Running Title: HGT and pH drive AMR in Salmonella infected chicks

Gene flux and acid-imposed selection are the main drivers of antimicrobial resistance in broiler chicks infected with *Salmonella enterica* serovar Heidelberg

Adelumola Oladeinde[1,2,10*], Zaid Abdo[2*], Maximilian O. Press[3¶], Kimberly Cook[4], Nelson A. Cox[1], Benjamin Zwirzitz[5,6¶], Reed Woyda[2¶], Steven M. Lakin[2¶], Jesse C. Thomas IV[7], Torey Looft[8], Douglas E. Cosby[1], Arthur Hinton Jr.[1], Jean Guard[1], Eric Line[1], Michael J. Rothrock[1], Mark E. Berrang[1], Kyler Herrington[9], Gregory Zock[10], Jodie Plumblee Lawrence[1], Denice Cudnik[1], Sandra House[1], Kimberly Ingram[1], Leah Lariscy[1], Martin Wagner[5,6], Samuel E. Aggrey[10], Lilong Chai[10], and Casey Ritz[10]

Authors' Affiliation

[1]U.S. National Poultry Research Center, USDA-ARS, Athens, GA, USA. [2]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, USA. [3]Phase Genomics Inc, Seattle, WA, 98109, USA. [4]Office of National Programs, USDA-ARS, Beltsville, Maryland, USA. [5]Institute of Food Safety, Food Technology and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria. [6]Austrian Competence Centre for Feed and Food Quality, Safety, and Innovation FFoQSI GmbH, Tulln, Austria. [7]Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD and TB Prevention, Center for Disease and Control, Atlanta, Georgia, USA. [8]National Animal Disease Center, USDA-ARS, Ames, IA, USA, [9]Medical College of Georgia, Augusta, GA, USA. [10] Poultry Science Dept, University of Georgia, Athens, GA, USA

Corresponding authors* ade.oladeinde@usda.gov; zaid.abdo@colostate.edu

¶ These authors contributed equally to this work.

Abstract

Antimicrobial resistance (AR) spread is a worldwide health challenge, stemming in large part, from the ability of microbes to share their genetic material through horizontal gene transfer (HGT). Overuse and misuse of antibiotics in clinical settings and in food production have been linked to this increased prevalence and spread of AR. Consequently, public health and consumer concerns have resulted in a remarkable recent reduction in antibiotics used for food animal production. This is driven by the assumption that removing this selective pressure will favor the recovery of antibiotic susceptible taxa and will limit AR sharing through HGT, allowing the currently available antibiotic arsenal to be effective for a longer period. In this study we used broiler chicks raised antibiotic-free and *Salmonella enterica* serovar Heidelberg (SH), as a model food pathogen, to test this hypothesis. Our results show that neonatal broiler chicks challenged with an antibiotic susceptible SH strain and raised without antibiotics carried susceptible and multidrug resistance SH strains 14 days after challenge. SH infection perturbed the microbiota of broiler chicks and gavaged chicks acquired antibiotic resistant SH at a higher rate. We determined that the acquisition of a plasmid from commensal *Escherichia coli* population conferred multidrug resistance phenotype to SH recipients and carriage of this plasmid increased the fitness of SH under acidic selection pressure. These results suggest that HGT of AR shaped the evolution of SH and that antibiotic use reduction alone is insufficient to limit antibiotic resistance transfer from commensal bacteria to *Salmonella*.

46 ## Importance

47 The reported increase in antibiotic resistant bacteria in humans have resulted in a major
48 shift away from antibiotics use in food animal production. This has been driven by the
49 assumption that removing antibiotics will select for antibiotic susceptible bacterial taxa,
50 and this in turn will allow the currently available antibiotic arsenal to be more effective.
51 This shift in practice has highlighted new questions that need to be answered to assess
52 the effectiveness of antibiotic removal in reducing the spread of antibiotic resistance
53 bacteria. This research demonstrates that antibiotic susceptible *Salmonella* Heidelberg
54 strains can acquire multidrug resistance from commensal bacteria present in the gut of
55 neonatal broiler chicks, even in the absence of antibiotic selection. We demonstrate that
56 exposure to acidic pH drove the horizontal transfer of antimicrobial resistance plasmids
57 and suggests that simply removing antibiotics from food-animal production might not be
58 sufficient to limit the spread of antimicrobial resistance.
59
60
61

62 # Introduction

63 Antimicrobial resistance (AR) spread is a worldwide health challenge[1,2]. A major aspect
64 of this challenge stems from the ability of microbes to share their genetic material through
65 horizontal gene transfer (HGT)[3]. Exposure to antibiotics can select for a microbiota of
66 commensals and pathogens that can withstand antibiotic selective pressure, creating a
67 potential reservoir of resistance genes that are shared with susceptible taxa allowing for
68 their survival and proliferation[3]. Overuse and misuse of antibiotics in a clinical setting[4]
69 and in food production[5] have been shown to result in such selective environments
70 contributing to the increased prevalence and spread of AR. These public health concerns
71 have resulted in a major shift away from antibiotics use in food production. This was driven
72 by the assumption that removing this selective pressure will favor the recovery of
73 susceptible taxa, and that this in turn will allow the currently available antibiotic arsenal to
74 be effective for a longer period[6-9]. This shift in food production practices has highlighted
75 new questions that need to be answered to understand and assess the effectiveness of
76 antibiotic removal in reducing the spread and prevalence of AR in food borne pathogens.

77 There has been a remarkable reduction in antibiotics sold or distributed in the United
78 States (US) for use in food-producing animals since the publication of the US Food and
79 Drug Administration's guidance for industry #209 (2012) and #213 (2013) and the
80 Veterinary Feed Directive[10]. Chicken production accounted for the largest reduction in
81 medically and nonmedically important antibiotics sold (~47% from 2016 to 2017), also
82 driven by consumer concerns over food safety related to antibiotics use[11]. *Salmonella*
83 *enterica* serovar Heidelberg (SH) strain is one of the prolific serovars causing
84 salmonellosis in the US and poultry is by far the major vector for SH infections[12-14] . One

85 of the largest food-borne outbreaks in US history was associated with the consumption
86 of chicken contaminated with SH[15]. During this outbreak, SH was present in blood
87 samples of case-patients (15%) and resulted in an overall hospitalization rate of 38% (200
88 case-patients). Poultry and clinical strains of SH can be resistant to at least 1 antibiotic in
89 3 or more drug classes. Therefore, we focused on studying antibiotic-free broiler chicken
90 production, the most consumed meat in the US, with a goal to evaluate the prevalence of
91 AR and its potential transfer to SH, an important model pathogen.

92 Our results suggested that *Salmonella* can acquire multidrug resistance from the
93 bacteria of neonatal broiler chicks raised without antibiotics. We determined that *E. coli*
94 strains carrying IncI1 plasmids were the main reservoir of transferable AR in broiler chicks
95 and confirmed that *E. coli* was the donor of IncI1-ST26 (pST26) carrying antibiotic, metal,
96 and disinfectant genes to SH. The presence of pST26 did not impose a fitness cost to the
97 SH host and exposure to acidic pH posed a selection pressure on pST26 plasmid
98 population. Commensals like *E. coli* are a reservoir of mobile genetic elements including
99 plasmids and bacteriophages. Therefore, we conclude that a simple reduction in antibiotic
100 use might not be sufficient to prevent transfer of these mobile elements to a major food-
101 borne pathogen like *Salmonella.*
102

103 # Results

104 **S. Heidelberg abundance in ceca and litter is influenced by the route of challenge.**
105 To investigate if the route of exposure to SH affected intestinal and litter colonization, we
106 challenged 150 one day old broiler chicks with a nalidixic (nal) resistant strain of SH (SH-
107 2813_{nal}^{R}) either by oral gavage (OG), intracloacally (IC) or by the seeder (SB) method
108 (i.e., a few chicks (n =5) were orally challenged and co-mingled with unchallenged chicks
109 (n = 20)) (Fig. 1a). Chicks were grown for 14 days on fresh pine shavings in four separate
110 floor pens (1.8 L ×1.16 W m or 5.9 L×3.8 W ft) including an unchallenged control (CTRL)
111 group (n=25). Chicks were not administered any medication or antibiotics for the duration
112 of the study. The challenge experiments, trial 1 and trial 2, were performed in September
113 2017 and April 2018, respectively and we have published the rate SH colonized the
114 ceca[16]. SH concentration in the ceca of gavaged chicks (SB and OG) was higher than IC
115 for trial 1 (H = 0.94, df =2, P = 0.62) and OG was higher than IC for trial 2 (H = 10.24, df
116 =2, P = 0.006) (Fig. 1b). In the litter, SH levels were higher for IC pens compared to SB
117 and OG in trial 1 and higher in OG pens compared to IC for trial 2 (Fig. 1c). The
118 percentage of chicks that had SH detected in their ceca was 80% for OG and SB and
119 100% for IC during trial 1. For trial 2, 100% of the OG and IC were positive and 40% for
120 SB[15]. The ceca and litter of CTRL were negative for SH by culture; however, one litter
121 sample was positive after 24 h enrichment in buffered peptone water. Litter pH and
122 moisture was higher for trial 1 (pH = 6.86 ± 0.27; moisture = 25± 0.03 %) compared to
123 trial 2 (6.54 ± 0.17; moisture = 21± 0.03 %) (Extended data Fig. 1a and b).

124 **S. Heidelberg perturbed the ceca and litter bacteria in a challenge route-specific**
125 **manner**. Next, we questioned if SH gut colonization perturbed changes in the ceca and

126 litter microbiota. We profiled the microbial community present in the ceca and litter using
127 16S ribosomal RNA gene amplicon sequencing. The bacterial alpha diversity was higher
128 for litter samples compared to the ceca for OG for all alpha diversity measures (Observed,
129 Chao1, Shannon and InvSimpson) examined ($P < 0.01$) (Extended data Fig. 2a). In
130 contrast, ceca from OG had lowest alpha diversity for all measures used compared to
131 CTRL ($P < 0.05$). Observed and Chao1 alpha diversity indices were higher for litter
132 compared to ceca for CTRL and IC ($P = 0.007$). Beta diversity differed between litter and
133 ceca and the route of SH challenge affected the beta diversity of the litter (F = 43.7, df =
134 11, $P = 0.001$) more than the ceca (F = 2.21, df = 29 $P = 0.007$). Cecal samples were
135 more dispersed than litter samples and OG chicks had the highest beta diversity (Fig. 2a).
136 *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* were the dominant
137 phyla present in litter (Fig. 2b). Litter from OG and SB carried higher abundance of
138 *Bacteriodetes*, whereas IC and CTRL carried higher abundance of *Firmicutes* than OG
139 and SB. *Firmicutes* dominated the cecal bacteria of broiler chicks and *Ruminococcaceae*
140 and *Lachnospiraceae* (class *Clostridia*) were the major families. *Proteobacteria* was
141 higher in the ceca of CTRL than OG or IC (X^2= 0.6412, P=0.72) (Fig.2b). Amplicon
142 sequence variants (ASV) matching *Subdolinagrum*, *Lactobacillus* and
143 *Family_Lachnospiraceae* were the most abundant ASVs in OG and IC compared to CTRL
144 in the ceca (Extended data Fig.2b). In contrast, *E. coli/Shigella* (*E. coli* and *Shigella* are
145 not distinguishable by 16S rRNA gene sequencing) and *Klebsiella* were the most
146 abundant ASVs in litter. *E. coli* was higher in IC and CTRL compared to OG and SB and
147 *Klebsiella* was higher in CTRL compared to OG, IC and SB (*E. coli*: X^2= 9.9744, P=0.018;
148 *Klebsiella*: X^2=9.6667, P=0.021). This result suggested that SH perturbed the bacterial

149 community present in the litter and ceca of broiler chicks especially the
150 *Enterobacteriaceae*.

151 **S. Heidelberg developed multidrug resistance after IncI1 plasmid acquisition.** We
152 investigated if SH isolates developed AR after 14 days of challenge. We used the national
153 antimicrobial resistance monitoring system sensititre ™ panel for Gram-negative bacteria
154 to conduct antibiotic susceptibility testing (AST) on 250 SH isolates recovered from either
155 the ceca or litter of challenged chicks. SH-2813_{nal}^{R} carries a *gyrA* mutation for nal
156 resistance, therefore all isolates recovered were resistant to nal. SH also carries a
157 chromosome-encoded *fosA7* gene that confers resistance to fosfomycin[17]. In trial 1, only
158 2 % of SH isolates (n = 92) developed resistance to an antibiotic. These two isolates were
159 resistant to gentamicin, streptomycin and sulfisoxazole. For trial 2, 40 % of the isolates
160 developed resistance to at least one antibiotic (Fig. 1d) and > 86 % of them were resistant
161 to 2 or more drug classes like the isolates from trial 1 (Extended data Fig. 3a). However,
162 trial 2 isolates developed AR to tetracyclines instead of sulfisoxazole. A disc diffusion
163 assay on a selected number of multidrug resistant (MDR) isolates (n = 4) revealed that
164 MDR isolates were also resistance to tobramycin, netilmicin and kanamycin (data not
165 shown). Additionally, OG chicks carried a higher percentage (46 %) of MDR isolates than
166 IC (24 %) or SB (8%) chicks (X^2 = 29.2, df = 2, P < 0.001) (Fig. 1e).

167 To find AR determinants responsible for the acquired AR phenotype, we performed
168 whole genome sequencing (WGS) using illumina short read sequencing technology on
169 sixty-nine SH isolates from trial 2 and two isolates from trial 1. We focused our resources
170 on trial 2, for which AR acquisition was higher than trial 1. The isolates sequenced were
171 either susceptible (n = 33) or antibiotic resistant (n = 38). The ancestral SH-2813_{nal}^{R}

172 (isolate used for challenge) harbors an IncX1 plasmid and carriage of this plasmid does
173 not confer AR in SH. The IncX1 *inc* region was not detected by plasmidFinder[18] in 42 of
174 the 71 isolates sequenced. Thirty-nine percent of SH isolates acquired Col plasmids, but
175 none carried a known antibiotic resistance gene (ARG). ARGs known to confer resistance
176 to aminoglycosides (*aadA1, aac(3)-Via*) and tetracyclines (*tetA*) were detected in all AR
177 isolates. The AR isolates from trial 1 harbored a sulphonamide resistance gene (*sul1*) in
178 addition to aminoglycoside genes but no *tet* gene. The ARGs were found on a plasmid
179 belonging to *inc* group IncI1 (Fig. 1f) and plasmid Multi Locus Sequence Type 26 (pST26).
180 A change in AR profile was observed in four SB isolates - for these isolates, AST
181 confirmed them to be MDR but after WGS, the AR determinants were not detected. Upon
182 AST re-test, these isolates were found to be susceptible. Acquired ARG was absent in
183 four isolates with resistance to either tetracycline or streptomycin.

184 **HGT and chromosome rearrangement shaped the genome of *S*. Heidelberg**. After
185 introduction of SH to the broiler gut microbiota, we hypothesized that successful
186 colonization will be dependent on gene flux (gain and loss of genes) and mutations. To
187 this end, we reconstructed a maximum-likelihood (ML) tree based on the pan genome
188 and mutations of SH strains recovered after 2 weeks in the gut or litter. The core genome
189 (genes present in ≥ 95% of the strains) and accessory genome (genes present in < 95%
190 of the strains) composed of 3729 and 1531 genes, respectively. A total of 91 new
191 mutations (SNPs and Indels) were on the chromosome of evolved strains and no single
192 mutation was present in every strain. Rather, mutations were unique to individual strains
193 or shared between 2-26 isolates (Supplementary data 1). The accessory genome tree
194 grouped the strains based on AR phenotype, plasmid and ARG carried (Fig. 3a).

195 Contrastingly, the core genome and SNP-based trees did not provide a clear division of
196 strains based on these characteristics and clades had low bootstrap support values
197 (Extended data Fig. 3b and c). Acquisition of IncI (IncI1 and IncI2) plasmid, Col plasmids
198 and the presence/absence of IncX1 plasmids defined the clades seen on the accessory
199 genome tree. Susceptible strains carrying only IncX1 plasmids represented Clade I and
200 MDR strains dominated Clade II. Clade II strains carried either IncI1 only or IncI1 plus a
201 Col plasmid or both were present with an IncX1 plasmid (Fig. 3a).

202 A few susceptible and strains with no plasmid (n = 12) were nested within clade II
203 and the genome of these strains exhibited signs of substantial gene loss (Fig. 3a;
204 Extended data Fig. 4a). Moreover, clade IIa strains had higher number of contigs, higher
205 misassemblies and lower genome size than the rest of the strains sequenced for this
206 study (H = 18.82, df =1, P <0.001; Extended data Fig. 4b-d). To determine if erroneous
207 assembly affected the clustering of this clade, we performed long read sequencing
208 (Pacific Biosciences Inc.) on one strain from this clade and used it in a hybrid approach
209 with Illumina short reads. This resulted in a partially complete chromosome (longest
210 contig - 4.7 Mbp) but 30 non- circular contigs were not scaffolded. An alignment of the
211 ordered contigs with the ancestor showed that the strain did not suffer significant gene
212 loss as suggested through short or long reads only assembly (Supplementary Table 1),
213 however a 3.2 Mbp chromosomal region was inverted (Fig. 3b). This strain also carried
214 an untypeable circular episome that carried uncharacterized proteins and two genes
215 encoding NADH-quinone oxidoreductase (*nuo*) (~22 kbp) (Extended data Fig.4e), and an
216 IncX1 plasmid with divergent protein sequences from the IncX1 present in the ancestor
217 (Extended data Fig.4f). Gene inversions can increase the diversity and virulence of

218 pathogens[19], hence we questioned if chromosomal inversion was present in other
219 evolved strains. To do this, we aligned protein sequences of two complete circular
220 chromosome from trial 1 and 2 with the ancestor. This analysis showed that the isolate
221 from trial 1 harbored a ~2 Mbp chromosomal inversion (Extended data Fig. 5a).

222 Gene inversion changes the leading or lagging strand sequence to its reverse
223 complement, thus altering the GC skew of the affected gene or genome from a positive
224 value to a negative value or negative to a positive[19]. Accordingly, we confirmed that there
225 was a GC skew inversion in these regions (Extended data Fig. 5b and c). Gene inversions
226 occur after rejoining of DNA breaks and inverted genes can introduce sequencing bias
227 and misassembly[20]. Here, deletions affecting the cytochrome c maturation gene cluster
228 (*ccm*) contributed to the inversion of genes in SH strain *og8-05a* (Fig. 3b; Extended data
229 Fig. 5b). Further, the *oriC* of *og8-05a* was reoriented when compared to the ancestor. On
230 the other hand, deletion of a putative YebC/PmpR transcriptional regulator and a deletion
231 between *cysK* and *cysZ* led to the reorientation of *oriC* in strain *ic9b* (Extended data Fig.
232 5a and c). The inverted region in these genomes encoded 34 - 60 % of the 168 virulence
233 genes present in the ancestor including *Salmonella* pathogenicity islands 1 and 2, fimbrial
234 and adherence genes, type 1 and 2 secretion system proteins and prophages
235 (Supplementary data 2; Extended data Fig. 6). Taken together, this result suggested that
236 gene flux and homologous recombination shaped the genome of SH strains after entry
237 into the broiler chick gut.

