 37°C and monitored for a period of 5
121 days. Ten larvae were injected with a sterile PBS, and 10 were incubated without
122 any form of injection or treatment. Cessation of movement and changes in larvae
123 cuticle colour were checked to distinguish dead larvae. All experiments were
124 repeated in triplicate independently. The LD₅₀ value (the lethal dose required to
125 kill 50% or more larvae after 5 days incubation) was calculated, and statistical
126 significance tests were performed using two-sample T-tests.

127 For experiments enumerating number of bacteria surviving inside *Galleria*
128 larvae, individual larvae were sacrificed by incision with a scalpel and then
129 ground with a sterile mortar and pestle. The material was then resuspended in

130 10ml sterile PBS and used for bacterial enumeration using CIN *Yersinia* selective
131 agar.

132 **Mutagenesis of the *cdt* and YGT loci**

133 All mutagenesis studies were performed in the genome sequenced type PG 1
134 strain YE5303 (McNally *et al.*, 2007; Reuter *et al.*, 2014). A cytolethal distending
135 toxin (CDT) mutant was made by PCR amplifying *cdtB* using primers CDTFor and
136 CDTRev (Table 2) and cloning into pCRTopo2.1 (Invitrogen) to create pAD5. The
137 *cat* gene was PCR amplified from pAM6 using primers CmFor and CmRev (Table
138 2) and cloned into the *AgeI* site of pAD5. The inactivated *cdtB* gene was then PCR
139 amplified and subcloned into the *SmaI* site of pKNG101. The resulting plasmid
140 was used to transform *E. coli* S17-1 Pir cells, and these were used as donor cells
141 in a filter mating conjugation with YE5303 (McNally *et al.*, 2007). A functional
142 *Yersinia* Genus Type III secretion system (YGT) mutant was constructed by PCR
143 amplifying the apparatus encoding gene *ygtV* (Reuter *et al.*, 2014) using primers
144 *ygtvFor* and *ygtvRev* (Table 2) and cloning into pCRTopo2.1 to create pAR1. The
145 *cat* gene was PCR amplified from pAM6 using primers CmFor and CmRev and
146 cloned into an *NheI* site. The inactivated *ygtV* gene was then PCR amplified and
147 subcloned into the *SmaI* site of pKNG101. The resulting plasmid, pAR3, was used
148 to transform *E. coli* S17-1 Pir cells, and these were used as donor cells in a filter
149 mating conjugation with YE5303 (McNally *et al.*, 2007). Complementation of the
150 *cdtB* and *ygtV* mutants was performed by transforming the mutated strains with
151 pAD5 and pAR1 respectively.

152 **Results**

153 **Phylogroup 1 *Y. enterocolitica* show high pathogenicity towards *Galleria***
154 ***mellonella***

155 To determine variation in the pathogenic potential of *Y. enterocolitica*
156 phylogroups to *G. mellonella*, strains YE5303 (PG 1), YE8081 (PG 2), YE1203 (PG
157 3), YE14902 (PG 4), YE5603 (PG 5) and YE3094/96 (PG 6) were used to
158 inoculate groups of larvae. Bacteria were pre-grown at 25°C and 37°C prior to
159 inoculation, and larvae were also incubated at both temperatures post-infection.
160 The LD₅₀ of each strain in each infection condition was calculated and plotted
161 (Fig 1, Fig S1). The data clearly shows that YE8081 of PG 2, the highly pathogenic
162 phylogroup in mouse infection models is the least pathogenic in the *Galleria*
163 assay ($p = 0.001$), and that YE5303 belonging to PG 1, which is considered to be
164 non-pathogenic to mammalian hosts is the most virulent in all conditions tested
165 ($p < 0.00001$) with virulence enhanced at 25°C compared to 37°C, and occurring
166 when larvae were incubated down to as low as 15°C (data not shown). The
167 mammalian low-pathogenic PG 3-6 strains all showed very low levels of
168 virulence to *Galleria*, with the exception of the PG 4 strain YE14902. To confirm
169 the findings a further 23 strains were tested in the *Galleria* assay (Fig S2) with
170 bacteria pre-grown at 25°C and the infected *Galleria* incubated at 37°C, which
171 show PG 1 strains significantly more virulent in the assay ($p = 0.03$).

172 **Virulence of PG1 *Y. enterocolitica* in *Galleria* is an active process**
173 **characterised by rapid death**

174 We sought to determine the kinetics of infection by YE5303 in the *Galleria* assay.
175 First we determined the time-to-death for the larvae in all combinations of pre
176 and post inoculation incubation and doses of bacteria (Fig 2). The results show
177 that the vast majority of killing occurs rapidly between 10 and 24 hours after
178 infection, with the exception being doses at or around sub-lethal levels where
179 small numbers of larvae may die after 24-48 hours. We then took lethal doses (1

180 x 10⁹ cfu) and sub lethal doses of YE5303, YE1203, YE14902 and YE8081 (1 x
181 10² cfu, 1x 10⁶ cfu, 1 x 10⁴cfu, and 1 x 10⁷ cfu, respectively) and inoculated
182 *Galleria*. At time intervals we sacrificed 5 x larvae in each group and counted the
183 number of recovered *Yersinia* from each larva (Fig 3). Our data shows that with
184 the mammalian pathogenic strains (YE1203, YE14902, and YE8081) the number
185 of bacteria is unchanged regardless of fate of the larvae. However, in the PG 1
186 YE5303 the number of bacteria surviving inside the *Galleria* drops dramatically
187 in the 24 hours leading to death, whilst in a sub-lethal dose there is rapid and
188 complete clearance of bacteria. To confirm that the fatal virulence of YE5303 was
189 an active process we prepared serial dilutions of overnight cultures of YE5303
190 and then heat killed the cells at 60°C for 1 hour (Autenrieth *et al.*, 1994) before
191 injecting larvae. No killing of *Galleria* larvae was observed after injection with
192 any dose of heat killed bacterial cells.

