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Running title: ESBL plasmid carriage in the intestine

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ABSTRACT

Objectives. To assess stability and contribution of a large extended spectrum β-lactamase (ESBL)-containing IncI1 plasmid to intestinal colonization by *Escherichia coli* O104:H4 in two different mammalian hosts.

Methods. Specific-pathogen-free 3-day old New Zealand White rabbits and conventionally-reared 6-week-old weaned lambs were orally infected with wild-type *E. coli* O104:H4 or the ESBL-plasmid cured derivative, and the recovery of bacteria in intestinal homogenates and faeces monitored over time.

Results. Carriage of the ESBL plasmid had differing impacts on *E. coli* O104:H4 colonisation of the two experimental hosts. The plasmid cured strain was recovered at significantly higher levels than wild type during late-stage colonization of rabbits, but at lower levels than wildtype in sheep. Regardless of the animal host, the ESBL plasmid was stably maintained in virtually all *in vivo* passaged bacteria that were examined.

Conclusions. These findings suggest that carriage of ESBL plasmids has distinct effects on the host bacterium depending upon the animal species it encounters and demonstrates that, as for *E. coli* O157:H7, ruminants could represent a potential transmission reservoir.

INTRODUCTION

In 2011, *Escherichia coli* O104:H4 caused a large outbreak of haemolytic uraemic syndrome centred in northern Germany, in which more than 50 people died.[1] Subsequent genotypic and phenotypic analyses demonstrated that this strain was unusual, with features of both enterohaemorrhagic (EHEC) and enteroaggregative *E. coli* (EAEC) pathotypes.[2, 3] Typical of all EHEC, the strain contained genes coding for Shiga toxin (Stx), specifically Stx2a, which is associated with severe clinical outcome in patients.[4] However, it also adhered to tissue culture cells in a characteristic 'stacked-brick' manner, a trait that defines the EAEC pathotype and is associated with aggregative adherence fimbriae, the genes for which are carried on a plasmid (pAA). Unusual for both pathotypes, *E. coli* O104:H4 harboured a large plasmid carrying genes coding for extended-spectrum β-lactamases (ESBLs). While several studies have focused on the contribution of pAA to *E. coli* O104:H4 pathogenesis[5-7], none have considered the significance of ESBL plasmid carriage beyond its immediate impact limiting antibiotic options.

ESBLs are enzymes that confer resistance to many front-line β-lactam antibiotics and are a dominant mechanism of antimicrobial resistance in Gram negative bacteria.[8] Many types of ESBL have been described, but those belonging to the CTX-M type, have become widespread in the UK and elsewhere.[9-12] Genes encoding ESBLs are most often located on large, conjugative plasmids, in part providing a mechanistic explanation for their spread.[8] Bacterial carriage of resistance plasmids such as those encoding ESBLs, is believed to confer a biological fitness cost to the host bacterium, although experimental evidence to support this is conflicting. *In vitro*-based growth assays have shown both detrimental[13] and no fitness cost[14-15] associated with carriage of CTX-M-containing plasmids. However, Schaufler and colleagues concluded that

ESBL plasmid carriage was only associated with a fitness cost when bacteria were grown on surfaces rather than as planktonic cultures.[16] In their study, surface-associated plasmid-cured variants of various pathogenic *E. coli* clonal lineages exhibited changes in fimbriae production, an ability to form biofilms or be motile; surface-associated attributes that may contribute to colonisation and persistence in the mammalian intestine.

