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## All Yersinia enterocolitica are pathogenic

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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 samples from cattle and sheep (McNally et al., 2004; Milnes et al., 2008). Human 38 39 versiniosis 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 classical serotyping (Bottone, 1999; Wauters et al., 1987). More recently whole 46 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 0:3), phylogroup 4 (serotype 05;27), phylogroup 51 5(serotype 0:9), 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 due to lethality in a mouse infection model, whilst PG 3-6 strains are considered
low-pathogenic due to the observed pathology in a mouse infection model
(Bottone, 1999). The major genetic difference between PG 1 and PG 2-6 that
accounts of the differences in observed pathogenesis are the presence of the
virulence plasmid pYV and the adhesion-encoding gene *ail* in PG 2-6 (Reuter *et al.*, 2014).

62 Despite lacking the key virulence factors involved in mammalian pathogenesis, there is still some debate as to the true pathogenic potential of PG 1 Y. 63 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 Virdi, 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 & Virdi, 2012; 75 Singh & Virdi, 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

PG 1 is the requirement of flagella to survive inside macrophages (McNally *et al.*,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 LD<sub>50</sub> of just 10 cfu, and that virulence is enhanced at 25°C compared to 37°C. We also show that the severe virulence of PG 1 isolates is in direct contrast 87 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 Yscp1 and Yscp2 to detect the YscP gene present on pYV. 112

113 Galleria infection assay: Galleria mellonella were infected as previously described (Fuchs et al., 2008). Galleria larvae were infected with a series of 114 115 bacterial suspensions containing 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup> & 10<sup>9</sup> 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 LD<sub>50</sub> 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.

For experiments enumerating number of bacteria surviving inside *Galleria* larvae, individual larvae were sacrificed by incision with a scalpel and then 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* selective131 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 Agel site of pAD5. The inactivated *cdtB* gene was then PCR 139 amplified and subcloned into the *Smal* 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 *vgtV* (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 *yqtV* gene was then PCR amplified and 147 subcloned into the *Smal* site of pKNG101. The resulting plasmid, pAR3, was used to transform *E. coli* S17-1 Pir cells, and these were used as donor cells in a filter 148 mating conjugation with YE5303 (McNally et al., 2007). Complementation of the 149 150 *cdtB* and *yqtV* mutants was performed by transforming the mutated strains with 151 pAD5 and pAR1 respectively.

152 **Results** 

Phylogroup 1 Y. enterocolitica show high pathogenicity towards Galleria
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 inoculation, and larvae were also incubated at both temperatures post-infection. 159 The LD<sub>50</sub> of each strain in each infection condition was calculated and plotted 160 161 (Fig 1, Fig S1). The data clearly shows that YE8081 of PG 2, the highly pathogenic phylogroup in mouse infection models is the least pathogenic in the Galleria 162 163 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 lows 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 show PG 1 strains significantly more virulent in the assay (p = 0.03). 171

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

We sought to determine the kinetics of infection by YE5303 in the *Galleria* assay. First we determined the time-to-death for the larvae in all combinations of pre and post inoculation incubation and doses of bacteria (Fig 2). The results show that the vast majority of killing occurs rapidly between 10 and 24 hours after infection, with the exception being doses at or around sub-lethal levels where small numbers of larvae may die after 24-48 hours. We then took lethal doses (1 180 x  $10^9$  cfu) and sub lethal doses of YE5303, YE1203, YE14902 and YE8081 (1 x  $10^2$  cfu, 1x  $10^6$  cfu, 1 x  $10^4$ cfu, and 1 x  $10^7$  cfu, respectively) and inoculated 181 182 *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 of bacteria is unchanged regardless of fate of the larvae. However, in the PG 1 185 186 YE5303 the number of bacteria surviving inside the *Galleria* drops dramatically in the 24 hours leading to death, whilst in a sub-lethal dose there is rapid and 187 188 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 LD<sub>50</sub> 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

(Fig 4). This shows that pYV was stable in all strains except the PG4 strain
YE14902 where 50% (6 of 12 colonies tested by PCR) of tested colonies had lost
the plasmid. PG4 strains curiously are also the most virulent of the pYV bearing
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 species diversity identified two putative virulence factors that are unique or 212 213 have PG 1 unique alleles (Reuter, et al., 2015). These are the YGT type III secretion system, and the cytolethal distending toxin CDT. A YGT mutant was 214 215 constructed by insertional inactivation of the *yqtV* 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  $LD_{50}$  calculations (Fig 5). 220 Our data show that mutations in the CDT operon or YGT secretion system have no discernable effect on virulence of YE5303 in Galleria larvae. However our 221 222 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

larvae) whilst supernatant from the *flgB* mutant showed 10% mortality (2/20injected 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., 2015; Reuter et al., 2014; Reuter et al., 2015). The pathogenic potential of each of 236 237 the phylogroups has been well characterised on the basis of epidemiological 238 studies of human infections (Fredriksson-Ahomaa & Korkeala, 2003; McNally et 239 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 & Virdi, 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.