193 **The *Yersinia* virulence plasmid pYV does not have a protective effect on**
194 ***Galleria* infection**

195 Given the clear difference in pathogenesis in the *Galleria* model between pYV
196 bearing strains and *Y. enterocolitica* PG 1, we sought to determine if pYV was
197 involved in the observed non-pathogenic phenotype of PG 2-6 strains. The pYV
198 plasmid was cured from YE8081, YE1203, and YE14902 by serial culture at 37°C
199 in the absence of calcium ions, and loss of pYV confirmed by PCR and Kado & Liu
200 gel electrophoresis. The plasmid + and plasmid - derivatives were then used to
201 perform larval infections and LD₅₀ compared (Fig 4). The data clearly shows that
202 the loss of pYV has no impact on the lack of pathogenesis of pYV bearing strains
203 on *Galleria* larvae. We also checked the stability of pYV during infections by PCR
204 amplification performed on bacteria recovered from dead and surviving larvae

205 (Fig 4). This shows that pYV was stable in all strains except the PG4 strain
206 YE14902 where 50% (6 of 12 colonies tested by PCR) of tested colonies had lost
207 the plasmid. PG4 strains curiously are also the most virulent of the pYV bearing
208 phylogroups in the *Galleria* assay.

209 **Targeted mutagenesis suggests a role for flagella and intracellular survival**
210 **in the pathogenesis of PG1 *Y. enterocolitica* to *Galleria* larvae**

211 Comparative analysis of 100 *Y. enterocolitica* genomes spanning the entire
212 species diversity identified two putative virulence factors that are unique or
213 have PG 1 unique alleles (Reuter, *et al.*, 2015). These are the YGT type III
214 secretion system, and the cytolethal distending toxin CDT. A YGT mutant was
215 constructed by insertional inactivation of the *ygtV* apparatus encoding gene, and
216 a CDT mutant by insertional inactivation of the *cdtB* gene. We also utilised a
217 functional flagella mutant made by insertional inactivation of *flgB* previously
218 described by our group (McNally *et al.*, 2007). The mutants and complemented
219 mutants were used to perform *Galleria* infections and LD₅₀ calculations (Fig 5).
220 Our data show that mutations in the CDT operon or YGT secretion system have
221 no discernable effect on virulence of YE5303 in *Galleria* larvae. However our
222 previously constructed and characterised flagella mutant has a significant
223 decrease ($p = 0.0014$) in virulence compared to the wild type, with restoration of
224 the phenotype upon complementation with the *flgB* gene on a high copy number
225 plasmid. To test if the lethality may be due to secretion of toxic effectors from
226 the flagella apparatus we tested the lethality of supernatant from overnight
227 cultures of YE5303 and the *flgB* mutant in *Galleria* larvae. Our results showed
228 that supernatant from the wild type YE5303 showed 100% mortality (20/20

229 larvae) whilst supernatant from the *flgB* mutant showed 10% mortality (2/20
230 injected larvae).

231 **Discussion**

232 *Yersinia enterocolitica* is a common causative agent of gastroenteritis in humans
233 and is a zoonotic infection (Valentin-Weigand, P. Heesemann, J. Dersch, 2014).

234 Recent population genomic studies have shown that *Y. enterocolitica* is a highly
235 diverse species composed of six genetically distinct phylogroups (Hall *et al.*,
236 2015; Reuter *et al.*, 2014; Reuter *et al.*, 2015). The pathogenic potential of each of
237 the phylogroups has been well characterised on the basis of epidemiological
238 studies of human infections (Fredriksson-Ahomaa & Korkeala, 2003; McNally *et al.*,
239 *et al.*, 2004) as well as the use of mouse models of infection (Handley *et al.*, 2004).

240 However there is still discordance between such data sets, with a prime example
241 being the frequent isolation of PG 1 *Y. enterocolitica* from symptomatic humans
242 (Mallik & Viridi, 2010; McNally *et al.*, 2004) despite this lineage lacking the
243 essential pYV virulence plasmid (Reuter *et al.*, 2014) and being completely non-
244 pathogenic in mouse infection models (Schiemann & Devenish, 1982). To further
245 investigate this dichotomy we utilised the *Galleria mellonella* infection model as
246 a novel infection model for representative strain of all *Y. enterocolitica*
247 phylogroups.

248 Our data shows that the PG 1 *Y. enterocolitica* strains are highly virulent to *G.*
249 *mellonella* larvae with a lethal dose as low as 10 cfu, with virulence enhanced
250 when the infection is incubated at 25°C compared to 37°C, though there was no
251 difference if the bacteria were pre-incubated at different temperatures prior to
252 infection. Conversely, the so called high-pathogenic PG 2 strains showed virtually
253 no virulence at all using any infection conditions. Additionally, the most

254 frequently encountered human-pathogenic phylogroups showed only trace
255 levels of virulence with infectious doses of $10^7 - 10^9$ cfu. These results appear
256 counterintuitive and may suggest that *G. mellonella* is a measure of virulence for
257 insects, but not for human disease, at least for *Y. enterocolitica*.