Given the widespread prevalence of ESBL-producing organisms that are also pathogens of animals and/or humans, it is perhaps surprising that more studies to examine their impact in the context of the host have not been performed. Moreover, evidence to indicate whether ruminants act as a reservoir for EAEC,[17] or Shiga toxin-producing EAEC isolates such as *E. coli* O104:H4[18] is lacking, although carriage following experimental infection of calves has recently been demonstrated.[19] Herein, we describe the impact of pESBL, the IncI1 plasmid of *E. coli* O104:H4, on the capacity of the pathogen to colonise the intestine of two different mammalian species. Plasmid carriage hindered late-stage colonisation of the intestine of infant rabbits, a model of EHEC-mediated intestinal disease in humans.[20] In contrast, *E. coli* O104:H4 persisted in the intestine and could be detected in faeces of weaned sheep for up to 4 weeks, independently of pESBL carriage. These studies indicate that carriage of a large ESBL plasmid mediated host-specific differences in the persistence of the host bacterium and demonstrate that, as for *E. coli* O157:H7,[21, 22] ruminants could represent a potential transmission source.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used in the study are listed in Table S1. BL211, a Stx2 deletion mutant of *E. coli* O104:H4 strain C227-11[5], was used in this study to

enable the animal experiments to be performed in the available facilities and reduce any risk of serious infection to research personnel. Bacteria were routinely grown in LB medium or on LB agar plates containing the appropriate antibiotics: gentamicin 10 µg/mL; tetracycline 10 µg/mL or cefotaxime 2 µg/mL.

Construction of the pESBL cured derivative. Strain BL211 harbours 3 plasmids including a large 88.5 kb ESBL-encoding plasmid that belongs to incompatibility group I1 (IncI1) and carries both the $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-15}}$ genes[2] (subsequently annotated as $bla_{\text{CTX-M-3}}$[23]). Incompatibility-based curing[13] was used to rid strain BL211 of this plasmid (originally referred to as pESBL-EA11 and herein called pESBL). Briefly, plasmid pIFM27, a *sacB*-containing plasmid, which encodes the IncI1 plasmid replication down-regulator RNAI that directly interferes with the replication of IncI plasmids, was introduced into BL211 by electroporation. Transformants were selected on LB agar supplemented with kanamycin (50 µg/mL) and were subsequently screened for an inability to grow on cefotaxime-containing LB media. Curing of plasmid pIFM27 from cefotaxime-susceptible colonies was accomplished by recovery of colonies on LB-agar supplemented with 5% sucrose. Subsequently, sucrose-resistant, cefotaxime-susceptible colonies were screened for loss of pESBL and pIFM27 by PCR and plasmid profiling (Figure S1A-B). Loss of pESBL did not impact the ability of the strain (herein called BL320) to grow *in vitro* in LB media in single strain growth assays (Figure S1C).

PCR analysis of colonies. Multiplex PCR was used to assess the chromosomal and plasmid gene content of BL211, BL320 and randomly selected colonies recovered from rabbits or sheep infected with the parental strain BL211. Primer sequences, expected product sizes and reaction conditions are reported in Table S1.

Infant rabbit studies. All experimental protocols were approved by the local Animal Welfare Ethical Review Body, and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Time-mated specific pathogen free adult New Zealand White rabbits were purchased from Harlan Laboratories (Derby, UK) at 2-3 weeks gestation. Following partition, mixed sex litters were kept together in a nesting box with the lactating doe and housed under standard conditions. Infant rabbit infections were performed on 3 to 4-day-old pups essentially as described previously.[5] Briefly, rabbits were administered ranitidine intraperitoneally (5 mg/kg body weight) and 2 hours later ~1 x 10^9 cfu bacteria re-suspended in sodium bicarbonate solution (2.5g $NaHCO_3$ in 100mL H_2O) was given via oral gavage. Following challenge, animals were monitored twice daily for signs of intestinal disease. Given that all bacterial strains lacked Stx, clinical signs were scored with the following revised scale: none (no adherent faecal material on fur and intestines appear normal with hard, formed digesta in the distal colon), intestinal disease (no adherent faecal material on fur but colon contains soft, poorly-formed digesta) and diarrhoea (adherent faecal material on fur and colon contains liquid or unformed digesta). Rabbits were euthanased by a Schedule 1 method at either day 3 or day 7 post infection. Tissues were aseptically sampled *post-mortem* for bacterial enumeration. Samples from the distal small intestine, caecum, mid- and distal- colon, and stool were homogenised in PBS, serially diluted and plated onto LB agar supplemented with gentamicin and tetracycline. Where no colonies were detected following plating of undiluted tissue homogenates, the number of bacteria recovered was set using the lower limit of detection as a value. Spread plates, which contained well-spaced colonies, were chosen for replica-plating to LB agar with