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

frequently encountered human-pathogenic phylogroups showed only trace levels of virulence with infectious doses of  $10^7 - 10^9$  cfu. These results appear counterintuitive and may suggest that *G. mellonella* is a measure of virulence for 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 species in insect models of infection (Fuchs et al., 2008) and that insect toxin 261 262 genes present in PG 3, 4 and 5 strains of Y. enterocolitica only contribute to virulence in insects infected via oral ingestion (Fuchs et al., 2008). Our data 263 264 clearly shows that PG 1 Y. enteroclitica 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).

A key difference between PG 1 *Y. enterocolitica* and the pathogenic phylogroups is the absence of the pYV virulence plasmid, the major virulence determinant in mammalian pathogenic *Yersinia* species (Reuter *et al.*, 2014). The plasmid contains the Ysc type III secretion system which is known to be used by *Yersinia* to disarm macrophages and dendritic cells to allow the bacteria to avoid phagocytosis (Cornelis & Wolf-Watz, 1997). Given that *G. mellonella* are known 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 haemoceol (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 interplay between flagella and the Ysc secretion system in Y. enterocolitica 313 314 (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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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-		YE5303 with	(McNally <i>et al.</i> , 2007)
floRMut		flaB gene	(101001) 00 00, 2007
JigDillat		inactivated	
YE5303-		YE5303 with	This study
cdtRMut		<i>cdtB</i> gene	inio occary
cuiDividi		inactivated	
YE5303-		YE5303 with	This study
vatVMut		vatV gene	This study
<i>ygi</i> v 1v1ut		inactivated	
<i>E. coli</i> S17-			Epicentre UK
1 Pir			
<i>E. coli</i> DH5α			Invitrogen UK
			-

### **Table 1: List of strains 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> / $\lambda$ Pir suicide vector	(Kaniga <i>et al.</i> , 1991)
pAR3	pKNG101 with Inactivated	This study
1	<i>ygtV</i> from pAR2 inserted	<i>,</i>
pAD5	pCR2.1 with <i>cdtB</i> inserted	This study
nAD6	nAD5 with <i>cat</i> inserted	This study
nAD9	nKNG101 with inactivated	This study
	<i>cdtB</i> from pAD6 inserted	
cdtFor	GGAAATAAATAAATCTGG	Tm 53ºC
cdtRev	GGGTGAGTAGAGTACGGT	
ygtFor	GCGCTATATCAGGTAGTTTC	Tm 57°C
ygtRev	CGGGAGAATACCGATGAGAG	
CmFor	ACCGAGCGTAGCGAGTCAGT	Tm 60°C
CmRev	ATTACGCCCCGCCCTGC	
YscP1	ATTAGAACCTGAGTATCAACC	Tm 52°C
YscP2	AACAAATAACTCATCATGTCC	

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

**Figure 1:** The calculated LD<sub>50</sub> values for infection of *Galleria mellonella* of a cross section of reference *Y. entercolitica* strains representing the diversity of the species. Results show values for strains pre-incubated at both 25°C and 37°C prior to inoculation into larvae. Black bars represent LD<sub>50</sub> values for infection at 37°C and grey bars represent values for infection at 25°C. Values are the mean for three independent experiments and error bars represent the standard error 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.

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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

**Figure 4:** (A) The effect of the loss of the pYV virulence plasmid on the pathogenesis of reference *Y. enterocolitica* strains to *Galleria mellonella*. Data shown are the mean of three independent experiments with the error bars indicating the standard error of the mean. (B) image showing the stability of pYV in reference strains of *Y. enterocolitica* during *Galleria mellonella* infection, as determined by PCR detection of the *yscP* gene. Lane M contains a 100bp marker; Lanes 1 & 2 show YE8081c 24 hrs and 120 hrs post infection; Lanes 3 & 4 show 12/03 24 hrs and 120 hrs post infection; Lanes 5 and 6 show 212/02 24 hrs &
120 hrs post infection; Lanes 7 & 8 show 149/02 24 hrs & 120 hrs post infection;
Lanes 9 & 10 show 56/03 24 hrs and 120 hrs post infection; Lanes 11 & 12 show
3094/96 24 hrs and 120 hrs post-infection. Lane marked -ve is a no template
negative control.

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

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YE8081