258 Our data raise more questions on our perceived knowledge of the ecology, life
259 style and evolution of pathogenesis of the *Y. enterocolitica* species. Previous
260 work has shown variation in pathogenesis of the human pathogenic *Yersinia*
261 species in insect models of infection (Fuchs *et al.*, 2008) and that insect toxin
262 genes present in PG 3, 4 and 5 strains of *Y. enterocolitica* only contribute to
263 virulence in insects infected via oral ingestion (Fuchs *et al.*, 2008). Our data
264 clearly shows that PG 1 *Y. enterocolitica* are acutely pathogenic to *Galleria*
265 *mellonella* via direct sub-cutaneous injection whilst the mammalian pathogenic
266 phylogroups are not, and it would now be interesting to test PG 1 strains via oral
267 ingestion by insects as these were not tested in the previous study. The variation
268 in pathogenesis suggests that different phylogroups are exposed to varying
269 predation threats, supporting recent population genomic analysis suggesting
270 that the phylogroups inhabit distinct ecological niches or micro-habitats on the
271 basis of limited gene sharing (Reuter *et al.*, 2015).

272 A key difference between PG 1 *Y. enterocolitica* and the pathogenic phylogroups
273 is the absence of the pYV virulence plasmid, the major virulence determinant in
274 mammalian pathogenic *Yersinia* species (Reuter *et al.*, 2014). The plasmid
275 contains the Ysc type III secretion system which is known to be used by *Yersinia*
276 to disarm macrophages and dendritic cells to allow the bacteria to avoid
277 phagocytosis (Cornelis & Wolf-Watz, 1997). Given that *G. mellonella* are known
278 to contain a functional non-specific immune response it seemed obvious that this

279 may be the reason for the lack of response of the larvae to the mammalian
280 pathogenic phylogroups. However our data shows that loss of pYV had no effect
281 on the virulence of the mammalian pathogenic phylogroups towards the larvae.
282 Indeed our data shows that the virulence of PG 1 *Y. enterocolitica* towards *G.*
283 *mellonella* is an active process that requires live cells. Our finding that heat killed
284 bacterial cells are unable to cause mortality also rule out the possibility that LPS,
285 which is phylogroup specific in *Y. enterocolitica* (Reuter, *et al.*, 2015), is the cause
286 of the severe toxicity of PG 1 strains.

287 Rather our results show a key role for flagella in the pathogenesis of phylogroup
288 1 *Y. enterocolitica* in *G. mellonella*. Previous work by our group showed that fully
289 functioning flagella are required for the ability of a PG 1 strain to survive inside
290 human cultured phagocytic cells for prolonged periods (McNally *et al.*, 2006,
291 2007a). This suggests that the pathogenesis of PG 1 *Y. enterocolitica* to *G.*
292 *mellonella* relies upon the ability of bacteria to survive the interaction with
293 phagocytic cells in the haemocoel (Fuchs *et al.*, 2008). The essential role of
294 flagella in the *G. mellonella* virulence process also explains the increased
295 pathogenesis when infected larvae are incubated at 25°C which is the permissive
296 temperature for flagella gene expression in *Y. enterocolitica* (Kapatral *et al.*,
297 1996). However, the fact that PG 1 strains still show toxicity at 37°C and that the
298 pre-infection incubation temperature has no effect on toxicity suggests that there
299 are other underlying molecular mechanisms of both flagella expression
300 regulation and *G. mellonella* pathogenesis. Indeed there are no apparent
301 differences in flagella structure or amino acid sequence between PG 1 and PG 2-5
302 strains (Reuter *et al.*, 2014; Reuter *et al.*, 2015). This means that the presence of
303 flagella alone is not sufficient to induce toxicity in the larvae, and that PG 1 *Y.*

304 *enterocolitica* utilise their flagella differently to other lineages of the species. It
305 may be that PG 1 strains utilise their flagella as a secretion system for lineage
306 specific effector proteins, something which has previously been proposed in the
307 species (Schmiel *et al.*, 2000) and is supported by the data showing that
308 supernatant from wild type PG1 bacteria is lethal to larvae but supernatant from
309 a flagella mutant is not . Alternatively it may be that there is co-ordinated
310 interaction between flagella and another as-yet-unidentified system in the
311 mammalian pathogenic phylogroups that down-regulates the pathogenic
312 phenotype of the flagella. It is known that there is transcriptional regulation
313 interplay between flagella and the Ysc secretion system in *Y. enterocolitica*
314 (Kapatral & Minnich, 1995), and so it is possible that flagella function is
315 differentially regulated in each lineage.

316 We therefore propose that it is no longer accurate to describe PG 1 *Y.*
317 *enterocolitica* as non-pathogenic, and that using an insect infection model we
318 show that all phylogroups of the *Y. enterocolitica* are capable of exhibiting high
319 levels of virulence in selected hosts. This emphasises our need to better
320 understand the true ecology of each lineage of this important bacterial species.
321 Furthermore there is now merit to fully investigate the differential functional
322 roles of flagella in each of the *Y. enterocolitica* phylogroups, as well as differences
323 that may exist in their regulatory control.

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329

330 **References**

331 **Autenrieth, I. B., Beer, M., Bohn, E., Kaufmann, S. H. & Heesemann, J. (1994).**

332 Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and
333 resistant C57BL/6 mice: an essential role for gamma interferon. *Infect*
334 *Immun* **62**, 2590–2599.

335 **Bottone, E. J. (1997).** *Yersinia enterocolitica*: the charisma continues. *Clin*
336 *Microbiol Rev* **10**, 257–276.

337 **Bottone, E. J. (1999).** *Yersinia enterocolitica*: overview and epidemiologic
338 correlates. *Microbes Infect* **1**, 323–333.

339 **Cornelis, G. R. & Wolf-Watz, H. (1997).** The *Yersinia* Yop virulon: a bacterial
340 system for subverting eukaryotic cells. *Mol Microbiol* **23**, 861–867.