and without cefotaxime. Colonies failing to grow on the antibiotic were subject to multiplex PCR to confirm the loss of $bla_{CTX\text{-}M}$. All infections were performed in at least 2 independent litters in order to limit any litter-specific effects.

Sheep colonisation studies. All experimental protocols were approved by the local Animal Welfare Ethical Review Body, and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Experimentally-inoculated, weaned sheep were used as a model of natural ruminant infection as previously described for *E. coli* O157:H7.[24,25] Conventionally-reared 6-week-old cross-bred commercial lambs were divided into mixed sex groups of 8 animals and housed under bio-secure conditions. Prior to challenge, individual sheep were confirmed as free of *E. coli* O104:H4 by screening faecal samples with an in-house *E. coli* O104-specific immunomagnetic separation (IMS) capture assay and an agglutination assay.[26] After one week acclimation, sheep were orally inoculated with ~5 $x10^9$ cfu of bacteria (BL211 or BL320) delivered in a volume of 11 ml using a worming gun (Novartis, UK). The inocula were prepared from 16 hr aerobically incubated cultures, which were grown in LB broth, pelleted by centrifugation and finally re-suspended in PBS. Faecal samples were collected *per rectum* from all animals on days 1 to 14 post infection (PI) and twice weekly thereafter until day 39. On days 4 and 39 PI, 3 and 5 animals respectively from each group were euthanased and tissue samples (1 g) were collected from the ileum, caecum, spiral colon, rectum and recto-anal junction. Prior to microbiological analysis, faecal and tissue samples were homogenised in buffered peptone water (BPW) at a ratio of 1:10 (weight/volume) using a vortex (faeces) or an Ystral D-79282 homogenizer (tissues). Ten-fold serial dilutions of the homogenised samples were plated

directly onto sorbitol MacConkey agar plates supplemented with tetracycline and gentamicin. If no colonies were observed after overnight incubation, samples were enriched by incubating the BPW homogenates at 37˚C for 18 h followed by re-plating to provide a qualitative result. Selected colonies from all faecal and tissue samples were screened by multiplex PCR as described above.

Statistical analysis. The presence or absence of disease in rabbits was expressed in a contingency table and analysed using Fisher's Exact test. Bacterial count data (cfu/g) were log transformed and differences in the number of wild-type or cured cells recovered in each intestinal section compared using Student's t-test. In the sheep infection studies, bacterial count data were log transformed and the total cfu shed over days 1 to 4 (8 animals per group) or days 5 to 39 (5 animals per group) were calculated using AUC following the trapezoidal rule (GraphPad Prism, version 5). Differences in the AUC of strains were compared using Student's t-test. All statistical analysis was performed using GraphPad Prism (version 5).

RESULTS

Previously, the *E. coli* O104:H4 outbreak strain was shown to colonize the infant rabbit intestine and cause diarrhoea in a manner that was dependent on Stx, but independent of pAA, the plasmid responsible for mediating aggregative adherence on cultured cells.[5] The contribution of the 88.5 kb β-lactamase-encoding plasmid (pESBL) of *E. coli* O104:H4 pathobiology was not explored. Stable maintenance of pESBL during *in vitro* growth[23] may indicate that pESBL plays an important role in the organism's biology. In order to investigate this further, we cured pESBL