238 **pST26 differed in their AR genetic context and genome architecture**. To compare
239 pST26 plasmids, we used hybrid assemblies to achieve circular plasmids for two MDR
240 SH isolates recovered from trial 1 and trial 2. The pST26 plasmids from trial 1 (p1ST26)

241 and trial 2 (p2ST26) were determined to be ~ 112 kbp long and ~ 87 % identical (Fig. 1f).
242 Both carried the atypical IncI1 backbone including regions encoding replication, stability,
243 leading and conjugative transfer[21]. To determine the IncI1-complex group, we used the
244 classical *traY* and *excA* protein sequences of plasmid R64 (IncI1) and R621a (IncI1-
245 gamma). A reconstructed tree confirmed that both plasmids belong to the IncI1 group
246 (Fig. 4a). A 19.8 kb variable region encoding transposons and AR was found in both
247 plasmids. For p1ST26, this region encoded *aadA1, aac(3)-Via*), *sul1* and quaternary
248 (quats) ammonium compound resistance *(qacEΔ1*) (Fig.1f; Extended data Fig. 7). In
249 contrast, this region carried *tetA* and *mer* operon in addition to *aadA1* and *aac(3)-Via* in
250 p2ST26 (Fig. 1f) .

251 To investigate whether gene flux influenced IncI1 genome architecture, we used
252 p1ST26 and p2ST26 as reference genomes for the rest of the MDR isolates carrying
253 IncI1. By aligning raw reads to complete pST26 genomes, we were able generate a
254 consensus IncI1 plasmid contig for each MDR isolate. A tree built with these plasmids
255 resulted in two clades represented by p1ST26 and p2ST26 plasmids, respectively (Fig.
256 4b). Multiple alignment of protein sequences revealed that pST26 plasmids differed in
257 number of genes carried and gene alleles. This was pronounced for regions encoding
258 AR, pilus/shufflon assembly proteins (*pil*) and IS66-family of transposases
259 (Supplementary data 3). A pangenome analysis revealed that the pST26 from this study
260 carried 70 core genes (genes present in ≥ 95% of the plasmids) and 90 accessory genes
261 (genes present in < 95% of the plasmids). A tree reconstructed with the core genes and
262 accessory genes divided the pST26 into 5 major groups (Fig. 4d and c). For both trees,
263 the p1ST26 formed one clade and the p2ST26 made up the other clades. No SNPs were

264 found between p2ST26 but p1ST26 differed from p2ST26 with 21 SNPs and 2 insertions
265 (Supplementary data 3; Supplementary Table 2). The insertions were present in only one
266 p1ST26 plasmid. One insertion was found on *tniA* (a DDE-type
267 integrase/transposase/recombinase protein) and the other was between tn3-like
268 transposon and *yadA*. Inserted DNA showed significant homology to *tniA* present in *E.*
269 *coli* plasmid EcPF5 (Genbank: CP054237) and IS5057 present in *E. coli* plasmid
270 pIOMTU792 (Genbank: C542972), respectively.

271 ***E. coli* was the major IncI1 reservoir in broiler chicks**. The application of proximity-
272 ligation method (Hi-C) has improved the assembly of metagenomes and made it possible
273 to detect plasmid-host associations[22]. Therefore, we used Hi-C to determine the bacterial
274 species carrying IncI1 plasmids in the broiler chicken microbiota. Hi-C was done on two
275 cecal samples from chicks challenged with SH via the cloaca (hereafter referred to as Hi-
276 IC-FL1 and Hi-IC-FL2). We chose IC samples since all chicks in this group were
277 successfully colonized with SH compared to OG or SB. Our goal was to determine if Hi-
278 C could find the bacterial host of IncI1 plasmids and detect inoculated SH strains.
279 *Salmonella enterica* was found in Hi-IC-FL1 and Hi-IC-FL2 using metagenome sequence
280 classification bioinformatic tools[23,24] (Fig. 5a). However, serovar determination with
281 cluster contigs (~ 1.4 and 1.2 Mbp total contig size, respectively) was not possible with
282 *Salmonella* serovar prediction tool[25] or after alignment of contigs to SH reference
283 genome. We detected IncI1 plasmid contigs in Hi-IC-FL1 that were linked to *E. coli*
284 clusters (Fig. 5b and c; Supplementary Table 3). In addition, we found contigs (n = 151,
285 mean contig size = 17,607 bp, smallest contig = 1,027 bp, largest contig = 282,389 bp)
286 with protein sequences identical to pST26 in both Hi-IC-FL1 and Hi-IC-FL2

287 (Supplementary data 4). Other bacterial clusters belonging to the *Firmicutes* showed a
288 low level of linkage to IncI1 (~100X less in Hi-IC-FL1). These results suggested that *E.*
289 *coli* was the main reservoir of IncI1 plasmids.

290 Next, we investigated if SH challenge affected *E. coli* and IncI1 plasmid abundance.
291 To answer this, we used qPCR primers targeting the glyceraldehyde-3-phosphate
292 dehydrogenase (*gapA*) gene of *E. coli*, the *inc*RNAi region of IncI1 and selected genes
293 carried on p2ST26 (*intI1*, *aadA* and *tetA*) to determine their concentration in the ceca and
294 litter. Litter carried higher concentration of *E. coli*, IncI1 and p2ST26 genes than ceca on
295 a per gram basis (W = 1, $P < 0.001$). (Fig. 6a; Extended data Fig.8a). In the ceca, IncI1
296 was higher in OG compared to IC or CTRL ($X^2 = 9.17$, df =2, $P = 0.01$), while *aadA* (H =
297 8.25, df = 2, $P = 0.016$) was lower in OG compared to IC or CTRL (Fig. 6a). CTRL chicks
298 carried a higher concentration of *E. coli* ($X^2 = 2.32$, df =2, $P = 0.31$), *tetA* ($X^2 = 5.74$, df
299 =2, $P = 0.05$) and *intI1* ($X^2 = 5.69$, df =2, $P = 0.05$) genes than OG and IC. A principal
300 component analysis with these genes and *Salmonella* tetrathionate reductase (*ttr*)
301 revealed that samples within the control and challenge groups clustered with their
302 respective groups and that the challenge and control groups were separated. (Extended
303 data Fig. 8b). A correlation analysis between *Salmonella* and *E. coli* for challenged chicks
304 showed a significant positive correlation (S = 784, $\rho = 0.55$, $P = 0.007$) (Fig. 6b). These
305 results suggested that the presence of SH affected *E. coli*, IncI1 and ARG concentrations.

306 **Broiler chicks carried *E. coli* strains harboring pST26 IncI1 plasmids.** To confirm if
307 broiler chicks from this study harbored *E. coli* populations that could serve as a reservoir
308 of pST26 plasmids, we retrospectively screened the cecal contents of the broiler chicks
309 used for Hi-C on CHROMagar™ supplemented with gentamicin and tetracycline (see

310 supplementary methods) and selected five colonies for whole genome sequencing. As
311 expected, we found pST26 in two strains (phylogroup F, MLST 6858), while the other
312 three strains (phylogroup D, MLST 69) carried an IncI1 that harbored no ARG, and an
313 unknown MLST (Table 1). Next, we compared the pST26 in SH to that found in *E. coli*
314 using complete plasmids. The p1ST26 and p2ST6 IncI1 plasmids of SH were determined
315 to be 86.8% and 99.4 % identical to pST26 present in *E. coli*. The main difference was is
316 in the region encoding AR and transposases in p1ST26 (Extended data Fig 9).
317 **pST26 carriage did not pose a fitness cost under selection pressure**. pST26 carried
318 accessory genes for AR, metal resistance or disinfectants (i.e., quaternary ammonium
319 compounds) Furthermore, SH strains carrying pST26 differed by route of exposure e.g.,
320 higher proportion in oral vs. cloacal. To this end, we questioned if any of these factors
321 could exert a selective pressure for pST26. We did not administer antibiotics to broiler
322 chicks throughout study, therefore we did not consider antibiotic selective pressure.
323 However, we detected metals including nickel, chromium, copper, and zinc in the starter
324 feed in parts per million and we found contaminants such as arsenic, lead and cadmium
325 in trace amounts (Supplementary Table 4). Disinfectants including quaternary ammonium
326 compounds were used for the cleaning of broiler houses and equipment, thus we
327 considered disinfectant as another source of selective pressure in this study. To
328 determine their effect on SH fitness, we used phenotype MicroArray™ (PM) 96-well plates
329 supplemented with selected metal chlorides and disinfectants at varying concentrations
330 to compare a pST26 carrying strain to a susceptible evolved strain. There was no
331 significant difference in metabolic activity for SH strain carrying pST26 compared to its
332 susceptible counterpart for metals and disinfectant but pST26 exhibited higher

333 metabolism for benzethonium chloride, chromate, tellurite, and zinc, whereas the
334 susceptible stain showed higher metabolism for dequalinum chloride, chromium, and
335 cesium (X^2= 1.83, df = 1, *P* = 0.1762) (Extended data Fig. 10a and b).

336 Cox and colleagues have suggested that the acidic pH of the upper GI tract was
337 associated with the lower number of chicks positive for *Salmonella* and *Campylobacter*
338 after oral gavage compared to cloacal inoculation[26-28]. Based on this premise, we
339 hypothesized that SH strains carrying pST26 will survive better under exposure to acidic
340 pH. To answer this question, we first compared the survival of the strains under different
341 pH levels (3.5 -10) with or without nitrogen sources and amino acids using PM plates. SH
342 strain carrying pST26 exhibited lower metabolic activity compared to the susceptible
343 strain at pH 3.5 – 4 (V = 3; *P* = 0.5), pH 4.5 with and without nitrogen sources (V = 599;
344 *P* = 3.426e-06) and at pH 9.5-10 with and without sources of nitrogen (V = 714; *P* =
345 6.547e-07) (Fig. 7a). Next, we compared the survival of the susceptible ancestor,
346 susceptible evolved strains without pST26 and antibiotic resistant evolved strains carrying
347 pST26 in pine shaving extract (PSE) adjusted to pH – 2.5. We acclimatized the bacterial
348 population to PSE (pH – 6.5) for 2 h before exposure to acidified PSE (Extended data
349 Fig. 10c). We screened for pST26 carrying populations using the gen^R marker on pST26.
350 After 2h of exposure to acidified PSE, the SH population carrying pST26 (resistant plus
351 susceptible cells) did not suffer a fitness cost, and cells carrying pST26 exhibited higher
352 fitness than their susceptible counterparts (X^2= 4.35, df = 2, *P* = 0.113) (Fig. 7b).
353 Notwithstanding, pST26 SH cells had a lower population size than the ancestor and
354 evolved susceptible strains (X^2 = 6.37, df =3, *P* = 0.095) (Fig. 7c). This result suggested

355 that pST26 carriage does not pose a fitness cost on SH host but benefits the host when
356 exposed to acidic pH.

357 Discussion

358 In this study, neonatal Cobb 500 broiler chicks challenged with a susceptible *S*.
359 Heidelberg (SH) strain carried susceptible and multidrug resistance SH population 2-
360 weeks after in their ceca and litter. This resistance phenotype was conferred by the
361 acquisition of IncI1-pST26 plasmids. pST26 plasmids are present in *S*. Heidelberg, *S*.
362 Typhimurium, *S*. Anatum, *S*. Derby, *S*. Schwarzengrund, *S*. Saintpaul and *E. coli* strains
363 isolated from animal and human sources (Extended data Fig. 11). The closest IncI1
364 plasmids to pST26 from this study are pST26 present in *E. coli* (CP018625) and *S*.
365 Typhimurium (CP027409). The plasmids carry aminoglycoside resistance genes with or
366 without tetracycline, sulphonamide, *qac* and *mer* resistance genes, making it a
367 multidrug/disinfectant/metal resistance plasmid. Additionally, pST26 transfer was higher
368 in chicks challenged orally compared to chicks inoculated via the cloaca. This route
369 specific rate of conjugation suggested that the selection for pST26 in our study was
370 associated with the differential pH in the upper GI versus lower GI tract. This was
371 corroborated by in vitro experiments, where acidic pH reduced the metabolism of SH cells
372 carrying pST26 compared to cells with no pST26. Furthermore, carriage of pST26
373 increased the fitness of pST26-carrying cells compared to the susceptible SH population
374 after exposure to acidic pine shaving extract. This type of acid-imposed selection for IncI1
375 alludes to the “negative frequency dependent selection hypothesis” - where the fitness of
376 the population is higher when a plasmid is present at a low frequency in the population[29].

377 This could also explain the higher levels of SH, but lower positivity rate found in the
378 ceca of broiler chicks gavaged compared to cloacally challenged chicks[16] (Fig. 1b). There
379 are multiple barriers that makes it challenging for SH to colonize the ceca of broiler chicks
380 gavaged including the acidity of the upper GI tract and the length of the GI tract to be
381 traveled. When we measured the distance of the cloaca and esophagus to the ceca of a
382 49-day old Cobb-500 broiler chicken (photo not shown), we estimated that the SH inocula
383 will have to travel ~20X farther to get to the ceca of gavaged chicks compared to cloacal
384 inoculated chicks. It is plausible that this longer "residence" time in the upper GI allows
385 more opportunities for SH to make contact and compete with other members of chicken
386 microbiota. In this study, such an event gave rise to SH cells that acquired pST26 from
387 *E. coli*. It is also noteworthy that AR acquisition occurred at a lower rate during trial 1
388 compared to trial 2. This could be due to several factors that warrants further study -
389 including the higher pH and moisture of the litter in trial 1 compared 2, seasonal
390 differences (trial 1 was done in the fall and trial 2 was in the spring) and the
391 antibiotic/disinfectant practices in place at the hatchery or breeder farms at time the
392 experiments were conducted.

393 The IncI1-complex are widespread in *Enterobacteriaceae* and have been reported to
394 be the major driver of AR in *Salmonella* and *E. coli*. Our understanding of their mode of
395 transfer and AR evolution have been limited to studies on their prevalence, mating
396 experiments and in vivo challenge with mice[21,30,31]. Fischer et al[32] showed that *E. coli*
397 populations in broiler chickens acquired β-lactam resistance after 4-days old broiler chicks
398 were gavaged with an *E. coli* strain carrying $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid. In a twin pair
399 experiment, Hagbo et al[33] revealed that IncI1 carrying $bla_{CMY\text{-}2}$, ColE1 and P1

400 bacteriophage are frequently transferred between microbiota present in pre-term infant
401 gut. Recently, Nyirabahizi,et al[34] used likelihood-ratios to show that *E. coli* AR predicted
402 *Salmonella* resistance to β-lactams in retail meat, and to gentamicin, ceftriaxone, and
403 amoxicillin-clavulanic acid in cecal samples and suggested that the horizontal transfer of
404 bla_{CMY-2} played a role. These studies have demonstrated that IncI1 is the most prevalent
405 plasmid carrying β-lactam in *Enterobacteriaceae* and that they are transferable from
406 *Salmonella* to *E. coli* and vice-versa. IncI1 plasmids can harbor transferable gene
407 cassettes with AR variants for β-lactam (bla_{TEM}, bla_{CMY}, bla_{CTX}), aminoglycoside (*aadA*,
408 *aacA*, *aph*), tetracycline (*tet*), sulphonamide (*sul*), fosfomycin (*fosA*), phenicol (*cmlA*, *floR*)
409 and trimethoprim (*dfrA*) resistance. Furthermore, mobile elements could carry AR genes
410 for disinfectants and metals including ammonium compounds (*qac, sug*), mercury (*mer*
411 operon) and arsenic (*ars* operon).

412 The nutrient-niche hypothesis suggests that SH will only be able to invade a new
413 niche if it can metabolize a growth-limiting resource or if it can outcompete a resident
414 species and a vacant niche arises due to a new component in diet or by eliminating the
415 competitor [35]. Competition for oxygen was shown to be the limiting factor that contributed
416 to the virulence of *S*. Enteritidis towards *E. coli* in neonatal chickens[36]. Competition within
417 and between SH and *E. coli* strains played a key role selecting for antibiotic resistant SH
418 in our study. SH challenge perturbed the bacterial community and ARGs of broiler chicks
419 and SH population was positively correlated with *E. coli*. Consequently, gene flux and
420 genome inversion shaped the evolution of SH in broiler chicks. For SH and *E. coli* to co-
421 evolve and persist in the same new niche, recombination of genes occurred and led to
422 the emergence of new SH strains. In our study, pST26 emerged after conjugation

423 between *E. coli* donors from broiler chicks raised on fresh litter and SH. As expected, SH
424 genomes were grouped into nine core genome MLST clusters with eight clusters
425 representing new strains (Extended data Fig. 12). These species/strains are expected to
426 be fitter in the new niche and persist longer.

427 Materials and Methods

428 **SH inoculum preparation.** We have previously described the characteristics of the SH
429 strain (SH-2813nal^R) used for this study and how the inoculum was prepared[16]. Briefly,
430 the strain belongs to the multilocus sequence type 15, carries a 37-kb conjugative
431 plasmid, and is resistant to erythromycin, tylosin, and fosfomycin. The strain was
432 recovered from broiler chicken carcass in 2013 and made resistant to 200 ppm of nalidixic
433 acid (nal^R) for selective enumeration. The nal^R phenotype is conferred by a serine to
434 tyrosine substitution at position 83 of DNA gyrase subunit A protein (GyrA). The SH
435 inoculum was grown overnight in poultry litter extract, centrifuged, and resuspended in
436 1X phosphate buffered saline (PBS). The resuspended cells were used as inocula. The
437 genome of the ancestor to SH-2813nal^R was sequenced using Illumina and PacBio
438 sequencing to achieve a complete circular chromosome and plasmid (Genbank). SH-
439 2813nal^R carries six mutations (Supplementary data 5) including the mutation affecting
440 *gyrA* that are **not present in the ancestor.**

441 **Challenging broiler chicks with SH**. One-day-old Cobb 500 broiler chicks were
442 purchased from a commercial hatchery in Cleveland, GA, USA. Upon purchase, chicks
443 were placed in plastic crates lined with brown paper and transported to the University of
444 Georgia, Poultry Research Center (33.90693362620281, -83.37871698746522). One
445 hundred chicks were either uninoculated, gavaged or cloacally inoculated with 100 µl-

446 volume of SH inoculum (Fig.1a). We included a seeder-bird (SB) colonization method,
447 whereby five orally gavaged chicks were mingled with twenty uninoculated chicks. Each
448 inoculated chick received ~10^6 colony forming unites (CFU) of SH-2813nal^R. Afterwards,
449 chicks were placed in floor pens at a stocking density of 0.65 m^2/chick on fresh pine
450 shavings litter. Broiler chicks were given water and feed ad libitum and raised antibiotic-
451 free on starter diet for 2-weeks (starter feed was synthesized by the University of
452 Georgia's poultry research center's feed mill). The concentration of metals and priority
453 pollutants in feed was determined by the University of Georgia's feed and environmental
454 water laboratory (Athens, GA, USA) (Supplementary Table 4). Husbandry and
455 management followed commercial broiler chicken industry guidelines. After 2-weeks, ten
456 chicks from OG, IC and control groups and fifteen chickens from SB (five seeder chicks
457 and ten uninoculated pen mate) were sacrificed to determine the extent of SH colonization
458 in ceca. The experiments were done in two trials conducted in September 2017 (Trial 1)
459 and April 2018 (Trial 2). The study was approved by the University of Georgia Office of
460 Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.