341 **Drummond, N., Murphy, B. P., Ringwood, T., Prentice, M. B., Buckley, J. F. &**
342 **Fanning, S. (2012).** *Yersinia enterocolitica*: a brief review of the issues
343 relating to the zoonotic pathogen, public health challenges, and the pork
344 production chain. *Foodborne Pathog Dis* **9**, 179–189. United States.

345 **Farmer, J.J., Carter, G. P., Miller, V. L., Falkow, S. & Wachsmuth, I. K. (1992).**
346 Pyrazinamidase, CR-MOX agar, salicin fermentation-esculin hydrolysis, and
347 D-xylose fermentation for identifying pathogenic serotypes of *Yersinia*
348 *enterocolitica*. *J Clin Microbiol* **30**, 2589–2594.

349 **Fredriksson-Ahomaa, M. & Korkeala, H. (2003).** Low occurrence of
350 pathogenic *Yersinia enterocolitica* in clinical, food, and environmental
351 samples: a methodological problem. *Clin Microbiol Rev* **16**, 220–+.

352 **Fuchs, T., Bresolin, G., Marcinowski, L., Schachtner, J. & Scherer, S. (2008).**
353 Insecticidal genes of *Yersinia* spp.: taxonomical distribution, contribution to
354 toxicity towards *Manduca sexta* and *Galleria mellonella*, and evolution. *BMC*
355 *Microbiol* **8**, 214.

356 **Gaspar, F., Teixeira, N., Rigottier-Gois, L., Marujo, P., Nielsen-LeRoux, C.,**
357 **Crespo, M. T. B., Lopes, M. de F. tima S. & Serror, P. (2009).** Virulence of
358 *Enterococcus faecalis* dairy strains in an insect model: the role of *fsrB* and
359 *gelE*. *Microbiology* **155**, 3564–3571.

360 **Gierczynski, R., Szych, J., Rastawicki, W., Wardak, S. & Jagielski, M. (2009).**
361 Molecular Characterization of Human Clinical Isolates of *Yersinia*
362 *enterocolitica* Bioserotype 1B/O8 in Poland: Emergence and Dissemination
363 of Three Highly Related Clones. *J Clin Microbiol* **47**, 1225–1228.

364 **Grant, T., Bennett-Wood, V. & Robins-Browne, R. M. (1999).** Characterization
365 of the Interaction between *Yersinia enterocolitica* Biotype 1A and
366 Phagocytes and Epithelial Cells In Vitro. *Infect Immun* **67**, 4367–4375.

367 **Hall, M., Chattaway, M. A., Reuter, S., Savin, C., Strauch, E., Carniel, E.,**
368 **Connor, T., Van Damme, I., Rajakaruna, L. & other authors. (2015).** Use
369 of whole-genus genome sequence data to develop a multilocus sequence
370 typing tool that accurately identifies *Yersinia* isolates to the species and
371 subspecies levels. *J Clin Microbiol* **53**, 35–42.

372 **Handley, S. A., Dube, P. H., Revell, P. A. & Miller, V. L. (2004).** Characterization
373 of Oral *Yersinia enterocolitica* Infection in Three Different Strains of Inbred
374 Mice. *Infect Immun* **72**, 1645–1656.

375 **Kado, C. I. & Liu, S. T. (1981).** Rapid procedure for detection and isolation of
376 large and small plasmids. *J Bacteriol* **145**, 1365–1373.

377 **Kaniga, K., Delor, I. & Cornelis, G. R. (1991).** A wide-host-range suicide vector
378 for improving reverse genetics in Gram-negative bacteria: inactivation of the
379 blaA gene of *Yersinia enterocolitica*. *Gene* **109**, 137–141.

380 **Kapatral, V. & Minnich, S. A. (1995).** Co-ordinate, temperature-sensitive
381 regulation of the three *Yersinia enterocolitica* flagellin genes. *Mol Microbiol*
382 **17**, 49–56.

383 **Kapatral, V., Olson, J. W., Pepe, J. C., Miller, V. L. & Minnich, S. A. (1996).**
384 Temperature-dependent regulation of *Yersinia enterocolitica* Class III
385 flagellar genes. *Mol Microbiol* **19**, 1061–1071.

386 **Kumar, P. & Viridi, J. S. (2012).** Identification and distribution of putative
387 virulence genes in clinical strains of *Yersinia enterocolitica* biovar 1A by
388 suppression subtractive hybridization. *J Appl Microbiol* **113**, 1263–1272.
389 England.

390 **Mallik, S. & Viridi, J. (2010).** Genetic relationships between clinical and non-
391 clinical strains of *Yersinia enterocolitica* biovar 1A as revealed by multilocus
392 enzyme electrophoresis and multilocus restriction typing. *BMC Microbiol* **10**,
393 158.

394 **Martinez, P. O., Fredriksson-Ahomaa, M., Pallotti, A., Rosmini, R., Houf, K. &**
395 **Korkeala, H. (2010).** Variation in the Prevalence of Enteropathogenic
396 *Yersinia* in Slaughter Pigs from Belgium, Italy, and Spain. *Foodborne Pathog*
397 *Dis.*

398 **McNally, A., Cheasty, T., Fearnley, C., Dalziel, R. W., Paiba, G. A., Manning, G.**
399 **& Newell, D. G. (2004).** Comparison of the biotypes of *Yersinia*
400 *enterocolitica* isolated from pigs, cattle and sheep at slaughter and from
401 humans with yersiniosis in Great Britain during 1999 - 2000. *Lett Appl*
402 *Microbiol* **39**, 103–108.

403 **McNally, A., Ragione, R. M. La, Best, A., Manning, G. & Newell, D. G. (2007a).**
404 An aflagellate mutant *Yersinia enterocolitica* biotype 1A strain displays
405 altered invasion of epithelial cells, persistence in macrophages, and cytokine
406 secretion profiles in vitro. *Microbiology* **153**, 1339–1349.