149 from the Shiga toxin negative derivative of *E. coli* O104:H4 and examined its contribution to
150 colonisation of rabbit and sheep intestines.
151
152 **pESBL hinders *E. coli* O104:H4 long term colonisation of infant rabbits.** Consistent with earlier
153 findings[5], oral infection of infant rabbits with the Shiga toxin negative derivative caused few
154 visible signs of disease. Loose stools were detected in 18% (3 of 17) and 11% (2 of 19) of
155 animals infected with the wildtype and plasmid-cured strain respectively, between days 2-3
156 post inoculation (PI) (Table S2). Since the majority of animals did not exhibit any manifestations
157 of diarrhoea, we focused on the role of the plasmid in bacterial colonisation of the rabbit
158 intestine.
159 The distribution and number of challenge *E. coli* present in the intestine of rabbits
160 infected with BL211 or BL320 were determined at days 3 and day 7 PI (Figure 1A-D). Regardless
161 of the infecting strain, there were no differences in the number of BL211 or BL320 cfu
162 recovered from these regions at day 3 PI. In contrast, by day 7 PI, 1-2 logs fewer BL211 than
163 BL320 were recovered in all regions of the intestine. Specifically, colonisation of the parent
164 strain BL211 was significantly reduced compared to the pESBL-cured strain in the ileum (160-
165 fold; $P<0.01$), caecum (215-fold; $P<0.01$) and was lower but did not reach statistical significance
166 in the colon (mid colon 95-fold; $P=0.07$) and distal colon (50-fold; $P=0.13$) of infected rabbits.
167 These findings suggest that carriage of pESBL hinders the longer-term persistence of *E. coli*
168 O104:H4 particularly in the upper regions of the rabbit intestine.
169 In order to investigate whether pESBL was stably maintained in the wild type strain
170 during infection, representative colonies recovered from each animal were replica-plated onto

media supplemented with/without cefotaxime. Loss of ESBL activity was rarely found, even after 7 days growth in the intestine. In all, approx. 2.5 x10^3 colonies recovered at either day 3 or day 7 PI were screened for growth on cefotaxime-containing media, and only 1 colony (recovered at day 3) failed to grow on the antibiotic-containing media. Loss of $bla_{CTX\text{-}M}$ in this colony was confirmed by PCR (see Figure S1A). Together these observations suggest that while pESBL hinders the ability of the host bacterium to persist in the rabbit intestine, the plasmid is stably maintained in the cell.

pESBL aids persistence in the ruminant intestine. In order to investigate the role of pESBL in colonisation of the ruminant intestine, we orally infected groups of 6-week-old conventional lambs with BL211 or BL320, and monitored the presence of bacteria up until day 39 PI. Due to the lower number of bacteria usually recovered following *E. coli* O157:H7 challenge of sheep[27], an additional enrichment step was included when necessary during sample processing in these experiments.

As expected, none of the infected lambs showed gross signs of disease after challenge and at *post-mortem* all intestinal tissues appeared normal. Regardless of the infecting strain, most animals shed high numbers of cells (>10^7 cfu/g) the day after challenge, declining thereafter (Figure 2A). Within each group, some animals continuously shed the challenge bacteria whereas others showed intermittent shedding or only shed for a few days. However, the magnitude and duration of faecal shedding in BL211-infected animals was generally greater than for BL320-infected animals (Figure 2A). The median time before the number of shed bacteria fell below experimental detection limits in two consecutive samples collected from the same animal was 35 (range 4 to 35) and 18 (range 10 to 21) days for BL211 and BL320,

193 respectively (Figure 2A, inset). As a result, higher numbers of bacteria were recovered from
194 animals infected with BL211 compared to BL320 in the later stages of the infection. Moreover,
195 bacteria were more likely to be recovered by direct plating rather than following overnight
196 enrichment of faecal samples from animals infected with BL211 versus BL320 (Figure 2B).
197 Despite these trends, the magnitude and duration of shedding when expressed as the area
198 under the curve (AUC) for each strain approached but did not reach statistical significance
199 (P=0.08). Thus, the presence of pESBL appears to prolong the duration of *E. coli* O104:H4
200 shedding in sheep, albeit with high inter-animal variability.