461 **Cecal and litter bacteriological analyses**. Ceca were aseptically removed from the
462 eviscera of 2-week-old broiler chicks, placed in a stomacher bag and transported on ice
463 to the US National Poultry Research Center for analysis. Ceca were weighed and
464 buffered peptone water (BPW) (BD Difco, MD, USA) was added 3X volume to the weight
465 (v/w) and stomached for 60s. Serial dilutions were made and plated onto Brilliant Green
466 Sulfa agar (BGS) (BD Difco, MD, USA) containing 200-ppm nal. In addition, a 10 µl
467 inoculating loop was used to streak cecal slurry (cecal contents in BPW) onto Xylose
468 Lysine Tergitol-4 agar (BD Difco, Sparks, MD). supplemented with 4 ppm tetracycline for

469 trial 1 and BGS supplemented 32 ppm ampicillin (amp) or 32 ppm streptomycin for trial
470 2. All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise
471 noted. Plates were incubated along with the cecal slurry for 24 h. All bacterial incubations
472 were carried out at 37°C, unless otherwise noted. After incubation, colonies were
473 manually counted and serial dilution plates with 2 – 100 colonies were used for CFU per
474 gram calculation. If no colonies appeared on serial dilution plates, cecal slurry was
475 streaked onto a new BGS plus nal plate and incubated overnight. After incubation, plates
476 were examined for the presence/absence of *Salmonella* colonies.

477 Broiler chicken litter was collected as grab samples from seven locations (4 corners
478 of the pen and under the waterer) in each pen after chicks were removed. The litter
479 samples were pooled and 30g was processed in duplicates from each pen as previously
480 described[37]. Serial dilutions of the litter slurry were made and plated onto BGS agar
481 containing 200 ppm nal. Plates were incubated overnight, and colonies were manually
482 counted and reported per gram litter dry weight. Litter pH and moisture was determined
483 as described previously[37]. We selected randomly 2 – 6 single colonies from BGS plates
484 supplemented with nal from each ceca and litter sample and archived them in 30% LB
485 glycerol at -80 ^{o}C. In addition, cecal slurry was saved at a 4:1 ratio in Luria Bertani broth
486 (BD Difco, MD, USA) containing 30% glycerol at –80°C, whereas litter samples were
487 stored in vacuum sealed whirl pak bags at -20°C.

488 **Determining SH metabolism under selective pressure.** To determine if exposure to
489 metals, disinfectants or acidic pH poses a selection on SH strains carrying IncI1 plasmids,
490 we used 96-well Phenotype Microarray (PM) ™ MicroPlates (PM10, PM12B, PM13B,
491 PM15B and PM16A) (Biolog, Inc., Hayward CA) to compare the metabolic profile of one

492 gentamicin resistant strain harboring IncI1 and one susceptible strain. PM™ plates uses
493 cell respiration via NADH production to determine cell metabolic activity. If the phenotype
494 is positive in a well, the cells respire actively, reducing a tetrazolium dye and forming a
495 strong color (Biolog, Inc.). Briefly, SH strains were sub-cultured twice in universal growth
496 agar (BUG+B) for 24 h at 37°C. Cells were removed with a sterile swab and transferred
497 to 16 ml IF-0 to achieve a final turbidimetric transmission of 42%T. Afterwards, 15 ml of
498 the 42%T cell suspension was transferred to 75 ml of IF-0 +dye to achieve a final
499 transmission of 85% T before 600 µl of the cell suspension was transferred to 120 ml IF-
500 10+ dye. The suspension was mixed and each well in a microplate was inoculated with
501 100 µl of the suspension. After inoculation, microplates were covered with a sterile plastic
502 film and monitored automatically for color development every 15 min for 48 h at 37 °C
503 using and OmniLog reader for 48 h. To identify phenotypes, the kinetic curves of the
504 gentamicin resistant and susceptible strains were compared using OmniLog PM software.
505 For each PM plate, the respiratory unit for each well at 22 h was extracted and $\log_{10}$
506 transformed. All supplies used were purchased from Biolog, Inc.
507 **Exposing SH to acidic pH.** To determine the fitness of evolved SH strains that acquired
508 antibiotic resistance compared to evolved susceptible strains after exposure to acidic pH,
509 we exposed gentamicin resistant (n = 3) and susceptible (n =3) strains recovered from
510 the litter of chicks gavaged with SH to acidified filter sterilized pine shaving extract. Pine
511 shaving extract (PSE) was prepared as described for poultry litter extract[37], using fresh
512 pine shavings collected from the University of Georgia, Poultry Research Center, Athens,
513 GA, USA. Three single colonies of each strain including the ancestral SH-2813nal^R were
514 selected from overnight cultures grown on sheep blood agar and transferred to a

515 microcentrifuge tube containing 900 µl of PSE (pH = 6.52) i.e., one tube per strain. After
516 transfer, tubes were vortexed, covered with a gas permeable paper strip and incubated
517 at 41°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for 2 h. After
518 incubation, tubes were vortexed and 100 µl of the suspension was transferred to a
519 microcentrifuge tube containing 900 µl of PSE (pH of PSE was adjusted to 2.54 using 1M
520 HCl (Spectrum Chemical Mfg. Corp., CA, USA) and 1M NaOH (Fisher Chemical, NJ,
521 USA) Afterwards, tubes were vortexed, and incubation continued for another 2 h as was
522 done for PSE at pH 6.5. To determine SH population in PSE, one replicate tube per strain
523 was removed and serially diluted in 1X PBS at timepoints 0 and 2 h for pH 6.5 and 0.5, 1
524 and 2 h for pH 2.5. Serial dilutions were plated onto BGS agar plates supplemented with
525 or without 16 ppm gentamicin. SH colonies were counted 18 - 24 h after incubation using
526 a calibrated automated colony counter.

527 The fitness of each evolved SH population relative to the SH-2813nal^R population
528 was determined after 2h at pH 2.54 as described by San Millan et al[38]:

529
$$W_{strain} = \frac{log_e \frac{N_{final,evol}}{N_{initial,evol}}}{log_e \frac{N_{final,anc}}{N_{initial,anc}}}$$

530 where W_{strain} is the fitness of the evolved susceptible (total susceptible) or gentamicin
531 resistant populations (total resistant and gen^R resistant), $N_{initial,\ evol}$ and $N_{final,evol}$ are the
532 numbers of cells (in CFU) of the evolved susceptible or gentamicin resistant population
533 at timepoint 0 and 2 h after exposure to PSE, and $N_{initial,anc}$ and $N_{final,anc}$ are the numbers
534 of cells of SH-2813nal^R population at timepoint 0 and 2 h.

535 **Antimicrobial susceptibility testing**. We performed antimicrobial susceptibility testing
536 (AST) on two hundred and fifty SH isolates recovered from the ceca and litter of broiler

537 chicks following National Antimicrobial Resistance Monitoring System (NARMS) protocol
538 for Gram-Negative bacteria. Minimum inhibitory concentration for isolates were
539 determined by broth microdilution using the Sensititre semiautomated antimicrobial
540 susceptibility system (Thermo Fisher Scientific, Inc., MA, USA) and interpreted according
541 to clinical and laboratory standards institute guidelines when available; otherwise,
542 breakpoints established by NARMS were used. AST was also done on *E. coli* isolates (n
543 = 5) recovered from the ceca of two broiler chicks challenged intracloacally with SH. The
544 Kirby-Bauer disc diffusion assay for tobramycin, kanamycin, neomycin, netilmicin was
545 done on four gentamicin resistant SH isolates as previously described[37].

546 **DNA extraction.** DNA was extracted and purified from bacterial colonies using FastDNA
547 spin Kit (MP Biomedicals, LLC, CA, USA), whereas 250 mg of cecal and litter were
548 extracted with Qiagen DNeasy Power-Soil DNA kit (Hilden, Germany). The modifications
549 we made to the manufacturers protocol for DNA extraction have been reported [37,39].

550 **Whole genome sequencing and processing.** Illumina short read sequencing was
551 performed on DNA extracted from SH isolates recovered from ceca and litter. In addition,
552 five *E. coli* isolates recovered from two cecal samples were sequenced. Libraries were
553 prepared using either Nextera™ XT or Nextera™ DNA Flex library preparation kits
554 (Illumina, Inc., San Diego, CA) following the manufacturers protocol. Libraries were
555 sequenced on the Illumina MiSeq platform with 150 or 250-bp paired end reads.
556 Additionally, five SH and two *E. coli* isolates were selected for long read sequencing using
557 Sequel II System (PacBio Biosciences Inc.) or MinION (Oxford Nanopore technology).
558 Preparation and sequencing of long read libraries were done by next generation

559 sequencing core centers of University of Georgia and Colorado State University (see
560 supplementary methods for read quality control and demultiplexing).

561 Genome assembly, resistome characterization, and quality assessment of long reads
562 was done using Reads2Resistome pipeline v.1.1.1[40]. Reads2Resistome performed
563 hybrid assemblies using either Illumina reads and PacBio or Illumina and MinION reads,
564 using both Unicycler[41] and SPAdes[42]. In addition, hierarchical genome assembly process
565 (HGAP) assembly was performed using PacBio's SMRT Link v9.0 analysis software suite
566 using default settings. Assembly quality was assessed by QUAST v5.0.2[43], using default
567 settings) and genome annotation was done using Prokka[44], Rapid Annotation using
568 Subsystem Technology (RAST)[45] and BlastKOALA[46]. We confirmed that all SH isolates
569 were *Salmonella enterica* serovar Heidelberg using *Salmonella* In Silico Typing Resource
570 (SISTR)[25], and used ClermonTyping[47] and Multi-Locus Sequence Typing (MLST)[48] for *E.*
571 *coli* strains. For resistome characterization reads2resistome used ARG-ANNOT[49], the
572 Comprehensive Antibiotic Resistance Database[50], MEGARes[51], AMRFinderPlus[52],
573 PlasmidFinder[18], ResFinder[53] and VirulenceFinder database[54]. For plasmid typing and
574 IncI1 clonal complex determination, we used plasmid MLST[18]. PHAST[55] was used to
575 identify prophages present in chromosomal contigs and predicted prophage DNA
576 sequence was annotated with RAST and BlastKOALA. ProgressiveMAUVE v. 1.1.1[56] and
577 MAFFT v. 1.4.0[57] implemented in Geneious Prime® v 2020.0.1 were used for aligning
578 and comparing sequences. In addition, a pan genome analysis of annotated assemblies
579 was conducted with Roary[58]. Phylogenetic trees based on core genome and accessory
580 genome were reconstructed using the maximum likelihood (ML) method implemented in
581 RAxML-NG v 2.0.0[59]. When computationally possible, the best model of sequence

582 evolution predicted by jModelTest[60] was used for tree reconstruction, otherwise the GTR
583 + GAMMMA model was implemented. Lastly, we used the bacterial genomes sequenced
584 in the study to create a BLAST database that can be searched for sequences of interest
585 in Geneious Prime®.

586 Illumina short reads were assembled de novo into contigs using Unicycler v.0.4.7 and
587 characterized with bioinformatic tools described for long reads. Illumina reads were used
588 to determine single nucleotide polymorphisms (SNPs) and indels present in SH isolates.
589 Alignment of raw FASTQ reads to the genome of SH-ancestor was done using Burrows-
590 Wheeler Aligner (BWA)[61] and SNPs/indels were called using Genome Analysis Toolkit[62]
591 as described previously[39]. Variant call format (VCF) files of identified SNPs/indels and the
592 Linux/Unix shell script used have been deposited in Dryad Digital
593 Repository: https://doi.org/10.5061/dryad.4tmpg4f8d. SNP based ML tree was
594 reconstructed by converting merged VCF files to PHYLogeny Inference Package format
595 using PGDSpider[63]. Afterwards, isolates with duplicated SNPs/indels were removed, and
596 a ML tree was drawn using the Jukes-Cantor model of nucleotide substitution and
597 GAMMA model of rate heterogeneity. For other bioinformatic analyses performed
598 including pST26-IncI1 consensus determination through FASTQ alignment, see
599 supplementary methods. All raw FASTQ reads for sequenced bacterial genomes are
600 publicly available under NCBI accession numbers:

601 **Hi-C and cecal metagenome library preparation and analysis** A shotgun and Hi-C
602 DNA library of two cecal samples was created using Nextera™ XT library preparation kit
603 and Phase Genomics (Seattle, WA) ProxiMeta Hi-C Microbiome Kit following the
604 manufacturer's instructions. Library sequencing was performed by Novogene corporation

605 (Sacramento, CA, USA) on the Illumina HiSeq platform using 150bp paired end reads.
606 Two libraries were sequenced per HiSeq flowcell lane resulting in a total of ~125 million
607 shotgun reads, and ~206 million Hi-C reads per sample. Metagenomic FASTQ files were
608 uploaded to the Phase Genomics cloud-based bioinformatics portal for subsequent
609 analysis. Shotgun reads were filtered and trimmed for quality using bbduk[64] and
610 normalized using bbtools[64] and then assembled with metaSPAdes[65] using default
611 options. Hi-C reads were then aligned to the assembly following the Hi-C kit
612 manufacturer's recommendations (https://phasegenomics.github.io/2019/09/19/hic-
613 alignment-and-qc.html). Briefly, reads were aligned using BWA-MEM with the -5SP and
614 -t 8 options specified, and all other options default. SAMBLASTER[66] was used to flag
615 PCR duplicates. Alignments were then filtered with samtools[67] using the -F 2304 filtering
616 flag to remove non-primary and secondary alignments. Lastly, Hi-C read alignments to
617 the assembly were filtered using Matlock (https://github.com/phasegenomics/matlock)
618 with default options (removing all alignments with MAPQ<20, edit distance greater than
619 5, and read duplicates). Metagenome deconvolution was performed with ProxiMeta[68,69],
620 resulting in the creation of putative genome and genome fragment clusters.

621 Clusters were assessed for quality using CheckM[70] and assigned preliminary
622 taxonomic classifications with Mash[71] and Kraken2 (v2.0.8-beta)[23]. To search the
623 metagenome for ARG and plasmids of interest, we used Geneious Prime® to create a
624 BLAST metagenome database. Metagenome contigs with significant homology (E value
625 < 0.05 and at least 1000 bp of aligned sequence) were considered contigs of the
626 respective plasmid or ARG. Hi-C data were then used to link identified sequences to host
627 genomes and genome fragments within ProxiMeta[22]. Phylogenetic visualizations of Hi-C

628 linkages used the placement of clusters in a large prokaryotic phylogeny as estimated by
629 CheckM[70]. Shotgun and Hi-C reads are publicly available under NCBI accession
630 numbers:

631 **16S rRNA gene sequence processing and analysis.** Cecal and litter DNA were used
632 for bacterial community analysis through the sequencing of the V4 hypervariable region
633 of the 16S rRNA gene of bacterial genomes. Sequencing was done using the paired-end
634 (250 × 2) method on the Illumina MiSeq platform. Raw 16S rRNA gene sequences were
635 processed with dada2 (version 1.14) in the R environment[72]. Briefly, the reads were
636 trimmed where the quality profile dropped below Q30 (250bp for forward and 240bp for
637 reverse reads) and all reads with more than 2 expected errors were removed entirely.
638 The remaining reads were used to learn the error rates and the samples were pooled
639 prior to sample inference. After merging of forward and reverse reads with the default
640 parameters, chimeric sequences were removed with the removeBimeraDenovo
641 command in the consensus method. High quality reads were then classified against the
642 SILVA database version 132[73]. To avoid bias introduced by spurious amplicon sequence
643 variants (ASVs) or from samples with low sequencing depth, any ASVs with less than 5
644 reads and samples with less than 5000 reads were removed from the dataset before
645 further analysis. Statistical analysis of microbial communities was performed in the R
646 environment using the packages “phyloseq”, “Ampvis2”, “vegan”, and “MaAsLin2”. Alpha
647 diversity indices were calculated with a dataset rarefied to the minimum sample size
648 (8740 sequences). After assessing normal distribution by qqplots, histograms, and the
649 Shapiro-Wilk normality test, the not normal distributed groups were compared using the
650 Wilcoxon Signed Rank test. Beta diversity was calculated with no initial data

651 transformation and ASVs that were not present in more than 0.1% relative abundance in
652 any sample have been removed. The principal coordinates analysis was based on Bray-
653 Curtis distances. The effect of the route of inoculation on individual members of the
654 bacterial community was computed with MaAsLin2 and a minimum prevalence threshold
655 of 0.1 and a minimum relative abundance threshold 0.01.Benjamini-Hochberg procedure
656 was applied as a correction method for computing the q-values. All raw FASTQ reads for
657 16S rRNA gene sequences have been deposited under NCBI accession number:

658 **Real-time qPCR.** Real-time qPCR amplification was performed on DNA extracted from
659 the ceca and litter of broiler chicks as described previously[37] using a CFX96 Touch Real-
660 Time PCR Detection System (Bio-Rad Inc., Hercules, CA). Reaction mixtures (20 µl) for
661 all assays contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Inc.,
662 Hercules, CA), 600 nM (each) primers (Supplementary Table 5), and 2 µl of cecal/litter
663 DNA. Calibration curves used for converting qPCR cycle threshold values to gene copies
664 per gram were determined using genomic DNA of *E. coli* or *Salmonella* strains harboring
665 the region or gene of interest

666 **Statistical analyses.** Continuous variables did not meet the assumption of a normal
667 distribution; therefore, non-parametric testing for direct comparisons was performed using
668 Wilcoxon rank sum and signed rank tests, and the Kruskal-Wallis rank sum test was used
669 for one-way analysis of variance tests. Furthermore, continuous variables were log-
670 transformed before any statistical tests were performed. Lastly, principal component
671 analysis was performed on litter and ceca samples using the copy number of genes
672 quantified by qPCR as features. The samples were then projected onto the first two
673 principal components and colored by route of SH challenge to visualize each route's

674 contribution to the data variance. Pearson correlation coefficients were calculated to
675 examine the correlation between *Salmonella ttr* and *E. coli gapA* gene copies present in
676 ceca and litter. Statistical analyses were performed using R (v 3.4.1).

677 **Data availability.** All raw FASTQ reads including short and long reads for sequenced
678 bacterial genomes are publicly available under NCBI accession numbers: PRJNA683658,
679 PRJNA684578 and PRJNA684580. Shotgun and Hi-C reads are publicly available under
680 NCBI accession number: PRJNA688069 and 16S rRNA gene sequences under NCBI
681 accession number: PRJNA669215. The whole genome assembly for *S*. Heidelberg
682 strains SH-2813-ancestor and ic9b harboring p1ST26 have been made available under
683 NCBI biosample number: SAMN12082795 and DDBJ/ENA/GenBank accession numbers
684 JAEMHU000000000, respectively. The whole genome assembly for *E. coli* strain Ec-FL1-
685 1X and Ec-FL1-2X carrying IncI1 are available under GenBank accession numbers
686 CP066836 and JAFCXR000000000, respectively. Variant call format (VCF) files of
687 identified SNPs/indels and the Linux/Unix shell script used have been deposited in Dryad
688 Digital Repository: https://doi.org/10.5061/dryad.4tmpg4f8d.