407 **McNally, A., Dalton, T., Ragione, R. M. La, Stapleton, K., Manning, G. &**
408 **Newell, D. G. (2006).** *Yersinia enterocolitica* isolates of differing biotypes
409 from humans and animals are adherent, invasive and persist in
410 macrophages, but differ in cytokine secretion profiles in vitro. *J Med*
411 *Microbiol* **55**, 1725–1734.

412 **McNally, A., Ragione, R. M. La, Best, A., Manning, G. & Newell, D. G. (2007b).**
413 An aflagellate mutant *Yersinia enterocolitica* biotype 1A strain displays
414 altered invasion of epithelial cells, persistence in macrophages, and cytokine
415 secretion profiles in vitro. *Microbiology* **153**, 1339–1349.

416 **Milnes, A. S., Stewart, I., Clifton-Hadley, F. A., Davies, R. H., Newell, D. G.,**
417 **Sayers, A. R., Cheasty, T., Cassar, C., Ridley, A. & other authors. (2008).**
418 Intestinal carriage of verocytotoxigenic *Escherichia coli* O157, *Salmonella*,
419 thermophilic *Campylobacter* and *Yersinia enterocolitica*, in cattle, sheep and
420 pigs at slaughter in Great Britain during 2003. *Epidemiol Infect* **136**, 739–
421 751.

422 **van Pelt, W., de Wit, M. A. S., Wannet, W. J. B., Ligtoet, E. J. J., Widdowson, M.**
423 **A. & van Duynhoven, Y. (2003).** Laboratory surveillance of bacterial
424 gastroenteric pathogens in The Netherlands, 1991-2001. *Epidemiol Infect*
425 **130**, 431–441.

426 **Reuter, S., Connor, T. R., Barquist, L., Walker, D., Feltwell, T., Harris, S. R.,**
427 **Fookes, M., Hall, M. E., Petty, N. K. & other authors. (2014).** Parallel
428 independent evolution of pathogenicity within the genus *Yersinia*. *Proc Natl*
429 *Acad Sci U S A* **111**, 6768–6773.

430 **Reuter, S., Corander, J., de Been, M., Harris, S., Cheng, L., Hall, M., Thomson,**
431 **N.R., McNally, A. (2015).** Directional gene flow and ecological separation in
432 *Yersinia enterocolitica*. *Microb Genomics* DOI: [10.1099/mgen.0.000030](https://doi.org/10.1099/mgen.0.000030)

433 **Rosner, B. M., Stark, K. & Werber, D. (2010).** Epidemiology of reported
434 *Yersinia enterocolitica* infections in Germany, 2001-2008. *BMC Public Health*
435 **10**, 337.

436 **Schiemann, D. A. & Devenish, J. A. (1982).** Relationship of HeLa cell infectivity
437 to biochemical, serological, and virulence characteristics of *Yersinia*
438 *enterocolitica*. *Infect Immun* **35**, 497–506.

439 **Schippers, A., Mateika, S., Prochnow, B., Gruber, A. D., Müller, W. &**
440 **Frischmann, U. (2008).** Susceptibility of four inbred mouse strains to a
441 low-pathogenic isolate of *Yersinia enterocolitica*. *Mamm Genome* **19**, 279–
442 291.

443 **Schmiel, D. H., Young, G. M. & Miller, V. L. (2000).** The *Yersinia enterocolitica*
444 Phospholipase Gene *yplA* Is Part of the Flagellar Regulon. *J Bacteriol* **182**,

445 2314–2320.

446 **Senior, N. J., Bagnall, M. C., Champion, O. L., Reynolds, S. E., Ragione, R. M. La,**
447 **Woodward, M. J., Salguero, F. J. & Titball, R. W. (2011).** *Galleria*
448 *mellonella* as an infection model for *Campylobacter jejuni* virulence. *J Med*
449 *Microbiol* **60**, 661–669.

450 **Singh, I. & Viridi, J. S. (2004).** Production of Yersinia stable toxin (YST) and
451 distribution of *yst* genes in biotype 1A strains of *Yersinia enterocolitica*. *J*
452 *Med Microbiol* **53**, 1065–1068.

453 **Tennant, S. M., Grant, T. H. & Robins-Browne, R. M. (2003).** Pathogenicity of
454 *Yersinia enterocolitica* biotype 1A. *FEMS Immunol Med Microbiol* **38**, 127–
455 137.

456 **Tennant, S. M., Skinner, N. A., Joe, A. & Robins-Browne, R. M. (2005).**
457 Homologues of Insecticidal Toxin Complex Genes in *Yersinia enterocolitica*
458 Biotype 1A and Their Contribution to Virulence. *Infect Immun* **73**, 6860–
459 6867.

460 **Thomson, N. R., Howard, S., Wren, B. W., Holden, M. T. G., Crossman, L.,**
461 **Challis, G. L., Churcher, C., Mungall, K., Brooks, K. & other authors.**
462 **(2006).** The Complete Genome Sequence and Comparative Genome
463 Analysis of the High Pathogenicity *Yersinia enterocolitica* Strain 8081. *PLoS*
464 *Genet* **2**, e206.

465 **Valentin-Weigand, P. Heesemann, J. Dersch, P. (2014).** Unique virulence
466 properties of *Yersinia enterocolitica* O:3 - An emerging zoonotic pathogen
467 using pigs as preferred reservoir host. *Int J Med Microbiol* **14**, 00090–3.

468 **Wauters, G., Kandolo, K. & Janssens, M. (1987).** Revised biogrouping scheme
469 of *Yersinia enterocolitica*. *Contrib Microbiol Immunol* **9**, 14–21.