201 While *E. coli* O157:H7 preferentially colonises the mid- to lower intestinal tract of
202 sheep,[24,27] the site of *E. coli* O104:H4 colonisation is less well-defined. Thus, samples from
203 different regions of the intestine (ileum, caecum, colon, rectum and anal-rectal junction) were
204 collected from infected animals at day 4 (n=3) and day 39 (n=5) PI. At day 4 PI, low levels of
205 challenge bacteria were recovered from all five sites of the intestine and all animals, regardless
206 of the infecting strain, yielded bacteria from at least two intestinal sites. While more bacteria
207 tended to be recovered from animals infected with the parent strain BL211, the tissue
208 distribution did not differ markedly between the two strains (Table S3), or from *E. coli*
209 O157:H7.[24] At day 39 PI, challenge bacteria could no longer be recovered, even with
210 enrichment, from any of the intestinal samples collected, even though the organisms could still
211 be detected in the animals' faeces. As found during the rabbit challenge experiments, the
212 ESBL-producing plasmid was stably maintained in colonies recovered from the sheep.
213 Consistent with reports from human infection[7], at least one colony was found to lack *aggR*
214 indicative of pAA loss.

DISCUSSION. While a considerable amount of work has been performed elucidating the genetic and biochemical basis of ESBL resistance, fewer studies have attempted to uncover the contribution of ESBL-containing plasmids to pathogen biology, particularly in context of bacterial survival and carriage in animal hosts. By generating a plasmid-cured derivative of Stx-negative ESBL-producing *E. coli* O104:H4, we were able to assess the impact of ESBL plasmid carriage in two complementary animal hosts: infant rabbits, which are used as a model of Stx-mediated intestinal disease[5, 20] and weaned sheep, which are a potential ruminant reservoir host of Stx-producing isolates.[28] We found that carriage of pESBL affected the fitness of the bacterium in the intestine of the two experimental hosts, with the cured strain being recovered at higher levels than wild type in rabbits but at lower levels (than wildtype) in sheep. Our findings challenge the idea that fitness costs are consistent across different assays as reported previously.[29] While Vogwill and colleagues[29] found a significant correlation between fitness scores assessed using *in vitro* and *in vivo* assays, the impact of the host was not fully ascertained in their analyses. All the *in vivo* studies examined in their study were performed in a single host species, mice. Our findings highlight the importance of the host context when considering the impact of resistance plasmids on the fitness of the bacterium during intestinal carriage.

Species-specific differences in the host can mitigate the requirement for particular bacterial factors and may explain the differing impact of pESBL on the host bacterium in this study. For example, *gltA*, encoding a citrate synthase and *mtlD*, encoding a mannitol metabolic protein, are important for effective *Vibrio cholerae* colonisation of the infant rabbit but not the infant mouse intestine.[30] Differences in the availability of carbon and energy sources within the

two animal intestines were hypothesised to explain the relative necessity of these genes. Likewise, differences between the rabbit and sheep gastrointestinal tracts could impact the contribution of plasmid-borne factors on BL211 fitness. In addition to the ESBL genes which are unlikely to confer a direct selective advantage in our experiments, pESBL contains 95 genes coding for factors of unknown and known function. One of these is Hha, a haemolysin expression-modulating protein that appears to affect a myriad of surface-associated cellular phenotypes in *E. coli* including bacterial motility, cell aggregation and biofilm formation.[31] Loss of Hha in laboratory K12 and some ESBL-producing *E. coli* isolates led to increased swimming activity and reduced biofilm formation compared to their parental strains[16]; phenotypes that may alter the ability of the organism to colonise and persist in the intestine.