689 **Ethics statement**. All animal experiments were approved by the University of Georgia
690 Office of Animal Care and Use under Animal Use Protocol: A2017 04-028-A2.

691 **Acknowledgements**. We are grateful to Marlo Sommers, Jasmine Johnson, Carolina
692 Hall, Jeromey Jackson, and Latoya Wiggins for their logistical and technical assistance.
693 This work was supported by USDA Agricultural Research Service (Project Number: 6040-
694 32000-010-00-D), non-assistance cooperative agreement (58-6040-6-030) between
695 USDA Agricultural Research Service and University of Georgia, Research Foundation,

696 and research service agreement (58-6040-8-035) between USDA Agricultural Research
697 Service and Colorado State University. This work was also partially supported by
698 Colorado State University through startup funds to ZA. BZ was supported by the
699 competence center. The competence center (FFoQSI) is funded by the Austrian
700 ministries BMVIT, BMDW, and the Austrian provinces Niederoesterreich, Upper Austria
701 and Vienna within the scope of COMET-Competence Centers for Excellent Technologies.
702 The Austrian Research Promotion Agency FFG handle the program COMET. This study
703 was supported in part by resources and technical expertise from the Georgia Advanced
704 Computing Resource Center, a partnership between the University of Georgia's Office of
705 the Vice President for Research and Office of the Vice President for Information
706 Technology. Any opinions expressed in this paper are those of the authors and do not
707 necessarily reflect the official positions and policies of the USDA or the National Science
708 Foundation, and any mention of products or trade names does not constitute
709 recommendation for use. The authors declare no competing commercial interests in
710 relation to the submitted work. USDA is an equal opportunity provider and employer.

711 **Author's contributions.** A.O., Z.A., K.C. and N.A.C. designed the study. A.O., G.Z.,
712 D.E.C. and C.R. performed live-broiler chicken studies. A.O., J.L., G.Z., S.H. and D.C.
713 performed bacteriological analyses, antibiotic susceptibility testing, DNA extraction and
714 qPCR. J.L., G.Z. and A.O. performed Illumina whole genome sequencing. A.O. performed
715 cecal shotgun and Hi-C library preparation. T.L. made 16S rRNA gene libraries and
716 sequencing. B.Z. performed 16S rRNA bacterial community analysis and interpretation.
717 K.I., A.H., and A.O. performed phenotype microarray analyses with the help of J.G. and
718 E.L. A.O., Z.A., M.O.P., J.C.T., S.M.L., R.W., J.L., D.C. and L.L performed bioinformatic

719 analyses and data curation. K.H. and A.O performed *Salmonella* Heidelberg fitness
720 testing and analysis. A.O., J.L. and D.C. isolated multidrug resistant *E. coli* donors from
721 cecal contents with help from M.J.R. A.O., B.Z. and M.E.B. performed statistical analyses.
722 A.O., Z.A., N.A.C., B.Z., R.W., M.P., S.M.L., K.I., G.Z., J.L., K.H. and L.L. drafted the
723 manuscript, which was reviewed and edited by all authors. A.O., Z.A., S.A.A., L.C. and
724 M.W. supervised the study.

725

726 **References**

727 1 Frieri, M., Kumar, K. & Boutin, A. Antibiotic resistance. *J Infect Public Health* **10**,
728 369-378, doi:10.1016/j.jiph.2016.08.007 (2017).

729 2 Akova, M. Epidemiology of antimicrobial resistance in bloodstream infections.
730 *Virulence* **7**, 252-266, doi:10.1080/21505594.2016.1159366 (2016).

731 3 Von Wintersdorff, C. J. *et al.* Dissemination of antimicrobial resistance in microbial
732 ecosystems through horizontal gene transfer. *Frontiers in microbiology* **7**, 173
733 (2016).

734 4 Ventola CL. The Antibiotic Resistance Crisis. P T. 2015;40: 277–283.
735

736 5 Angulo FJ, Baker NL, Olsen SJ, Anderson A, Barrett TJ. Antimicrobial use in
737 agriculture: controlling the transfer of antimicrobial resistance to humans1 1This
738 article is based in part on an appendix written for an Institute of Medicine workshop
739 summary by Anderson AD, McClellan J, Rossiter S, Angulo FJ entitled Public
740 Health Consequences of Use of Antimicrobial Agents in Agriculture in "The
741 Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications
742 for Human Health and Strategies for Containment-Workshop Summary," Stacey

743 L. Knobler, Stanley M. Lemon, Marian Najafi, and Tom Burroughs (Eds.), Institute
744 of Medicine of the National Academies, Washington, DC, The National Academies
745 Press, 231–243, 2003. Seminars in Pediatric Infectious Diseases. 2004;15: 78–
746 85. doi:10.1053/j.spid.2004.01.0106
747 6 Ventola, C. L. The antibiotic resistance crisis: part 2: management strategies and
748 new agents. *Pharmacy and Therapeutics* **40**, 344 (2015).
749 7 Scott, A. M. *et al.* Is antimicrobial administration to food animals a direct threat to
750 human health? A rapid systematic review. *International journal of antimicrobial*
751 *agents* **52**, 316-323 (2018).
752 8 Aidara-Kane, A. *et al.* World Health Organization (WHO) guidelines on use of
753 medically important antimicrobials in food-producing animals. *Antimicrobial*
754 *Resistance & Infection Control* **7**, 1-8 (2018).
755 9 Tang, K. L. *et al.* Restricting the use of antibiotics in food-producing animals and
756 its associations with antibiotic resistance in food-producing animals and human
757 beings: a systematic review and meta-analysis. *The Lancet Planetary Health* **1**,
758 e316-e327 (2017).
759 10 Singer, R. & Porter, L. Estimates of on-farm antimicrobial usage in broiler chicken
760 and turkey production in the united states, 2013-2017. Retrieved from
761 https://www.uspoultry.org/poultry-antimicrobial-use-
762 report/docs/USPOULTRY_Antimicrobial-Report.pdf. (2019)
763 11 Karavolias, J., Salois, M. J., Baker, K. T. & Watkins, K. Raised without antibiotics:
764 impact on animal welfare and implications for food policy. *Translational Animal*
765 *Science* **2**, 337-348, doi:10.1093/tas/txy016 (2018).

766 12 Bekal, S. *et al.* Usefulness of High-Quality Core Genome Single-Nucleotide Variant
767 Analysis for Subtyping the Highly Clonal and the Most Prevalent Salmonella
768 enterica Serovar Heidelberg Clone in the Context of Outbreak Investigations. *J*
769 *Clin Microbiol* **54**, 289-295, doi:10.1128/Jcm.02200-15 (2016).
770 13 Liakopoulos, A. *et al.* Extended-Spectrum Cephalosporin-Resistant Salmonella
771 enterica serovar Heidelberg Strains, the Netherlands(1). *Emerg Infect Dis* **22**,
772 1257-1261, doi:10.3201/eid2207.151377 (2016).
773 14 Edirmanasinghe, R. *et al.* A Whole-Genome Sequencing Approach To Study
774 Cefoxitin-Resistant Salmonella enterica Serovar Heidelberg Isolates from Various
775 Sources. *Antimicrob Agents Chemother* **61**, doi:10.1128/AAC.01919-16 (2017).
776 15 Gieraltowski, L. *et al.* National Outbreak of Multidrug Resistant Salmonella
777 Heidelberg Infections Linked to a Single Poultry Company. *PLoS One* **11**,
778 e0162369, doi:10.1371/journal.pone.0162369 (2016).
779 16 Cox, N. A. *et al.* Research Note: Evaluation of several inoculation procedures for
780 colonization of day-old broiler chicks with Salmonella Heidelberg. *Poult Sci* **99**,
781 1615-1617, doi:10.1016/j.psj.2019.10.020 (2020).
782 17 Rehman, M. A., Yin, X., Persaud-Lachhman, M. G. & Diarra, M. S. First Detection
783 of a Fosfomycin Resistance Gene, fosA7, in Salmonella enterica Serovar
784 Heidelberg Isolated from Broiler Chickens. *Antimicrob Agents Chemother* **61**,
785 doi:10.1128/AAC.00410-17 (2017).
786 18 Carattoli, A. *et al.* In Silico Detection and Typing of Plasmids using PlasmidFinder
787 and Plasmid Multilocus Sequence Typing. *Antimicrob Agents Ch* **58**, 3895-3903,
788 doi:10.1128/Aac.02412-14 (2014).

789 19 Merrikh, C. N. & Merrikh, H. Gene inversion potentiates bacterial evolvability and
790 virulence. *Nat Commun* **9**, 4662, doi:10.1038/s41467-018-07110-3 (2018).
791 20 Lucas Lledo, J. I. & Caceres, M. On the power and the systematic biases of the
792 detection of chromosomal inversions by paired-end genome sequencing. *PLoS*
793 *One* **8**, e61292, doi:10.1371/journal.pone.0061292 (2013).
794 21 Carattoli, A., Villa, L., Fortini, D. & Garcia-Fernandez, A. Contemporary IncI1
795 plasmids involved in the transmission and spread of antimicrobial resistance in
796 Enterobacteriaceae. *Plasmid*, doi:10.1016/j.plasmid.2018.12.001 (2018).
797 22 Stalder, T., Press, M. O., Sullivan, S., Liachko, I. & Top, E. M. Linking the resistome
798 and plasmidome to the microbiome. *ISME J* **13**, 2437-2446, doi:10.1038/s41396-
799 019-0446-4 (2019).
800 23 Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken
801 2. *Genome biology* **20**, 257 (2019).
802 24 Ondov, B. D. *et al.* Mash Screen: high-throughput sequence containment
803 estimation for genome discovery. *Genome Biol* **20**, 232, doi:10.1186/s13059-019-
804 1841-x (2019).
805 25 Yoshida, C. E. *et al.* The Salmonella In Silico Typing Resource (SISTR): An Open
806 Web-Accessible Tool for Rapidly Typing and Subtyping Draft Salmonella Genome
807 Assemblies. *PLoS One* **11**, e0147101, doi:10.1371/journal.pone.0147101 (2016).
808 26 Cox, N. A. *et al.* Fifty percent colonization dose for Salmonella typhimurium
809 administered orally and intracloacally to young broiler chicks. *Poult Sci* **69**, 1809-
810 1812, doi:10.3382/ps.0691809 (1990).

811 27 Bailey, J. S., Cox, N. A., Cosby, D. E. & Richardson, L. J. Movement and
812 persistence of Salmonella in broiler chickens following oral or intracloacal
813 inoculation. *J Food Prot* **68**, 2698-2701, doi:10.4315/0362-028x-68.12.2698
814 (2005).
815 28 Cox, N., Richardson, L., Cosby, D., Berrang, M. & Harrison, M. Recovery of
816 Campylobacter from External and Internal Spleen Samples from Baby Broiler
817 Chicks Following Various Routes of Inoculation. *Journal of Food Safety* **36**, 132-
818 135 (2016).
819 29 Dimitriu, T. *et al.* Negative frequency dependent selection on plasmid carriage and
820 low fitness costs maintain extended spectrum beta-lactamases in Escherichia coli.
821 *Sci Rep* **9**, 17211, doi:10.1038/s41598-019-53575-7 (2019).
822 30 Benz, F. *et al.* Plasmid- and strain-specific factors drive variation in ESBL-plasmid
823 spread in vitro and in vivo. *ISME J*, doi:10.1038/s41396-020-00819-4 (2020).
824 31 Wotzka, S. Y. *et al.* Escherichia coli limits Salmonella Typhimurium infections after
825 diet shifts and fat-mediated microbiota perturbation in mice. *Nat Microbiol* **4**, 2164-
826 2174, doi:10.1038/s41564-019-0568-5 (2019).
827 32 Fischer, E. A. J. *et al.* Competition between Escherichia coli Populations with and
828 without Plasmids Carrying a Gene Encoding Extended-Spectrum Beta-Lactamase
829 in the Broiler Chicken Gut. *Appl Environ Microbiol* **85**, doi:10.1128/AEM.00892-19
830 (2019).
831 33 Hagbo, M. *et al.* Experimental support for multidrug resistance transfer potential in
832 the preterm infant gut microbiota. *Pediatr Res* **88**, 57-65, doi:10.1038/s41390-019-
833 0491-8 (2020).

834 34 Nyirabahizi, E. *et al.* Evaluation of Escherichia coli as an indicator for antimicrobial
835 resistance in Salmonella recovered from the same food or animal ceca samples.
836 *Food Control*, 107280 (2020).
837 35 Pereira, F. C. & Berry, D. Microbial nutrient niches in the gut. *Environ Microbiol* **19**,
838 1366-1378, doi:10.1111/1462-2920.13659 (2017).
839 36 Litvak, Y. *et al.* Commensal Enterobacteriaceae Protect against Salmonella
840 Colonization through Oxygen Competition. *Cell Host Microbe* **25**, 128-139 e125,
841 doi:10.1016/j.chom.2018.12.003 (2019).
842 37 Oladeinde, A. *et al.* Hotspot mutations and ColE1 plasmids contribute to the fitness
843 of Salmonella Heidelberg in poultry litter. *PLoS One* **13**, e0202286,
844 doi:10.1371/journal.pone.0202286 (2018).
845 38 San Millan, A., Heilbron, K. & MacLean, R. C. Positive epistasis between co-
846 infecting plasmids promotes plasmid survival in bacterial populations. *ISME J* **8**,
847 601-612, doi:10.1038/ismej.2013.182 (2014).
848 39 Oladeinde, A. *et al.* Horizontal Gene Transfer and Acquired Antibiotic Resistance
849 in Salmonella enterica Serovar Heidelberg following In Vitro Incubation in Broiler
850 Ceca. *Appl Environ Microbiol* **85**, doi:10.1128/AEM.01903-19 (2019).
851 40 Woyda, R. R., Oladeinde, A. & Abdo, Z. Reads2Resistome: An adaptable and
852 high-throughput whole-genome sequencing pipeline for bacterial resistome
853 characterization. *bioRxiv*, 2020.2005.2018.102715,
854 doi:10.1101/2020.05.18.102715 (2020).

855 41 Wick, R. R., Judd, L. M., Gorrie, C. L. & Holt, K. E. Unicycler: Resolving bacterial
856 genome assemblies from short and long sequencing reads. *PLoS Comput Biol* **13**,
857 e1005595, doi:10.1371/journal.pcbi.1005595 (2017).
858 42 Antipov, D., Korobeynikov, A., McLean, J. S. & Pevzner, P. A. hybridSPAdes: an
859 algorithm for hybrid assembly of short and long reads. *Bioinformatics* **32**, 1009-
860 1015, doi:10.1093/bioinformatics/btv688 (2016).
861 43 Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. QUAST: quality assessment
862 tool for genome assemblies. *Bioinformatics* **29**, 1072-1075,
863 doi:10.1093/bioinformatics/btt086 (2013).
864 44 Seemann, T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **30**,
865 2068-2069, doi:10.1093/bioinformatics/btu153 (2014).
866 45 Brettin, T. *et al.* RASTtk: a modular and extensible implementation of the RAST
867 algorithm for building custom annotation pipelines and annotating batches of
868 genomes. *Sci Rep* **5**, 8365, doi:10.1038/srep08365 (2015).
869 46 Kanehisa, M., Sato, Y. & Morishima, K. BlastKOALA and GhostKOALA: KEGG
870 Tools for Functional Characterization of Genome and Metagenome Sequences. *J*
871 *Mol Biol* **428**, 726-731, doi:10.1016/j.jmb.2015.11.006 (2016).
872 47 Beghain, J., Bridier-Nahmias, A., Le Nagard, H., Denamur, E. & Clermont, O.
873 ClermonTyping: an easy-to-use and accurate in silico method for Escherichia
874 genus strain phylotyping. *Microb Genom* **4**, doi:10.1099/mgen.0.000192 (2018).
875 48 Larsen, M. V. *et al.* Multilocus sequence typing of total-genome-sequenced
876 bacteria. *J Clin Microbiol* **50**, 1355-1361, doi:10.1128/JCM.06094-11 (2012).

877 49 Gupta, S. K. *et al.* ARG-ANNOT, a New Bioinformatic Tool To Discover Antibiotic
878 Resistance Genes in Bacterial Genomes. *Antimicrob Agents Ch* **58**, 212-220,
879 doi:Doi 10.1128/Aac.01310-13 (2014).
880 50 Jia, B. *et al.* CARD 2017: expansion and model-centric curation of the
881 comprehensive antibiotic resistance database. *Nucleic Acids Res* **45**, D566-D573,
882 doi:10.1093/nar/gkw1004 (2017).
883 51 Lakin, S. M. *et al.* MEGARes: an antimicrobial resistance database for high
884 throughput sequencing. *Nucleic Acids Research* **45**, D574-D580,
885 doi:10.1093/nar/gkw1009 (2017).
886 52 Feldgarden, M. *et al.* Validating the AMRFinder Tool and Resistance Gene
887 Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in
888 a Collection of Isolates. *Antimicrob Agents Ch* **63**, doi:ARTN e00483-19
889 10.1128/AAC.00483-19 (2019).
890 53 Zankari, E. *et al.* Identification of acquired antimicrobial resistance genes. *J*
891 *Antimicrob Chemother* **67**, 2640-2644, doi:10.1093/jac/dks261 (2012).
892 54 Liu, B., Zheng, D., Jin, Q., Chen, L. & Yang, J. VFDB 2019: a comparative
893 pathogenomic platform with an interactive web interface. *Nucleic Acids Res* **47**,
894 D687-D692, doi:10.1093/nar/gky1080 (2019).
895 55 Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J. & Wishart, D. S. PHAST: a fast
896 phage search tool. *Nucleic Acids Res* **39**, W347-352, doi:10.1093/nar/gkr485
897 (2011).

898 56 Darling, A. E., Mau, B. & Perna, N. T. progressiveMauve: multiple genome
899 alignment with gene gain, loss and rearrangement. *PLoS One* **5**, e11147,
900 doi:10.1371/journal.pone.0011147 (2010).
901 57 Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version
902 7: improvements in performance and usability. *Mol Biol Evol* **30**, 772-780,
903 doi:10.1093/molbev/mst010 (2013).
904 58 Page, A. J. *et al.* Roary: rapid large-scale prokaryote pan genome analysis.
905 *Bioinformatics* **31**, 3691-3693, doi:10.1093/bioinformatics/btv421 (2015).
906 59 Kozlov, A., Darriba, D., Flouri, T., Morel, B. & Stamatakis, A. RAxML-NG: A fast,
907 scalable, and user-friendly tool for maximum likelihood phylogenetic inference.
908 *bioRxiv*, doi:10.1101/447110 (2018).
909 60 Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. jModelTest 2: more models,
910 new heuristics and parallel computing. *Nat Methods* **9**, 772,
911 doi:10.1038/nmeth.2109 (2012).
912 61 Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler
913 transform. *Bioinformatics* **25**, 1754-1760, doi:10.1093/bioinformatics/btp324
914 (2009).
915 62 McKenna, A. *et al.* The Genome Analysis Toolkit: a MapReduce framework for
916 analyzing next-generation DNA sequencing data. *Genome Res* **20**, 1297-1303,
917 doi:10.1101/gr.107524.110 (2010).
918 63 Lischer, H. E. & Excoffier, L. PGDSpider: an automated data conversion tool for
919 connecting population genetics and genomics programs. *Bioinformatics* **28**, 298-
920 299, doi:10.1093/bioinformatics/btr642 (2012).