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472 **Table 1: List of strains used in this study**

Strain Name	Phylogroup	Biological Origin	Source
YE8081	PG1	Human	(Thomson <i>et al.</i> , 2006)
YE0902	PG1	Sheep	(Reuter <i>et al.</i> , 2014)
YE0903	PG1	Human	(Reuter <i>et al.</i> , 2014)
YE3403	PG1	Human	(Reuter <i>et al.</i> , 2014)
YE3503	PG1	Pig	(Reuter <i>et al.</i> , 2014)
YE5303	PG1	Human	(McNally <i>et al.</i> , 2006)
NZ3	PG1	Sheep	(Reuter <i>et al.</i> , 2014)
YE11902	PG5	Sheep	(Reuter <i>et al.</i> , 2014)
YE21202	PG5	Pig	(McNally <i>et al.</i> , 2006)
YE21502	PG5	Pig	(Reuter <i>et al.</i> , 2014)
YE21802	PG5	Pig	(Reuter <i>et al.</i> , 2014)
YE2403	PG5	Human	(Reuter <i>et al.</i> , 2014)
YE5603	PG5	Human	(McNally <i>et al.</i> , 2006)
YE5803	PG5	Human	(Reuter <i>et al.</i> , 2014)
YE11102	PG4	Sheep	(Reuter <i>et al.</i> , 2014)
YE14902	PG4	Sheep	(McNally <i>et al.</i> , 2006)
YE15302	PG4	Cattle	(Reuter <i>et al.</i> , 2014)
YE22602	PG4	Pig	(Reuter <i>et al.</i> , 2014)
YE23102	PG4	Pig	(Reuter <i>et al.</i> , 2014)
YE23202	PG4	Pig	(Reuter <i>et al.</i> , 2014)
YE01/2012	PG3	Human	Claire Jenkins HPA
YE02/2012	PG3	Human	Claire Jenkins HPA
YE0303	PG3	Human	(Reuter <i>et al.</i> , 2014)
YE1203	PG3	Human	(McNally <i>et al.</i> , 2006)
YE20102	PG3	Pig	(Reuter <i>et al.</i> , 2014)
YE20402	PG3	Human	(Reuter <i>et al.</i> , 2014)
YE21302	PG3	Pig	(Reuter <i>et al.</i> , 2014)
NZ15	PG3	Pig	(Reuter <i>et al.</i> , 2014)
Y1	PG3	Human	Petra Dersch, HZI)
YE3094/96	PG6	Hare	(Reuter <i>et al.</i> , 2014)
YE5303- <i>flgB</i> Mut		YE5303 with <i>flgB</i> gene inactivated	(McNally <i>et al.</i> , 2007)
YE5303- <i>cdtB</i> Mut		YE5303 with <i>cdtB</i> gene inactivated	This study
YE5303- <i>ygtV</i> Mut		YE5303 with <i>ygtV</i> gene inactivated	This study
<i>E. coli</i> S17-1 Pir			Epicentre UK
<i>E. coli</i> DH5 α			Invitrogen UK

474 **Table 2: List of primers and plasmids used in this study**

Name	Description	Source
pCR2.1-TOPO	TA cloning vector	Invitrogen
pAR1	pCR2.1 with <i>ygtV</i> inserted	This study
pAM6	pCR2.1 with <i>cat</i> inserted	(McNally <i>et al.</i> , 2007a)
pAR2	pAR1 with <i>cat</i> inserted	This study
pKNG101	<i>sacB</i> /λPir suicide vector	(Kaniga <i>et al.</i> , 1991)
pAR3	pKNG101 with Inactivated <i>ygtV</i> from pAR2 inserted	This study
pAD5	pCR2.1 with <i>cdtB</i> inserted	This study
pAD6	pAD5 with <i>cat</i> inserted	This study
pAD9	pKNG101 with inactivated <i>cdtB</i> from pAD6 inserted	This study
cdtFor	GGAAATAAATAAATCTGG	Tm 53°C
cdtRev	GGGTGAGTAGAGTACGGT	
ygtFor	GCGCTATATCAGGTAGTTTC	Tm 57°C
ygtRev	CGGGAGAATACCGATGAGAG	
CmFor	ACCGAGCGTAGCGAGTCAGT	Tm 60°C
CmRev	ATTACGCCCCGCCCTGC	
YscP1	ATTAGAACCTGAGTATCAACC	Tm 52°C
YscP2	AACAAATAACTCATCATGTCC	

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481 **Figure 1:** The calculated LD₅₀ values for infection of *Galleria mellonella* of a cross
482 section of reference *Y. enterocolitica* strains representing the diversity of the
483 species. Results show values for strains pre-incubated at both 25°C and 37°C
484 prior to inoculation into larvae. Black bars represent LD₅₀ values for infection at
485 37°C and grey bars represent values for infection at 25°C. Values are the mean
486 for three independent experiments and error bars represent the standard error
487 of the mean.

488

489 **Figure 2:** Survival curves for *Galleria mellonella* infected with the phylogroup 1
490 *Y. enterocolitica* reference strain 5303. Data shown is a representative
491 experiment of three independent replicate experiments.

492

493 **Figure 3:** Infection kinetics graphs showing the numbers of bacteria recovered
494 from infected *Galleria mellonella* larvae infected with reference *Y. enterocolitica*
495 strains at lethal and sub-lethal doses. Data shown are the mean of three
496 independent experiments with the error bars indicating the standard error of
497 the mean.