The late-stage colonisation advantage of the pESBL cured strain was not evident in sheep, a ruminant host. Instead, it appeared that loss of pESBL resulted in more rapid clearance of the cured strain from the intestine than wild type (Figure 2). Only detailed molecular analyses involving deletion mutants and further *in vivo* experimentation will uncover the factors and/or mechanisms that explain these fitness outcomes. Like the prototypical *E. coli* O157:H7 serotype, we found that *E. coli* O104:H4 was able to persist in the ruminant intestine for at least 4 weeks. These findings are consistent with those recently reported following experimental infection of *E. coli* O104:H4 in weaned calves.[19]

Finally, we found that pESBL was stably maintained during infection, as most cells recovered from the rabbit or sheep intestine retained the ability to grow on cefotaxime-containing media. Similar observations were reported for calves infected with *E. coli* O104:H4[19], for pigs infected with *E. coli* carrying an IncI/ST12 $bla_{CTX\text{-}M}$-encoding plasmid[32] and most

recently, in streptomycin-treated mice infected with *E. coli* carrying a large non-conjugative virulence plasmid.[33] As noted by others, why these large plasmids are stably maintained in the host bacterium in the absence of obvious selection pressure is intriguing. Yamaichi and colleagues identified 6 regions of pESBL as essential for pESBL replication or segregation.[23] Intriguingly one of these regions mapped to *hha*. Thus, as well as *hha* potentially affecting the surface-expressed properties of the host bacterium, its' presence may also help to ensure that pESBL is stably maintained in the cell during intestinal growth. Unlike some other IncI ESBL plasmids circulating in the UK[10], pESBL also appears to contain a recognisable plasmid addiction system (PndAC). While not identified as playing a role in plasmid maintenance in *E. coli* O104:H4[23], PndAC has been found to play a role in the maintenance of other plasmids.[34, 35]

Collectively our studies suggest that the impact of plasmids bearing antibiotic resistance determinants on pathogen biology needs to be understood in the context of the host. For zoonotic food-borne pathogens, this should include intestinal environments that are healthy (i.e. reservoir hosts) or diseased, as host factors such as inflammatory cells[36], are known to affect AMR transmission and pathogen survival. Finally, our studies show that ruminants such as sheep can act as reservoir hosts for *E. coli* O104:H4, and thus they should be considered as a potential source of transmission to humans.

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TRANSPARENCY DECLARATIONS

None to declare.