921 64 Bushnell, B., Rood, J. & Singer, E. BBMerge - Accurate paired shotgun read
922 merging via overlap. *PLoS One* **12**, e0185056, doi:10.1371/journal.pone.0185056
923 (2017).
924 65 Nurk, S., Meleshko, D., Korobeynikov, A. & Pevzner, P. A. metaSPAdes: a new
925 versatile metagenomic assembler. *Genome Res* **27**, 824-834,
926 doi:10.1101/gr.213959.116 (2017).
927 66 Faust, G. G. & Hall, I. M. SAMBLASTER: fast duplicate marking and structural
928 variant read extraction. *Bioinformatics* **30**, 2503-2505,
929 doi:10.1093/bioinformatics/btu314 (2014).
930 67 Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics*
931 **25**, 2078-2079, doi:10.1093/bioinformatics/btp352 (2009).
932 68 Press, M. O. *et al.* Hi-C deconvolution of a human gut microbiome yields high-
933 quality draft genomes and reveals plasmid-genome interactions. *biorxiv*, 198713
934 (2017).
935 69 Stewart, R. D. *et al.* Assembly of 913 microbial genomes from metagenomic
936 sequencing of the cow rumen. *Nat Commun* **9**, 870, doi:10.1038/s41467-018-
937 03317-6 (2018).
938 70 Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. & Tyson, G. W.
939 CheckM: assessing the quality of microbial genomes recovered from isolates,
940 single cells, and metagenomes. *Genome Res* **25**, 1043-1055,
941 doi:10.1101/gr.186072.114 (2015).
942 71 Ondov, B. D. *et al.* Mash: fast genome and metagenome distance estimation using
943 MinHash. *Genome Biol* **17**, 132, doi:10.1186/s13059-016-0997-x (2016).

944 72 Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina
945 amplicon data. *Nat Methods* **13**, 581-583, doi:10.1038/nmeth.3869 (2016).
946 73 Quast, C. *et al.* The SILVA ribosomal RNA gene database project: improved data
947 processing and web-based tools. *Nucleic Acids Res* **41**, D590-596,
948 doi:10.1093/nar/gks1219 (2013).
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966

967

968 **Table 1: Multidrug resistant *E. coli* genomes found in the ceca of broiler chicks**

Isolate ID	Resistance phenotype†	Antibiotic resistance genes‡	Plasmids§	Phylogroup	MLST
Ec-FL1-1X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL2-2X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL2-5X	**Amp Gen Str Fis Tet**	***aac(3)-Iid aph(3')-Ia aph(6)-Id aph(3'')-Ib blaTEM-1B tet (B) sul2***	IncF:B:A-, IncI1	D	69
Ec-FL1-2X*	Gen Str Tet	*aadA1 aac(3)-Via tet(A)*	IncF:B:A- IncI1 IncI2 Col8282 ColRNAI	F	6858
Ec-FL1-5X*	Gen Str Tet	*aadA1 aac(3)-Via tet(A)*	IncF:B:A- IncI1 IncI2 Col8282 ColRNAI	F	6858

969 * *E. coli* isolates carrying IncI1-pST26 with >99% identical DNA sequence with p2ST26 acquired by SH.
970 † Amp – ampicillin, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole.
971 ‡ Boldness denotes antibiotic resistance genes encoded on the chromosome.
972 § Illumina short reads and PacBio long reads were combined to assemble the plasmids of E. coli strains Ec-FL1-1X and Ec-FL1-2X.
973

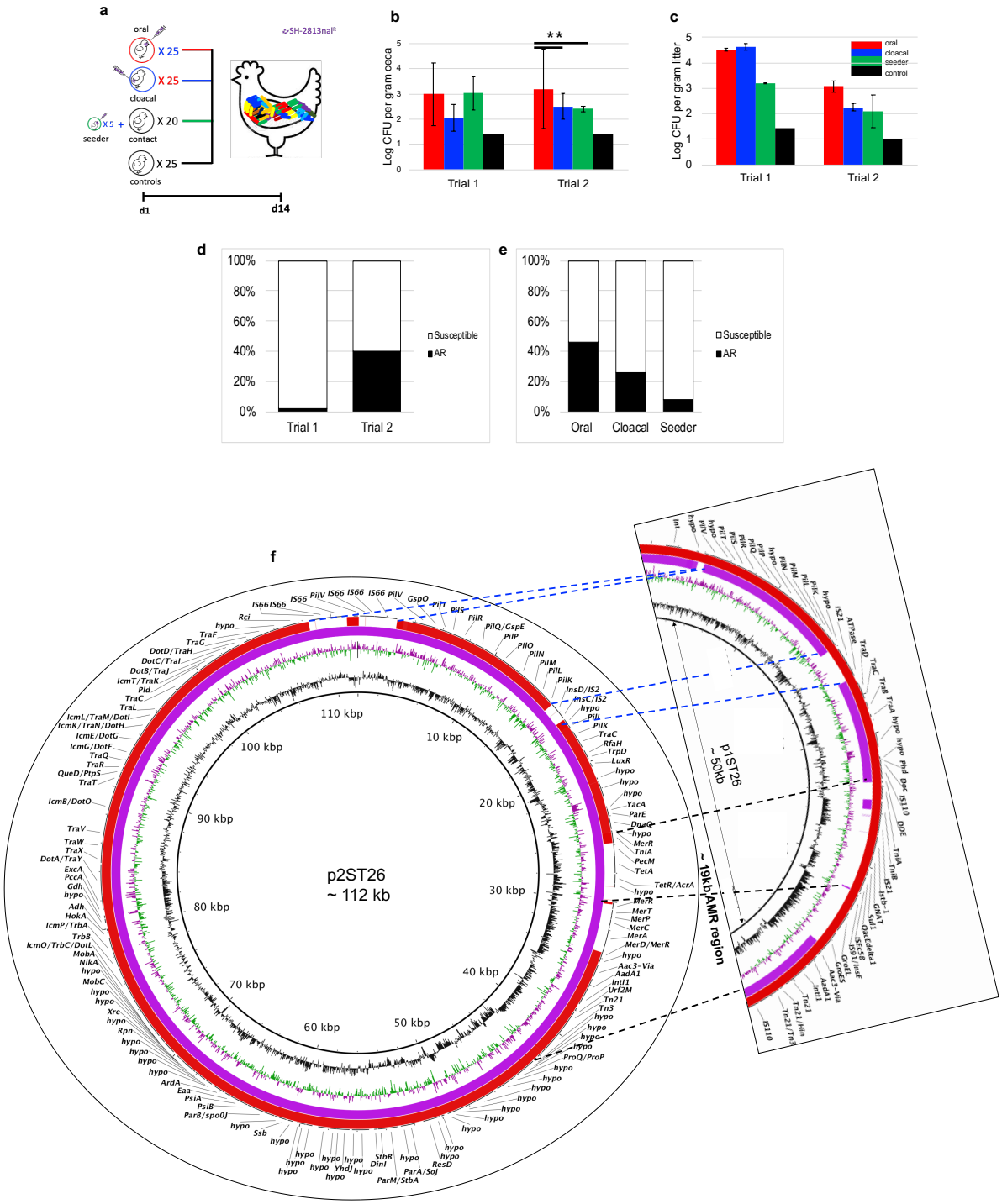


Figure 1. **Broiler chicks challenged with an antibiotic susceptible strain of SH carried antibiotic resistance SH strains 2-weeks after challenge**. **a**, Experimental design for trial 1 and trial 2 conducted in September 2017 and April 2018, respectively. **b,c** SH load in the ceca (n = 10 per trial) and litter (n = 2 per trial) 2 weeks after challenging 1-day broiler chicks (100 chicks/trial) with 10^6 colony forming units (CFU) of SH by oral gavage, cloacal or through the seeder method (*P < 0.01 (Kruskal-Wallis test, multiple comparison correction; error bar = Standard deviation; limit of quantification = Log 1.3 CFU/g litter). **d**, Percentage of SH isolates that acquired antibiotic resistance in trial 1 (n = 92) and trial 2 (n = 158) **e**, Percentage of SH isolates in trial 2 that acquired antibiotic resistance grouped by the route the broiler chicks got challenged (n = 54 62, and 42 for oral, cloaca and seeder, respectively. **f,** BLASTn alignment of the IncI1-complex plasmid acquired during trial 1 (p1ST26) and trial 2 (p2ST26) – dashed black lines highlights mobile region encoding antimicrobial resistance genes while blue dashed lines shows other regions that are different between p1ST26 and p2ST26.

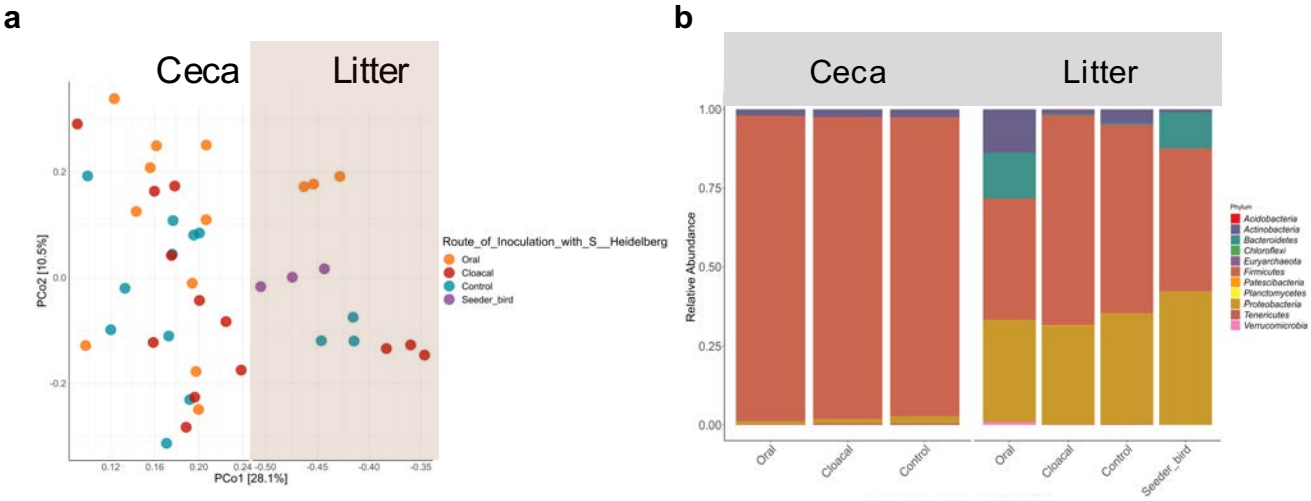
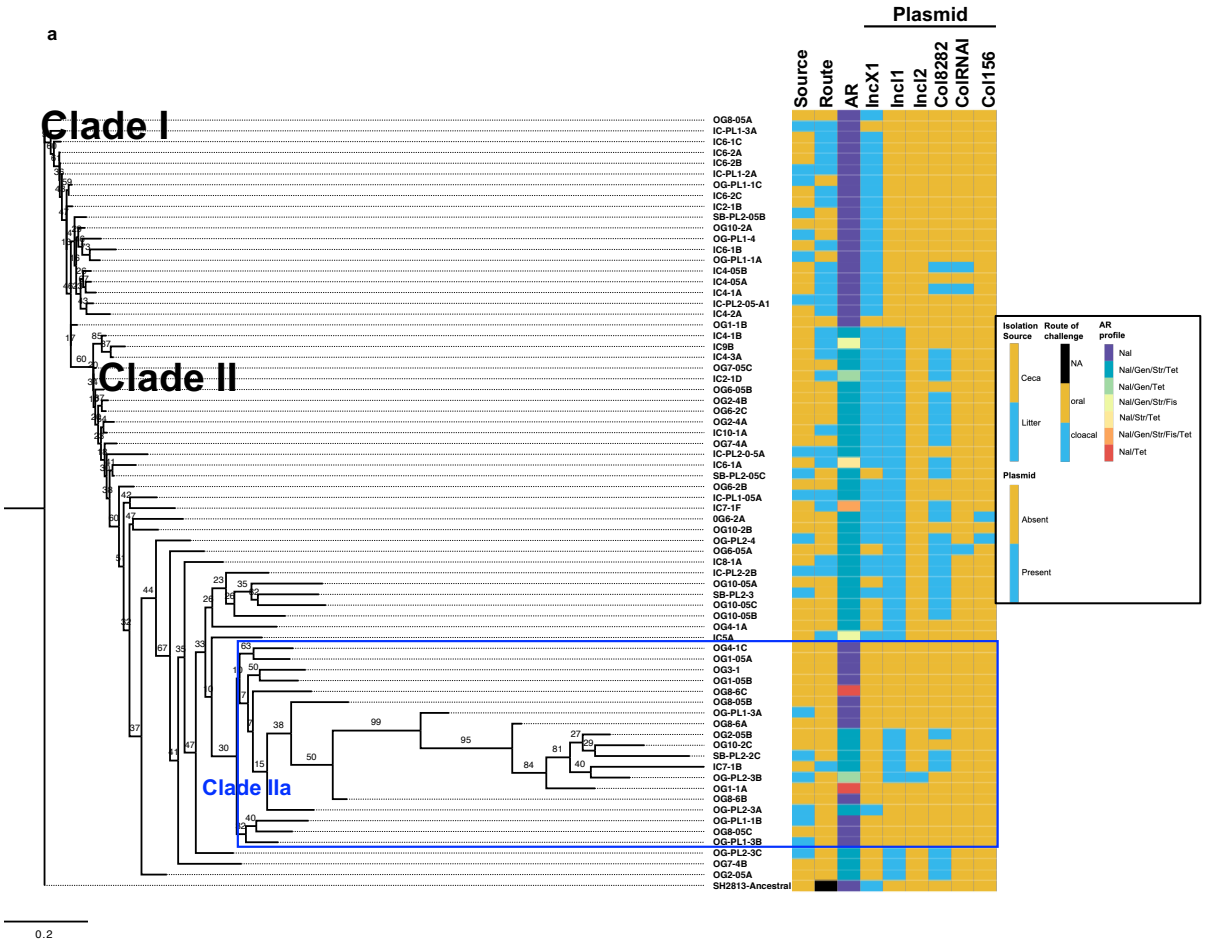


Figure 2. SH infection perturbed the bacterial community present in ceca and litter of broiler chickens. a, Principal coordinates analysis of Bray-Curtis distances based on 16S rRNA gene libraries obtained from ceca and litter samples. Each point represents values from individual libraries with colors expressing the route of inoculation of SH in for respective samples. b, Phylum-level classification of 16S rRNA gene sequence reads in ceca and litter grouped by route of SH infection. Data represent average of amplicon sequence variant (ASV) counts from replicate libraries for each category.



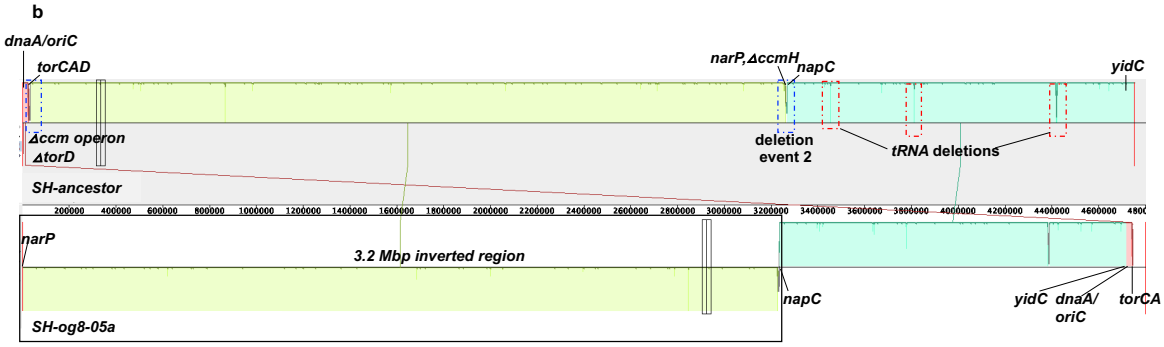


Figure 3. Horizontal gene transfer and genome inversion shaped the fitness of SH in broiler chicks. a, Maximum likelihood tree constructed using accessory genes present in SH isolates (n=72) recovered from the ceca and litter of broiler chicks colonized with SH. The GTR model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates. Tree was rooted with the ancestral susceptible SH strain. Clade numbers were arbitrary assigned to facilitate discussion on isolates that acquired antibiotic resistance. Blue rectangular box highlights the clade with susceptible strains nested within antibiotic resistance strains due to sequencing bias and misassembly. (Legend: Nal – nalidixic acid, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole, NA – Not applicable) **b,** Mauve visualization of the inverted genome of SH strain og8-05a. The chromosomal contigs of og8-05a was aligned and ordered to the complete chromosome of SH ancestor. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region. When the similarity plot points downward it indicates an alignment to the reverse strand of the SH ancestor genome i.e., inversion. Segment highlighted with solid black rectangular box shows the inverted region in og8-05a, while dashed blue and red rectangular boxes denotes segments with relevant mutations.