498

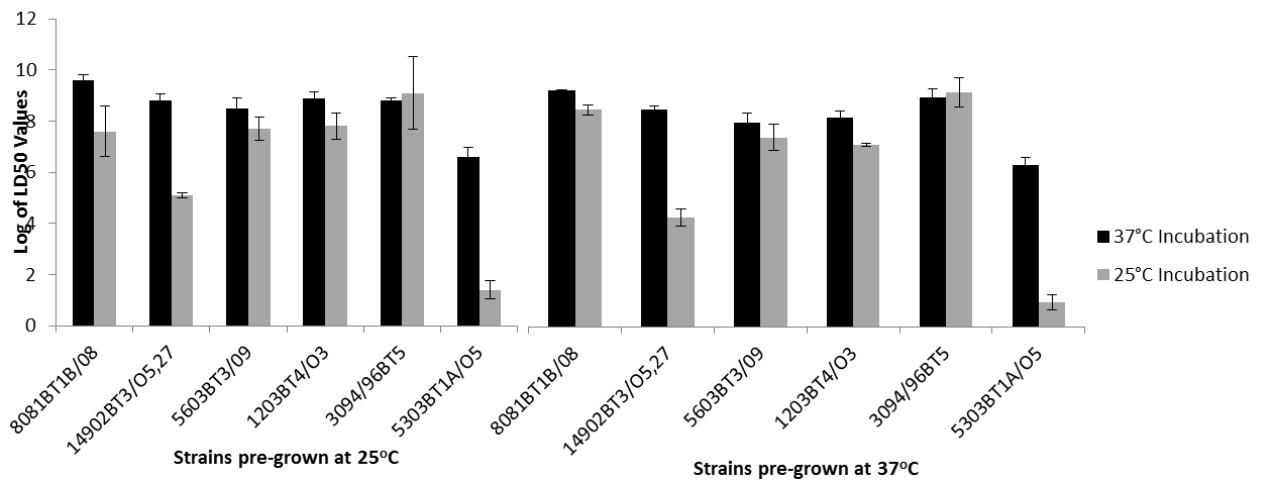
499 **Figure 4:** (A) The effect of the loss of the pYV virulence plasmid on the
500 pathogenesis of reference *Y. enterocolitica* strains to *Galleria mellonella*. Data
501 shown are the mean of three independent experiments with the error bars
502 indicating the standard error of the mean. (B) image showing the stability of pYV
503 in reference strains of *Y. enterocolitica* during *Galleria mellonella* infection, as
504 determined by PCR detection of the *yscP* gene. Lane M contains a 100bp marker;
505 Lanes 1 & 2 show YE8081c 24 hrs and 120 hrs post infection; Lanes 3 & 4 show

506 12/03 24 hrs and 120 hrs post infection; Lanes 5 and 6 show 212/02 24 hrs &
507 120 hrs post infection; Lanes 7 & 8 show 149/02 24 hrs & 120 hrs post infection;
508 Lanes 9 & 10 show 56/03 24 hrs and 120 hrs post infection; Lanes 11 & 12 show
509 3094/96 24 hrs and 120 hrs post-infection. Lane marked -ve is a no template
510 negative control.

511

512 **Figure 5:** Graph showing the effect of mutation and complementation in the CDT
513 operon (*cdtB*), the YGT type III secretion system (*ygtV*), and flagella (*flgB*) on the
514 pathogenesis of the phylogroup 1 *Y. enterocolitica* reference strain 5303. Data
515 shown are the mean of three independent experiments with the error bars
516 indicating the standard error of the mean.

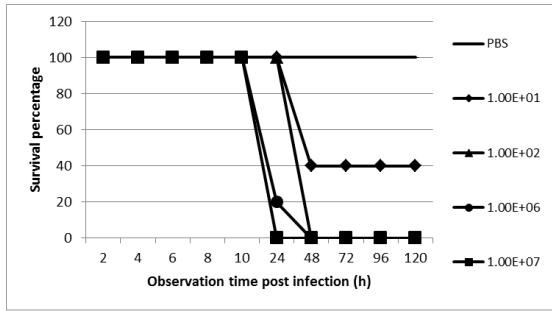
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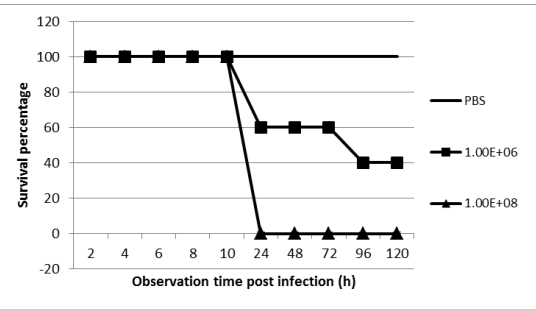
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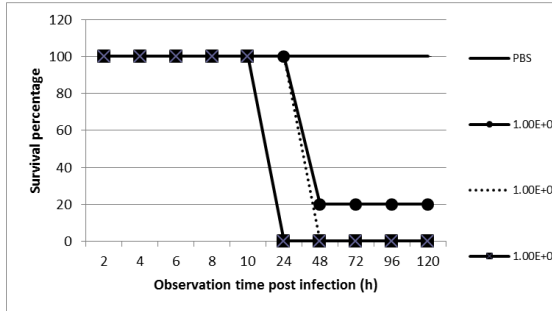
Yersinia 25°C – *Galleria* 25°C