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Figure 1

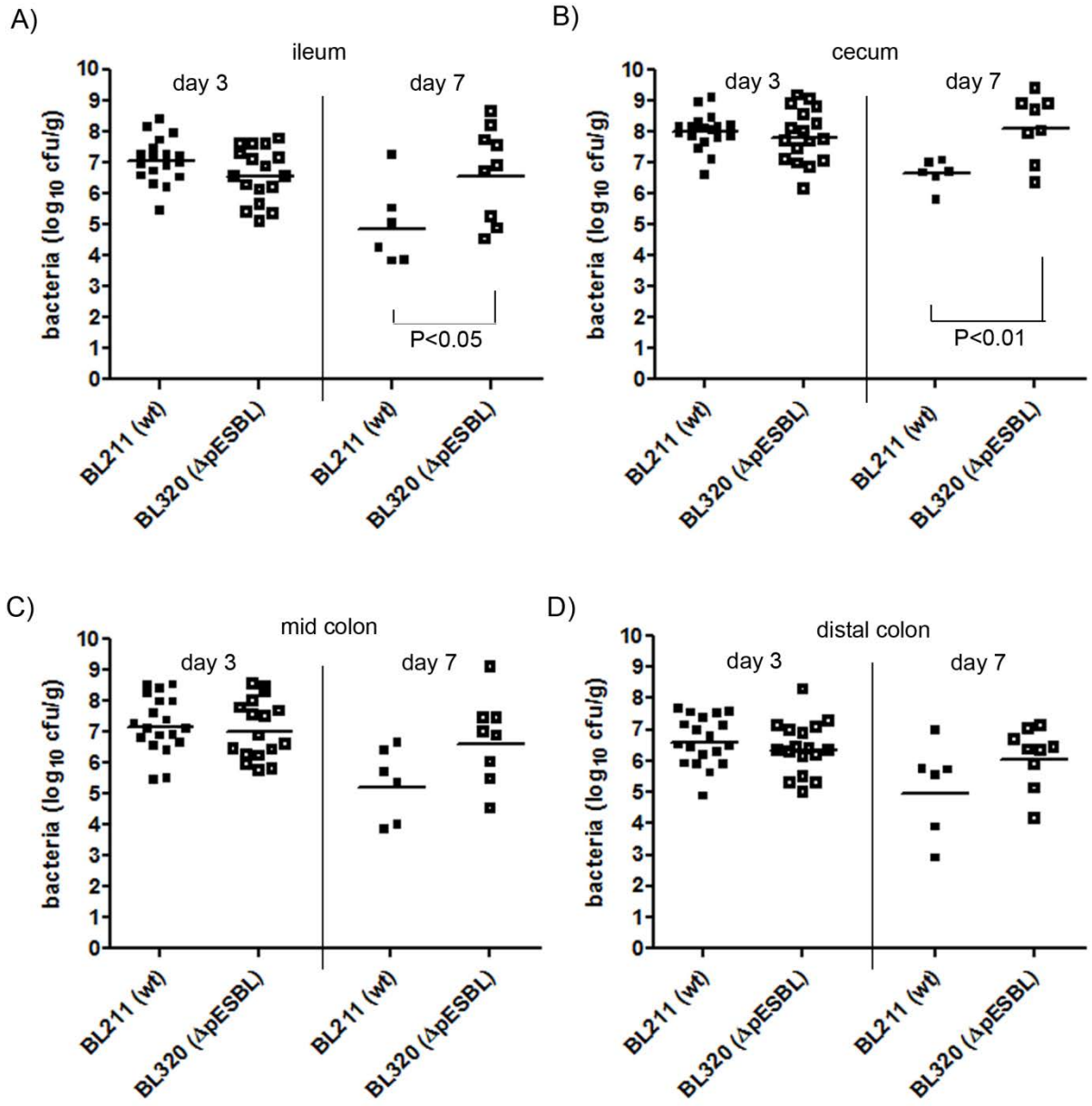

Figure 1. Recovery of bacteria in infant rabbits orally infected with *E. coli* O104:H4 or a derivative lacking pESBL. Concentration (cfu/g) of bacteria recovered at 3 and 7 days post infection in intestinal homogenates of rabbits infected with the indicated strain (wild-type BL211 and pESBL-cured strain, BL320). Data points represent individual animals (at day 3:

BL211, n=19 and BL320, n=17; at day 7: BL211, n=6 and BL320, n=9) and the bar represents the geometric mean. Statistical analysis was performed using Student's t-test with $P \leq 0.05$ deemed significant.