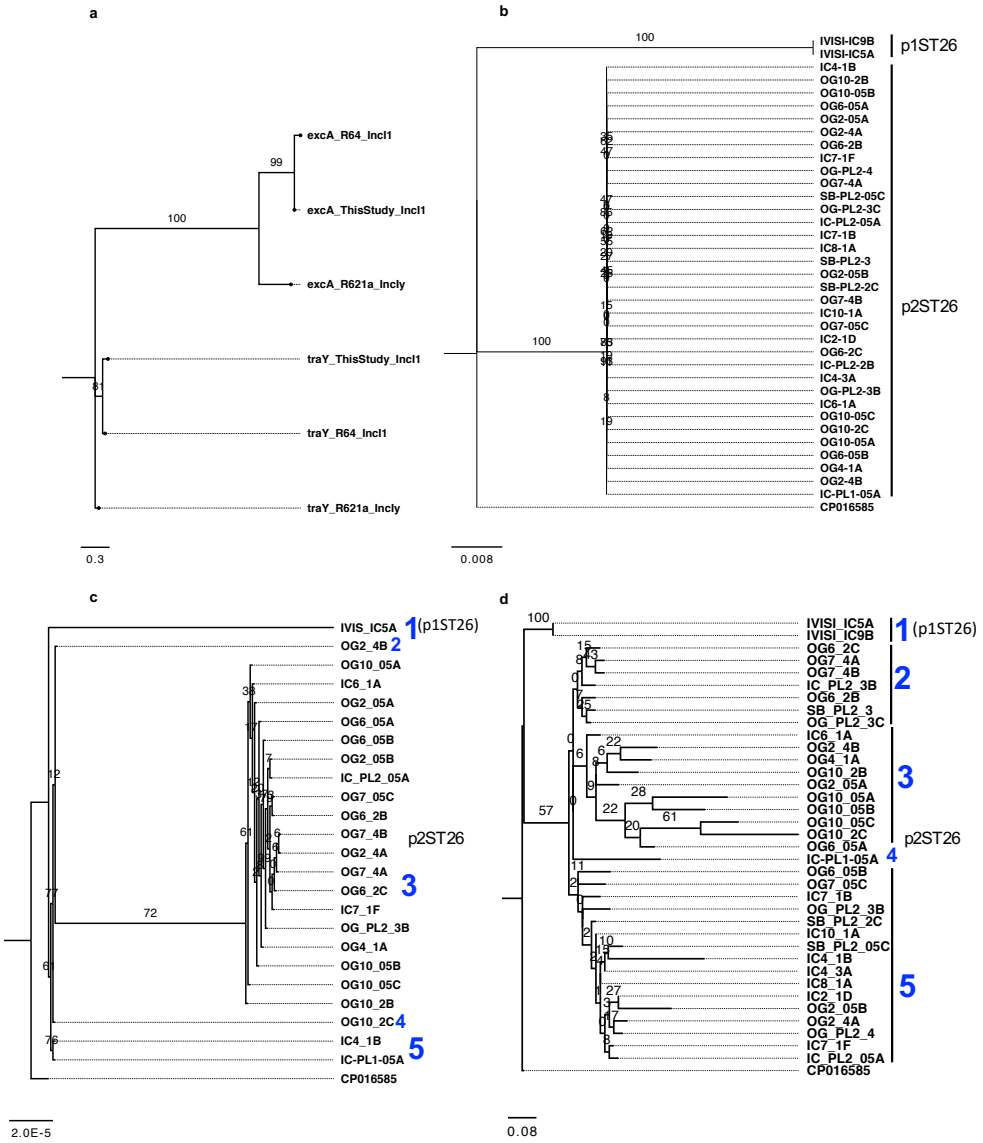


Figure 4. Gene flux contributed to the diversity of IncI1-pMLST26 plasmids. **a,** Maximum likelihood tree constructed using TraY and ExcA protein sequences from a representative IncI1 plasmid from this study, R64 (IncI1) and R621a (IncI1-gamma). The GTR model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction. Tree was rooted with traY sequence of R621a. **b - d,** Maximum likelihood tree of pST26 IncI1 plasmids from this study (n = 36) constructed using complete plasmid DNA sequences, core genes (**c**) and accessory genes (**d**). The GTR+I, GTR and JC+I model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction for b, c, and d, respectively (Tree was rooted with the closest relative (GenBank: CP016585) found through NCBI BLASTn)). Clade numbering were arbitrary assigned to show number of clades found and not order of evolution. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates.

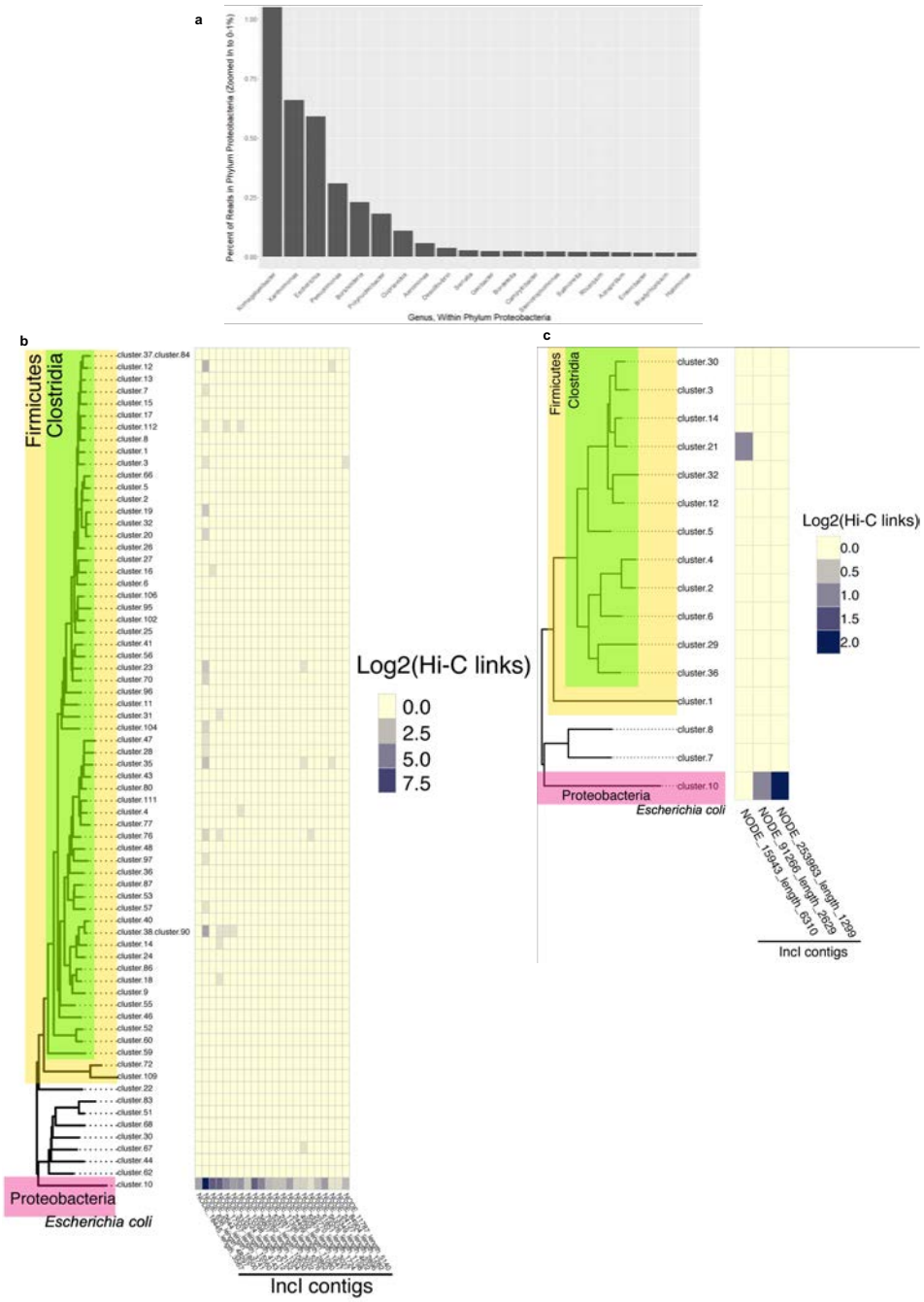
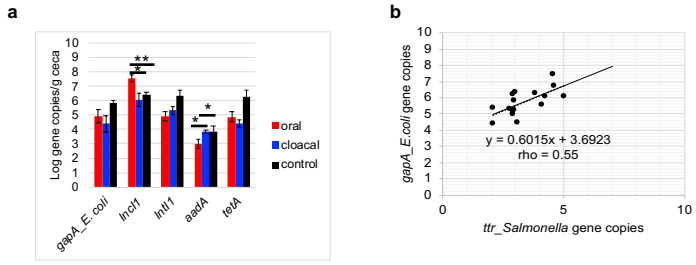


Figure 5. *Proteobacteria* was the reservoir of IncI1-plasmids in broiler chickens. a, Percentage of metagenomic reads assigned to the top 20 bacterial genera of the phylum Proteobacteria present in samples Hi-IC-FL1 and Hi-IC-FL2. Only reads assigned to phylum *Proteobacteria* (70.9 % of the metagenomic reads) were used and zoomed in to show 0-1% of total reads within phylum Proteobacteria (96.7% of reads within phylum *Proteobacteria* were assigned to *Komagataeibacter*). **b and c,** IncI1 contigs hosts found by Hi-C contacts. IncI1-derived contigs (horizontal axis of heatmap) show specific Hi-C associations with metagenome-assembled genomes (MAGs) present in samples Hi-IC-FL1 (**b**) and Hi-IC-FL2 (**c**) (vertical axis of heatmap). MAGs are derived from Hi-C deconvolution of the metagenome assembly and placed into a bacterial phylogeny using CheckM. Cluster.10 in each sample is an *Escherichia coli* genome. In Hi-IC-FL1, one MAG (cluster.82) representing an extremely fragmented and partially contaminated *Escherichia coli* genome is omitted for clarity (due to its contamination, this cluster was placed in *Clostridia* by CheckM). Heatmap values indicate transformed counts of Hi-C read contacts (indicating intracellular physical proximity of IncI1 contigs to those genomes. Heatmap values were pseudocounted to facilitate plotting of log-transformed data including zeroes.

1028



1029 **Figure 6. SH challenge affected the relative abundance of antibiotic resistance genes present in the ceca and litter of broiler chickens.** a, Concentration of selected antibiotic resistance determinants,
1030 IncI1 and *E. coli* present in the ceca of broiler chickens. Gene copies were determined using qPCR. Primers targeting the housekeeping gene (*gapA*) and *inc*RNAI was used for *E. coli* and IncI1 quantification,
1031 respectively. b, Pearson correlation between *Salmonella ttr* and *E. coli gapA* gene copies present in ceca and litter (n =15).

1032

1033

1034

1035

1036

1037

1038

1039

1040

1041

1042

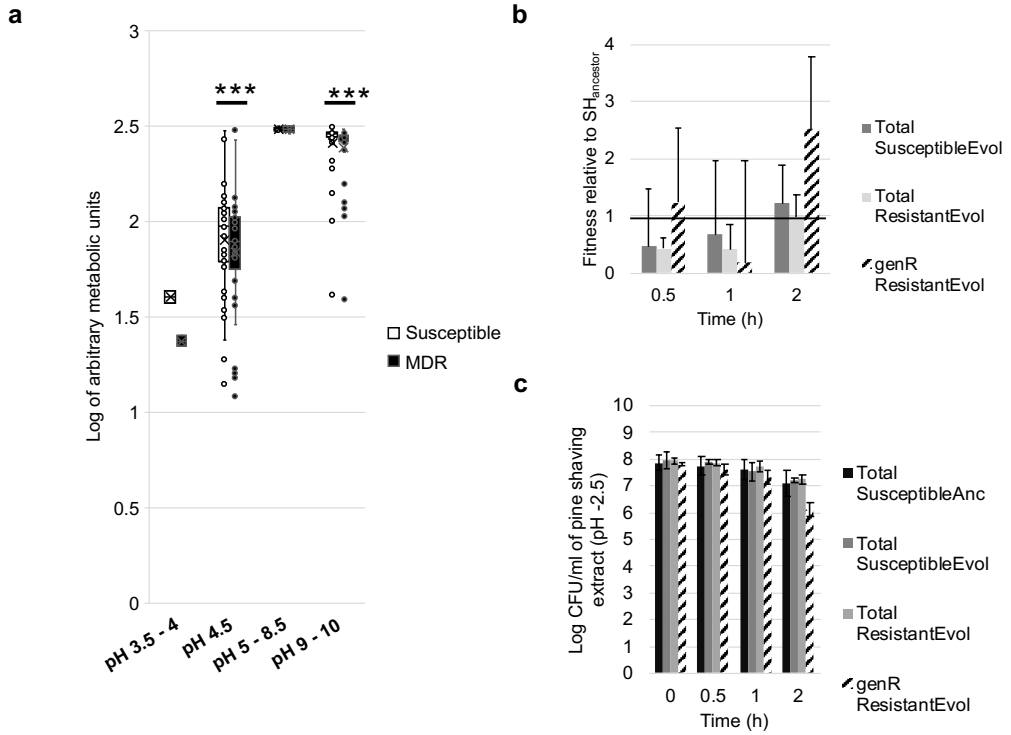


Figure 7. SH exposure to acidic pH imposes negative frequency dependent selection for IncI1 (pST26). a Box plot comparing the metabolic activity of one evolved susceptible (no IncI1) and one multidrug (MDR carrying pST26) SH strain using phenotype microarray (PM) plates (Biolog Inc). Pre-configured PM plates are composed of microtiter plates with one negative control well and 95 wells pre-filled with or without nitrogen and at pH of 3.5 – 10 (n = 1 plate per strain; no of wells per plate for pH 3.5-4, 4.5, 5-8.5 and 9-10 was 2, 6, 37, and 38 respectively; ***$P < 0.001$ (Wilcoxon signed rank test). **b and c,** Fitness and abundance of evolved susceptible and MDR SH strains when exposed to pine shaving extract of pH 2.5. Each bar represents the average fitness or abundance (colony forming units per ml of pine shaving extract) of three individual population that was established from three single bacterial colonies from three different strains (Error bar = Standard deviation). The horizontal line in **b** represents the fitness of three SH-Ancestor population established from three single bacterial colonies. (Anc = Ancestor, Evol – Evolved i.e., SH isolate recovered during *in vivo* experiment, genR – population resistant to gentamicin).

Gene flux and acid-imposed selection are the main drivers of antimicrobial resistance in broiler chicks infected with *Salmonella enterica* serovar Heidelberg

Adelumola Oladeinde[1,2,10*], Zaid Abdo[2*], Maximilian O. Press[3¶], Kimberly Cook[4], Nelson A. Cox[1], Benjamin Zwirzitz[5,6¶], Reed Woyda[2¶], Steven M. Lakin[2¶], Jesse C. Thomas IV[7], Torey Looft[8], Douglas E. Cosby[1], Arthur Hinton Jr.[1], Jean Guard[1], Eric Line[1], Michael J. Rothrock[1], Mark E. Berrang[1], Kyler Herrington[9], Gregory Zock[10], Jodie Plumblee Lawrence[1], Denice Cudnik[1], Sandra House[1], Kimberly Ingram[1], Leah Lariscy[1], Robert Wagner[5,6], Samuel E. Aggrey[10], Lilong Chai[10], and Casey Ritz[10]

Authors' Affiliation

[1]U.S. National Poultry Research Center, USDA-ARS, Athens, GA, USA. [2]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, USA. [3]Phase Genomics Inc, Seattle, WA, 98109, USA, [4]Office of National Programs, USDA-ARS, Beltsville, Maryland, USA. [5]Institute of Food Safety, Food Technology and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria. [6]Austrian Competence Centre for Feed and Food Quality, Safety, and Innovation FFoQSI GmbH, Tulln, Austria. [7]Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD and TB Prevention, Center for Disease and Control, Atlanta, Georgia, USA. [8]National Animal Disease Center, USDA-ARS, Ames, IA, USA, [9]Medical College of Georgia, Augusta, GA, USA. [10] Poultry Science Dept, University of Georgia, Athens, GA, USA

Corresponding authors* ade.oladeinde@usda.gov; zaid.abdo@colostate.edu

¶ These authors contributed equally to this work.

Supplementary Methods

Isolation of *E. coli* from ceca of broiler chicks

To isolate *E. coli*, we retrospectively screened two cecal slurry from intracloacally challenged chicks on CHROMagar™ plates supplemented with gentamicin (8 ppm) and tetracycline (8 ppm). Frozen vials of cecal contents were thawed on ice and vortexed. For each sample, a 10 µl aliquot was struck for isolation, and a 100 µl aliquot was spread plated on CHROMagar™ with relevant antibiotics. Plates were incubated overnight at 37°C. Blue green and blue-cream colonies were counted as presumptive *E. coli* and colonies was restruck for isolation on CHROMagar™ supplemented with gentamicin and tetracycline. After incubation, 2- 3 colonies from each sample were selected to represent different forms of blue green/blue-cream colonies and struck in parallel onto sheep blood agar (SBA) and Eosin Methylene Blue (EMB) (Remel Inc., KS, USA) and incubated overnight. Growth on EMB was the characteristic green metallic sheen after incubation. Colonies from SBA were stored in 30% LB glycerol at -80°C and were used for antimicrobial susceptibility testing and whole genome sequencing.

Long read sequencing quality control. PacBio sequencing of samples was done using using the Sequel II system. MinION sequencing of samples was done using R9.5 chemistry on a 1D flowcell. Lima (https://github.com/PacificBiosciences/pbbioconda) was used to demultiplex barcoded PacBio samples and to convert the split BAM files into fastq format. BBMap reformat.sh (https://github.com/BioInfoTools/BBMap) was used to randomly subsample generated PacBio reads down to 200X coverage. Porechop v0.2.4 (https://github.com/rrwick/Porechop) was used to demultiplex barcoded MinION samples. Raw Illumina reads were trimmed using Trimmomatic v0.39[1] (command line parameters: PE ILLUMINACLIP:NexteraPE-PE.fa:2:30:10:3 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36).

Comparative genomics. To determine IncI1-pST26 consensus, first we used BWA[2] or Bowtie v. 7.2.1[3] (implemented in Geneious Prime®) to align raw FASTQ files against a complete circular p1ST26 or p2ST26 and used the binary alignment map file generated for consensus determination in Geneious Prime®. To identify identical IncI1-pST26 plasmids, we performed a BLAST search against the NCBI non-redundant database and selected the top 50 plasmids matching p2ST26 from this study. To construct a whole plasmid

based maximum likelihood tree, we downloaded the FASTA files for the top 50 plasmids from NCBI and used them for whole genome alignment.

Tools used for visualizing and exploring high-throughput sequence data. Gene presence/absence heatmap, with gene presence/absence data obtained from Roary, was generated using the pheatmap v1.0.12, tidyverse v1.3.0 and viridis v0.5.1 packages in R v4.0.2. BLAST Ring Image Generator[4] was used for genome comparison visualization including GC skew change and Phandango[5] was used for visualizing phylogenetic trees with their associated metadata. PHYLOViZ 2.0[6] was used to generate a minimum spanning tree of SH isolates from core genome information obtained through SISTR (i.e., wgMLST_330:complete-alleles, wgMLST_330:missing-alleles, wgMLST_330:partial-alleles,closest-public-genome-alleles-matching and cgMLST_cluster_level). SnapGene® was used for drawing linear maps of plasmid, prophages, and other regions of interest in bacterial genomes. Geneious Prime® and SnapGene® were used to view MAFFT alignments of DNA and amino-acid sequences.

References:

1 Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120, doi:10.1093/bioinformatics/btu170 (2014).

2 Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760, doi:10.1093/bioinformatics/btp324 (2009).

3 Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**, 357-359, doi:10.1038/nmeth.1923 (2012).

4 Alikhan, N. F., Petty, N. K., Ben Zakour, N. L. & Beatson, S. A. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* **12**, 402, doi:10.1186/1471-2164-12-402 (2011).

5 Hadfield, J. *et al.* Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics* **34**, 292-293, doi:10.1093/bioinformatics/btx610 (2018).

6 Nascimento, M. *et al.* PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* **33**, 128-129, doi:10.1093/bioinformatics/btw582 (2017).