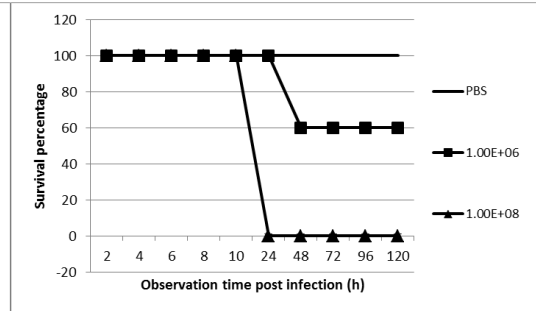
Yersinia 25°C – *Galleria* 37°C



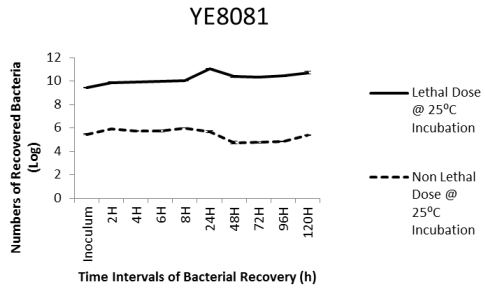
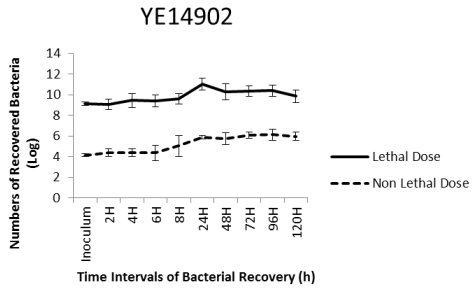
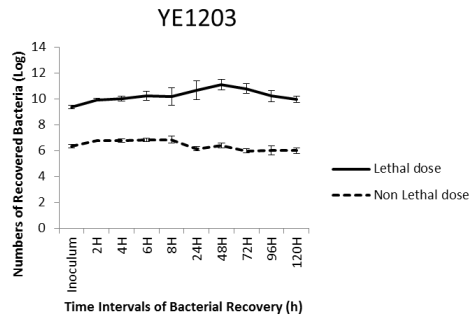
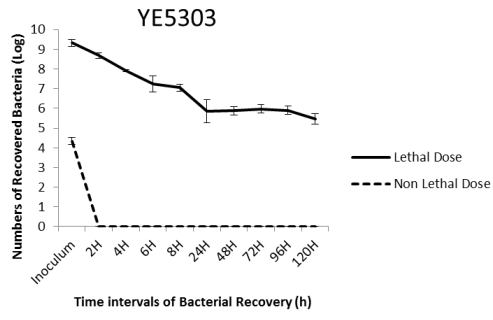
Yersinia 37°C – *Galleria* 25°C



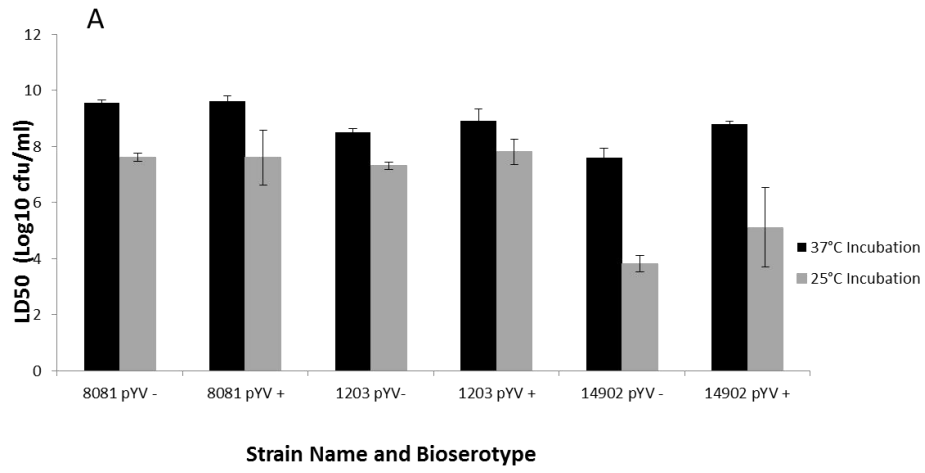
Yersinia 37°C – *Galleria* 37°C



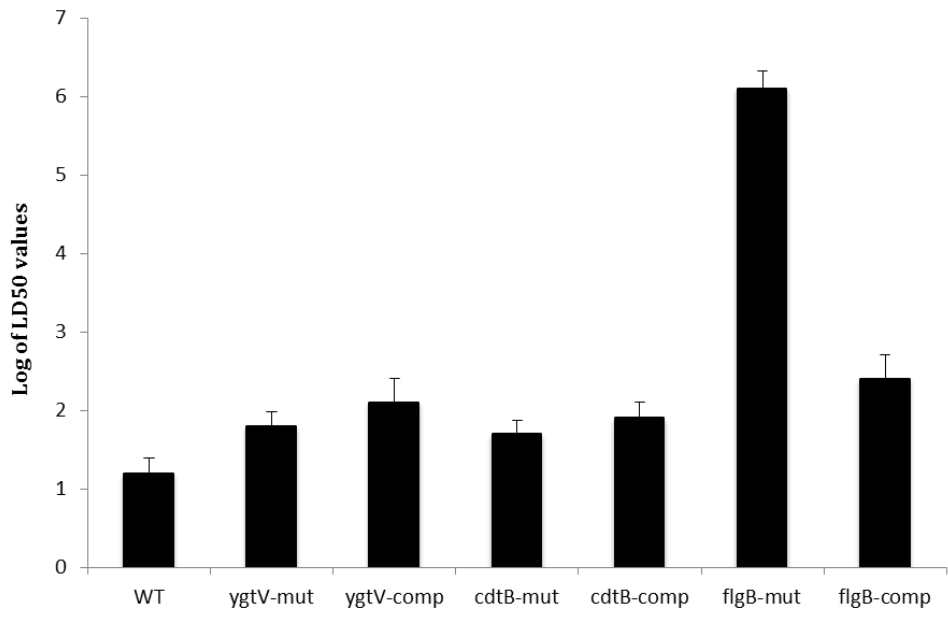
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