Figure 2

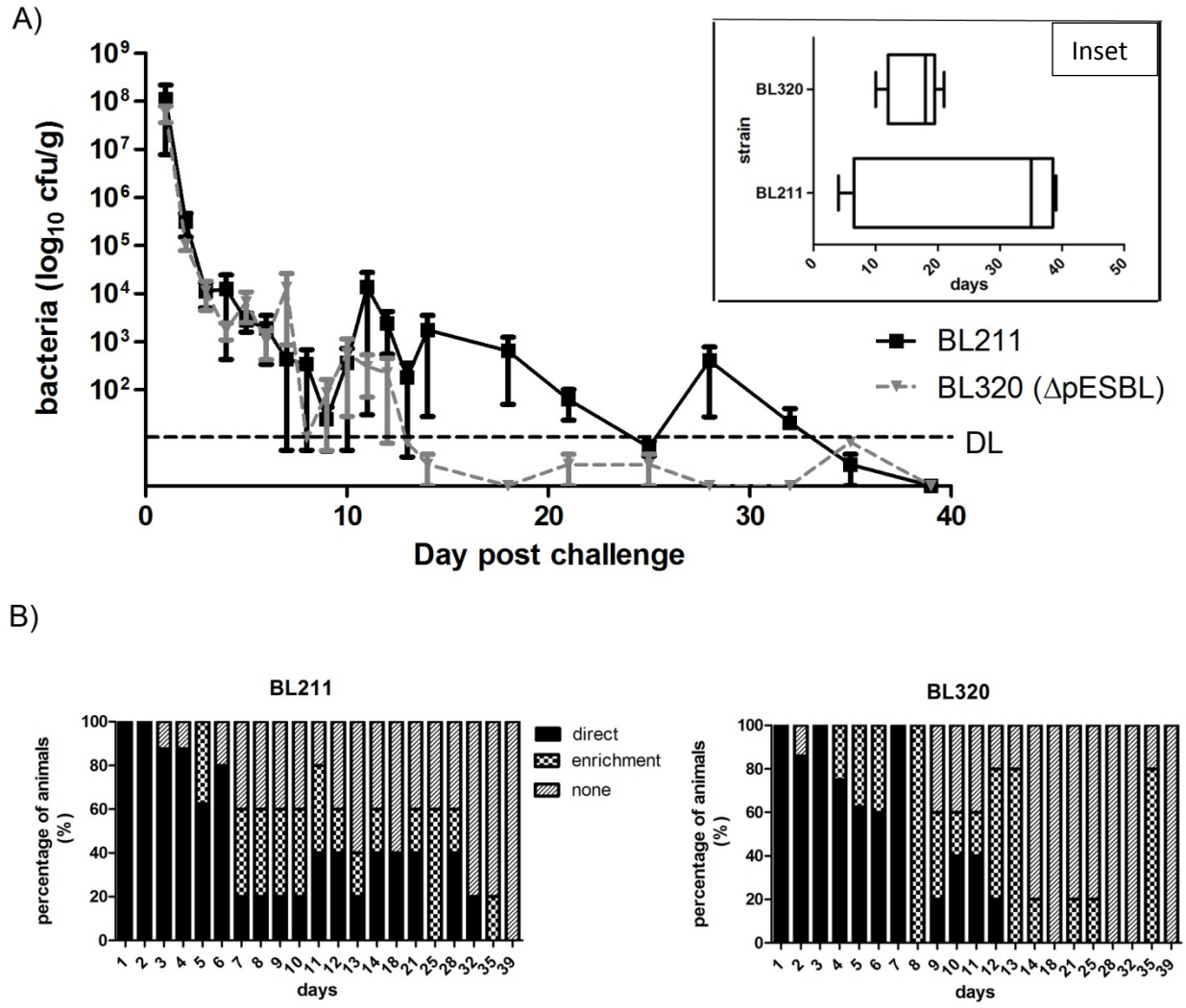

Figure 2. Faecal shedding of *E. coli* O104:H4 or the pESBL-cured derivative from orally infected six-week old conventional weaned lambs. Mixed sex lambs (n=8 per group) were group-housed and individual faecal samples collected per rectum. Samples were homogenised in buffered peptone water and plated directly, or after 18-hour enrichment, on SMAC media supplemented with appropriate antibiotics. Count data were log transformed and the mean (+/- standard error) number of bacteria recovered for each strain was expressed over time (A). Inset figure shows boxplots representing the median, lower and upper quartiles, and the

403 minimum and maximum duration of shedding for each strain. DL = experimental limit of
404 detection based on average weight of tissue. The proportion of faecal samples in which bacteria
405 were not found or recovered directly or following enrichment (B).
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