Supplementary Table 1: Assembly report for a *S*. Heidelberg strain with sequencing bias

Assembly statistics*	**Short read**†	**Long read** ‡	**Hybrid**§
Number of contigs	554	4	33
Total length (bp)	4314964	4703397	4865484
Reference length (bp)	4788855	4788855	4788855
Number of misassembled contigs	4	0	1
Misassembled contigs length (bp)	47430	0	4742328
Genome fraction of reference (%)	89.969	98.247	99.815
Largest alignment (bp)	60767	2669051	3233973
Total aligned length (bp)	4308655	4703397	4826756

* Assembly was done using Unicycler and SH-ancestor was used as the reference genome (4,788,855 bp)
† Illumina short technology was used for sequencing.
‡ PacBio long read sequencing technology was used.
§ Illumina short reads and PacBio long reads were combined.

Supplementary Table 2. Mutations found in p1ST26 but absent in p2ST26

Loci*	Location†	Product	Mutation‡
20998	Intergenic	Upstream replication protein	N/A
23599	CDS	*yacA*	Substitution
23637	CDS	*yacA*	Substitution
23661	CDS	*yacA*	Substitution
23695	CDS	*yacA*	Substitution
23698	CDS	*yacA*	Substitution
23704	CDS	*yacA*	Substitution
23731	CDS	*yacA*	Substitution
23734	CDS	*yacA*	Substitution
23826	CDS	*parE*	Substitution
25883	CDS	*tniA*	Insertion
35066	CDS	*aac3-Via*	Substitution
35608	CDS	*aadA1*	Substitution
35635	CDS	*aadA1*	Substitution
38685	CDS	*tn21*	Substitution
41737	Intergenic	downstream tn3; upstream hypothetical	N/A
42362	CDS	hypothetical	Substitution
49072	CDS	hypothetical	Substitution
70253	CDS	hypothetical	Substitution
70287	CDS	hypothetical	Substitution
70333	CDS	hypothetical	Substitution
70343	CDS	hypothetical	Substitution
92634	CDS	*icmG/dotF*	Substitution

*A circular and complete IncI1 plasmid from Trial 2 was used as the reference genome (111, 898 bp).
†CDS- Coding DNA sequence
‡N/A – Not applicable

Supplementary Table 3. Plasmids found in the cecal metagenome †

Hi-IC-FL1	**Hi-IC-FL2**
IncI1, IncI2, IncFIB, IncFII, IncFIC, IncHIB, p0111, Col8282, ColRNAI	p0111

† We searched the cecal metagenome of two cecal samples for plasmid *inc* groups that are commonly found in family *Enterobacteriaceae*

Supplementary Table 4: Concentration of metals and pollutants in starter feed.

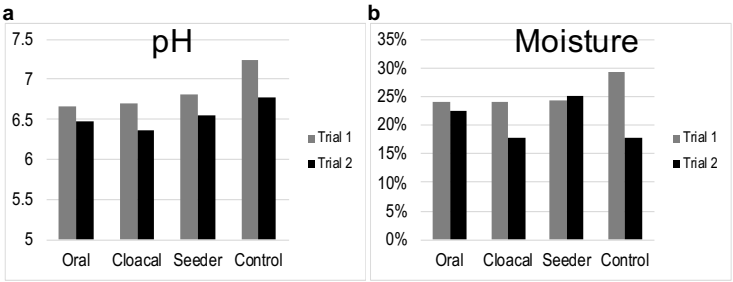
Starter feed*	Parts per million (ppm)	Percent (%)	Pounds per ton
Total Acid Digestion			
Antimony (Sb)	<0.4	negligible	negligible
Arsenic (As)	0.999	negligible	negligible
Beryllium (Be)	<0.05	negligible	negligible
Cadmium (Cd)	0.109	negligible	negligible
Chromium (Cr)	2.74	0.0003	0.01
Copper (Cu)	7.96	0.0008	0.02
Lead (Pb)	0.326	negligible	negligible
Nickel (Ni)	1.67	0.0002	0
Selenium (Se)	0.985	negligible	negligible
Silver (Ag)	<0.05	negligible	negligible
Thallium (Tl)	<0.4	negligible	negligible
Zinc (Zn)	82.7	0.0083	0.17

*Unmedicated starter feed was synthesized by University of Georgia's poultry research center's feed mill.

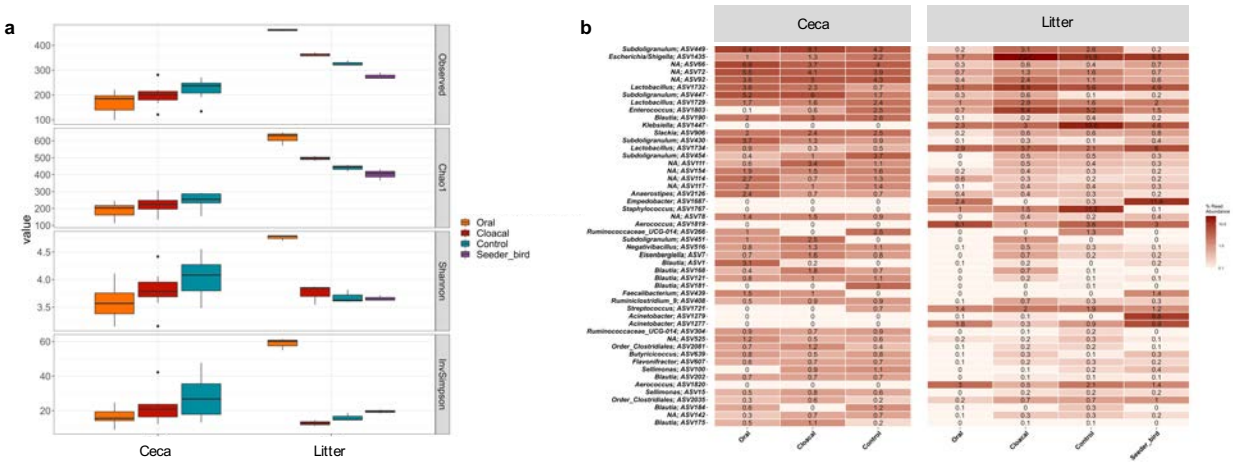
Supplementary Table 5. Primers used for the qPCR analysis of ceca and litter.

Gene	Target description	Primer Sequences (5' - 3')	Amplification factor*	Reference
aadA	Aminoglycoside resistance	F: CAGCGCAATGACATTCTTGC R: GTCGGCAGCGACAYCCTTCG	2.02	73
gapA	D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *E. coli*	F: CCGTTGAAGTGAAAGACGGTC R: AACCACTTTCTTCGCACCAGC	1.98	74
IncI1	*inc*RNAi	F: CAGGAGAGATGGCATGTA R: GGGTTTTCCTTTTATGGC	1.94	This study
intI1	Class 1 integron	F: CCTCCCGCACGATGATC R: TCCACGCATCGTCAGGC	1.98	75
tetA	Tetracycline resistance	F: TGTCCACCAACTTATCAG R: TGCTCAGAATTACGATCA	1.81	This study
ttr	*Salmonella*	F: CTCACCAGGAGATTACAACATGG R: AGCTCAGACCAAAAGTGACCATC	1.86	76

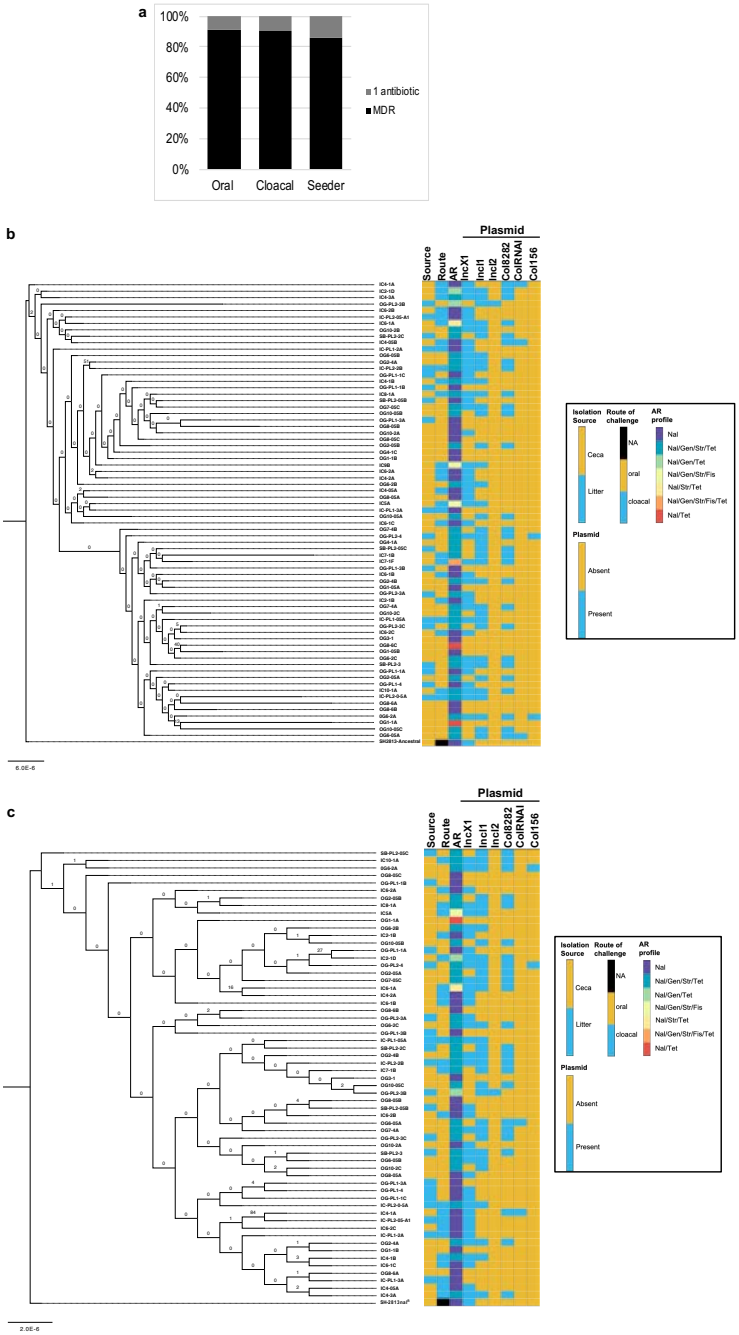
*Genomic DNA extracted from an *E. coli* strain carrying *aadA1, aac(3)-Via, tetA* and *IntI1* on IncI1-pST26 plasmid was used for the calibration curves of *aadA, tetA, IntI1,* IncI1 and *gapA* whereas DNA from an SH-2813-ancestor was used for the calibration curve of *ttr*.



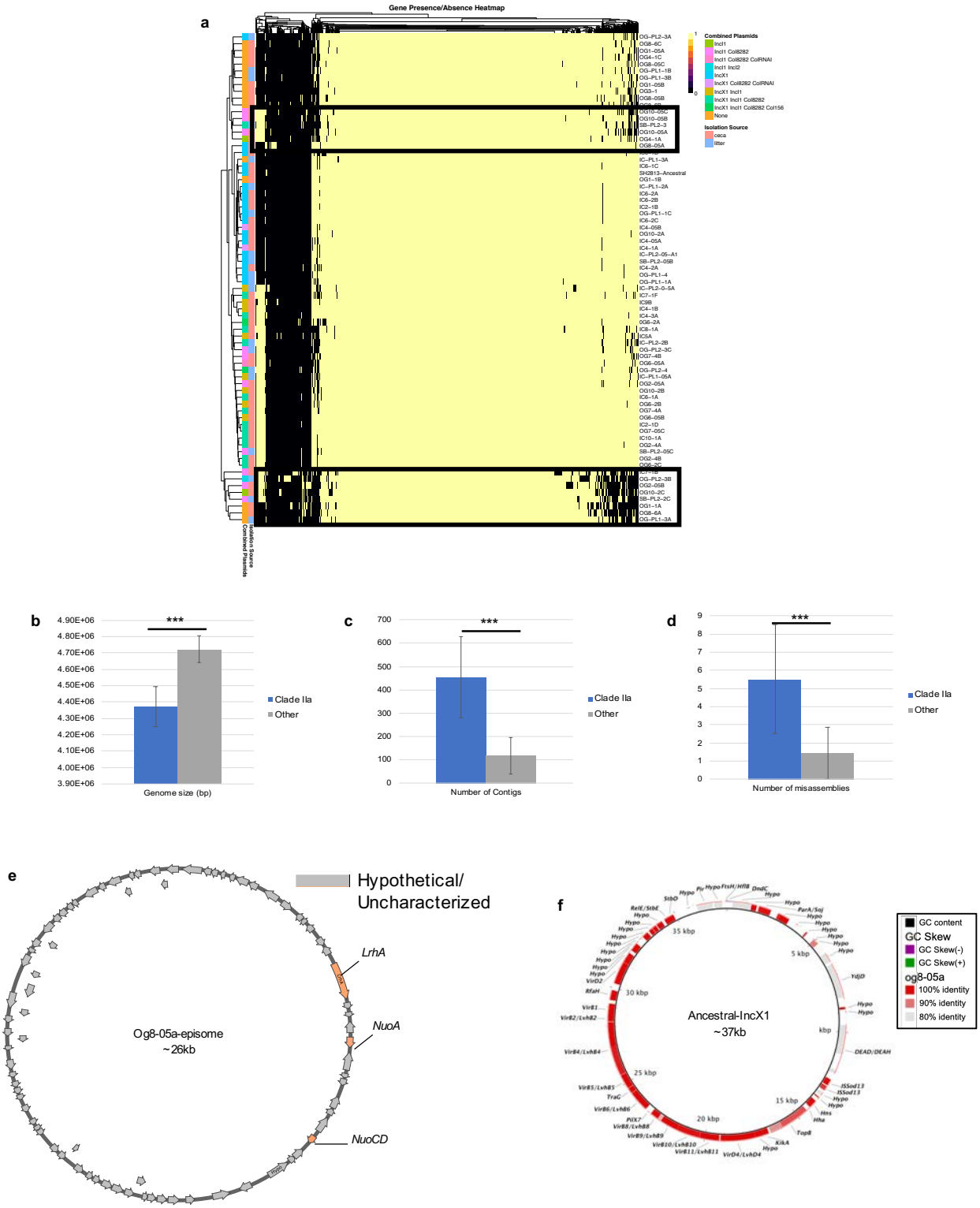
Extended Data Fig. 1. pH and moisture levels of pen litter. Litter was collected as grab samples (n = 7) and pooled into one whirl pak bag (n = 1 per pen). The pH of the litter was determined from the eluate made from a 1:5 dilution of litter in 1X PBS.



Extended Fig. 2 Challenging broiler chicks with SH affected the diversity of bacterial species in their ceca and litter. a, Average alpha diversity indices of ceca and litter samples grouped by route of SH infection. Boxes indicate the interquartile range (75th to 25th) of the data. Whiskers extend to the most extreme value within 1.5 * interquartile range and dots represent outliers beyond that range. d, Heatmap of the top 50 most abundant amplicon sequence variants (ASV) grouped by route of SH infection and split by sample type (Ceca, Litter). Data represents average of ASV counts from replicate 16S rRNA gene libraries for each category.

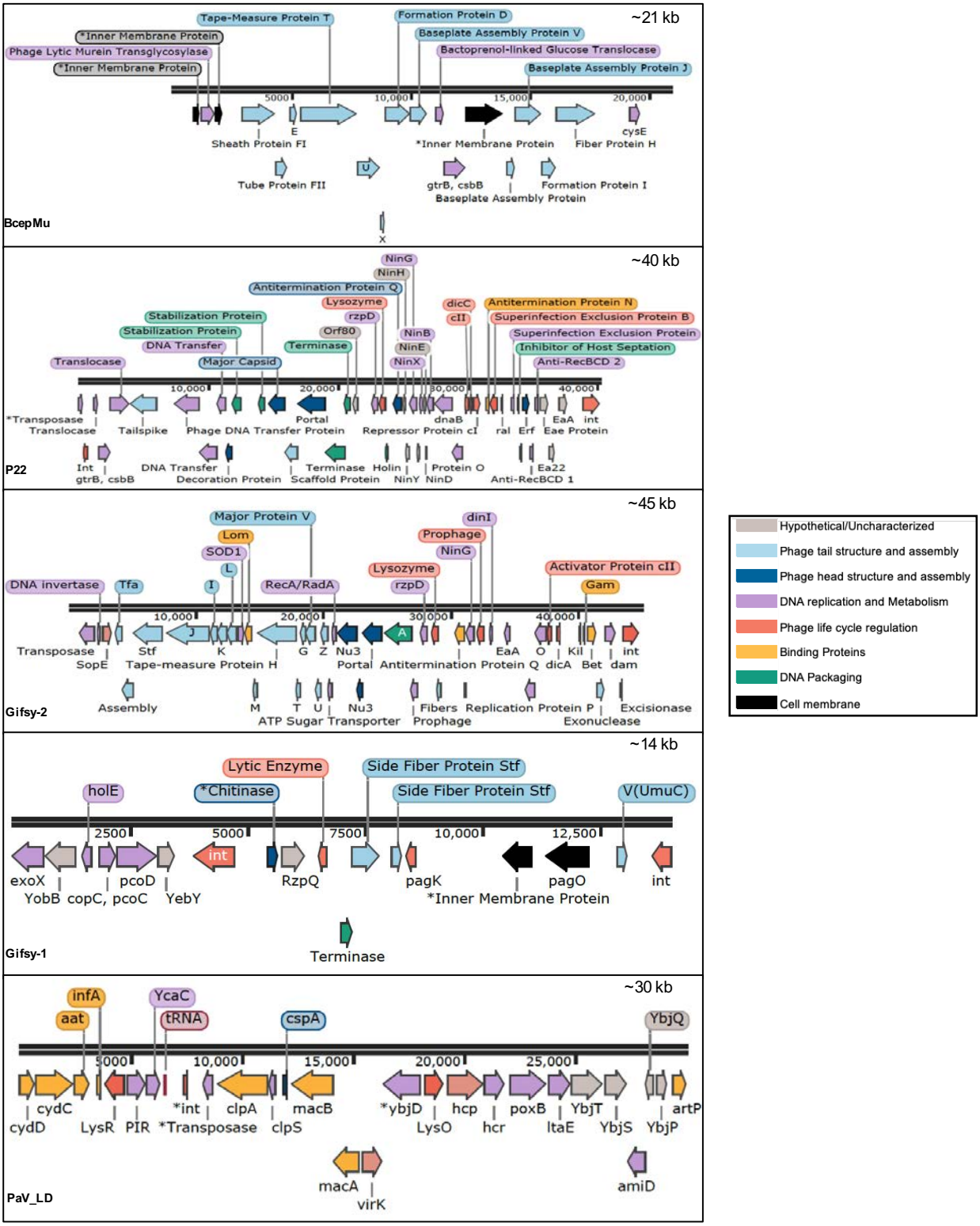


Extended Data Fig. 3. Acquisition of IncI1 plasmid conferred multidrug resistance on SH cells and influenced the contents of their genomes. Antimicrobial susceptibility and whole genome sequencing were performed on SH isolates recovered from the ceca and litter of broiler chicks 2-weeks after challenge. **a**, Percentage of SH isolates (n = 63) from Trial 2 that acquired resistance to two or more antibiotics compared to isolates resistance to only one antibiotic class. Core genome (**b**) and SNP (**c**) based maximum likelihood tree of SH isolates sequenced from Trial 1 (n =2) and Trial 2 (n=69). Isolates with duplicated SNPs/indels were removed before SNP based ML tree was reconstructed. GTR and JC model of nucleotide substitution was used for core and SNP tree, respectively and the GAMMA model of rate heterogeneity were used for sequence evolution prediction. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates. Tree was rooted with the susceptible SH ancestor for core genome and nal resistant SH ancestor (SH-2813nalR) for SNP tree. (Legend: Nal – nalidixic acid, Gen – gentamicin, Str – streptomycin, Tet – tetracycline, Fis – sulfisoxazole, NA – Not applicable) .

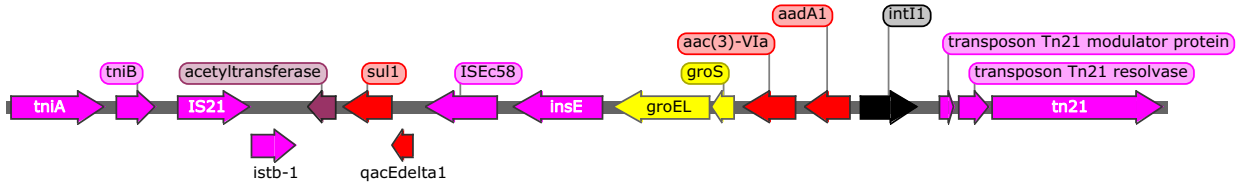


Extended Data Fig. 4. Gene gain and loss contributed to SH diversity in the ceca and litter of broiler chicks a, Heatmap of genes present and absent in SH genomes determined by pangenome analysis. Thick rectangular box highlights SH strains that exhibited higher gene flux and strains with no plasmids but were grouped with SH strains carrying IncI1. **b,c,d** SH genome assembly statistics for SH strains with higher gene flux (n = 12; clade IIa of ML tree reconstructed using accessory genes (see Fig. 2a)) compared to other SH (n=59). (***P < 0.001 (Binomial logistic regression; Error bar = Standard deviation). **e**, Circular map of novel episome present in SH strain og8-05a. **f**, BLASTn alignment of IncX1 present in SH ancestor and IncX1 present in SH strain og8-05a.

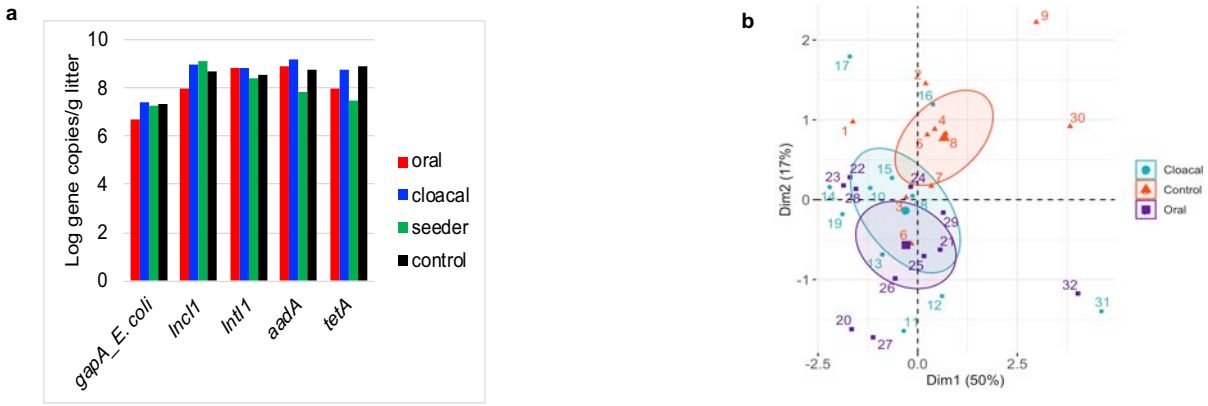
Extended Data Fig. 5. Chromosomal inversion re-oriented the origin of replication of SH strains and positively skewed their GC. Complete chromosome contigs from three SH strains recovered from ceca during Trial 1 (ic9b) and 2 (ic4-3a and og8-05a) were aligned and compared to the ancestor. a, ProgressiveMauve alignment of SH strains ic4-3a and ic9b. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region. When the similarity plot points downward it indicates an alignment to the reverse strand of the genome of SH-ancestor i.e., inversion. Segment highlighted with solid black rectangular box denotes the inverted 2Mbp region in ic9b. Segment highlighted with horizontal blue box denotes regions with relevant mutations. b and c, GC skew in og8-05a and ic9b compared to SH-ancestor. Red arrow points towards reoriented oriC or ter, whereas red and blue arcs show the inverted region. The number of guanine and cytosine in inverted area was used for GC skew calculation. Dashed blue and red rectangular boxes denotes segments with mutations.



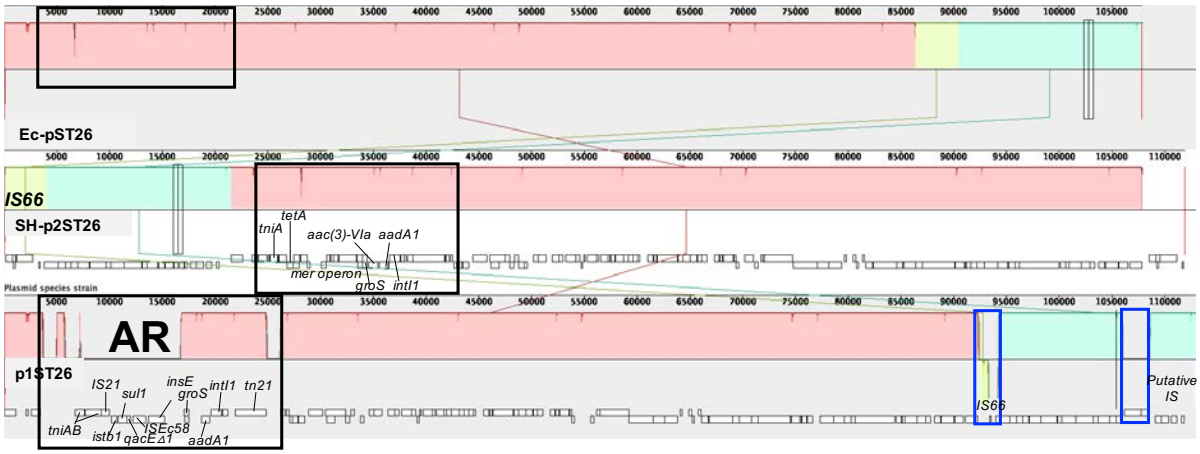
Extended Data Fig. 6 Linear maps of prophages found in SH ancestor.



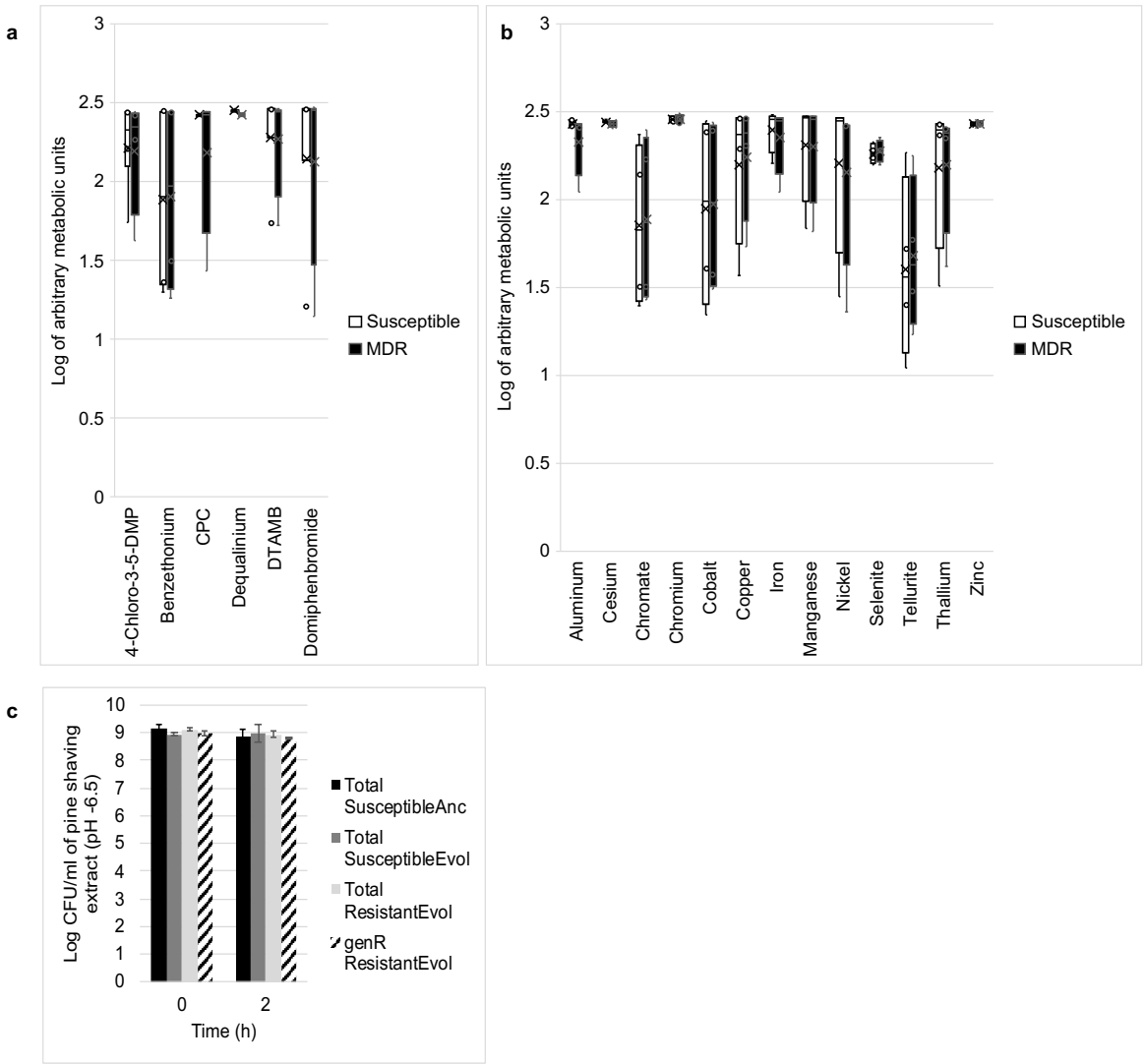
Extended Data Fig. 7 Linear map of the variable region encoding antimicrobial resistance genes in IncI1. The ~19kb DNA region of the p1ST26 plasmid (~113kb) present in one multidrug resistant SH isolate from Trial 1 was used for drawing the map.



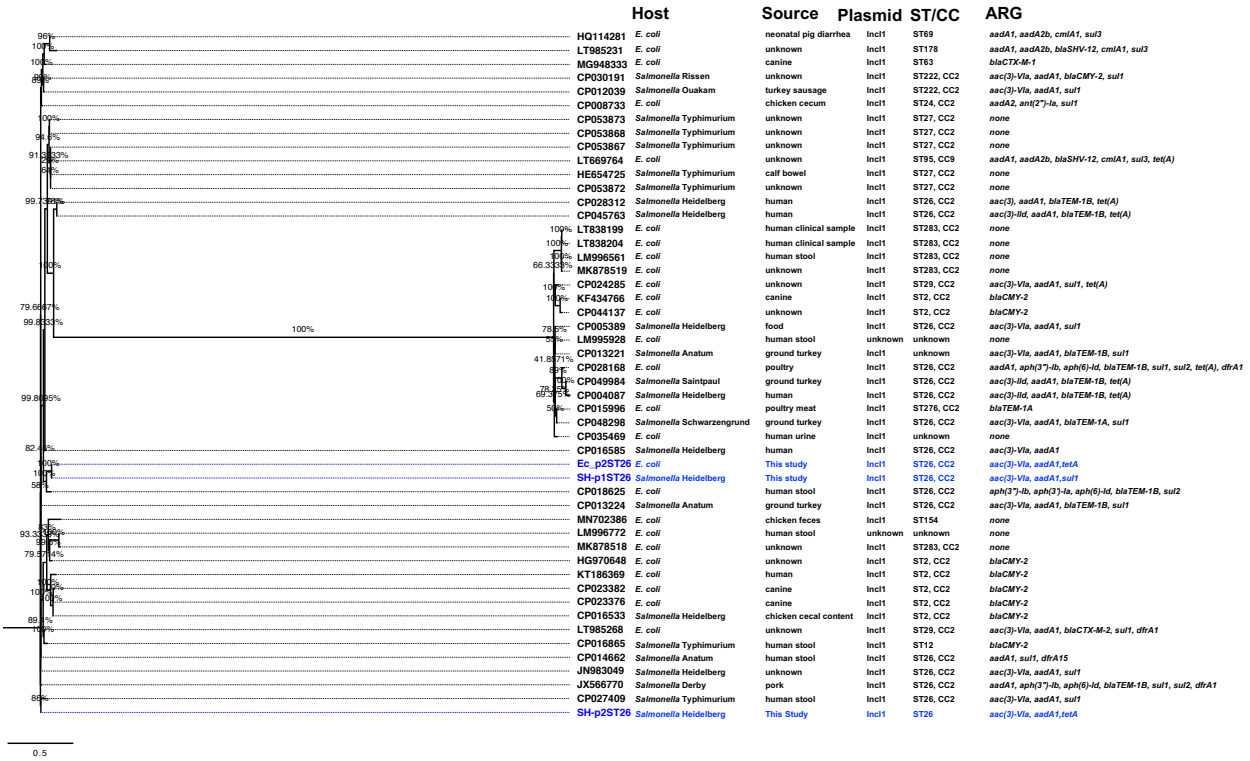
Extended Data Fig. 8. SH challenge affected the abundance of antimicrobial genes a, Concentration of *E. coli* and resistome (IncI1, IntI1 and antibiotic resistance genes) present in the litter of broiler chicks. Gene copies was determined using qPCR. b, Principal component analysis (PCA) characterizing the differences between samples grouped by route broiler chicks got challenged with SH. The PCA was constructed using the concentration of *E. coli, Salmonella* and resistome genes present in ceca and litter of broiler chicks challenged with SH and determined using qPCR. Each number and dot denote one sample, and data is from 32 samples. The farther apart two samples are from each other, the more different they are. Ellipses represents the mean/confidence interval if all the dots of the same color are represented by just one sample.



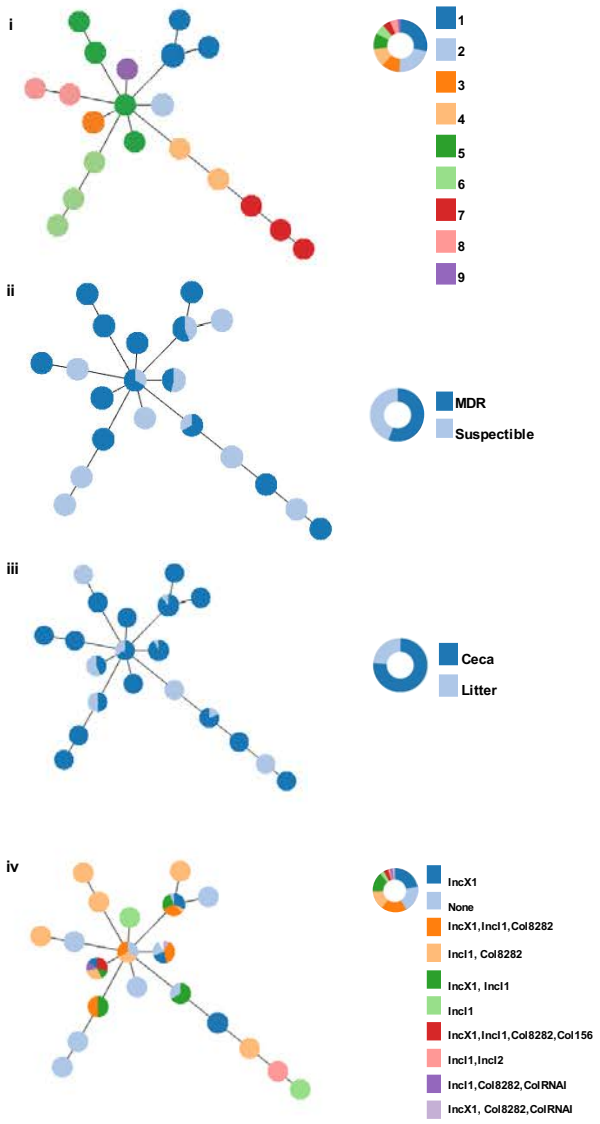
Extended Data Fig. 9. IncI1-pST26 acquired by SH are identical to the pST26 present in commensal *E. coli* strains. Complete pST26 contigs from two SH and one *E. coli* strain recovered from the ceca of broiler chicks were used for ProgressiveMauve alignment. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region. When the similarity plot points downward it indicates an alignment to the reverse strand of the genome of Ec-pST26 i.e., inversion. Segment highlighted with solid black rectangular box denotes the ~ 20kb region encoding antimicrobial resistance (AR), whereas blue horizontal box denotes region encoding transposons.



Extended Data Fig. 10. Exposing SH to metal chlorides, disinfecting compounds or neutral pH did not significantly change their metabolism. **a,b** Box plots comparing the metabolic activity of one evolved susceptible (no IncI1) and one multidrug (MDR carrying pST26) SH strain using phenotype microarray (PM) plates (Biolog Inc). Pre-configured PM plates are composed of microtiter plates with one negative control well and 95 wells pre-filled with or without nitrogen or amino acid nutrient source and at pH of 3.5 – 10 (n = 1 plate per strain; no of wells per plate per compound was 4) **c,** Concentration of evolved susceptible and MDR SH strains when exposed to pine shaving extract of pH 6.5. Each bar represents the average concentration (colony forming units per ml of pine shaving extract) of three individual population that was established from three single bacterial colonies from three different evolved strains. SH-Ancestor population was established from three single bacterial colonies of SH-2813nal^{R}. (Anc = Ancestor, Evol – Evolved i.e., SH isolate recovered during in vivo experiment, genR – population resistant to gentamicin). (Error bars = Standard deviation; $P > 0.05$ (Wilcoxon signed rank test and Kruskal-Wallis rank sum test).



Extended Data Fig. 11. IncI1 plasmids of multilocus sequence type 26 (pST26) are carried by strains of *Salmonella enterica* and *E. coli* isolated from animal and human sources. Maximum likelihood tree reconstructed using the complete DNA sequence of the fifty closest plasmids to pST26 found using NCBI BLASTn search. After multiple alignment was performed, duplicated plasmids (n =4) before ML tree were built. JC model of nucleotide substitution and GAMMA model of rate heterogeneity was used for sequence evolution prediction. Numbers shown next to the branches represent the percentage of replicate trees where associated isolates cluster together based on ~100 bootstrap replicates. Tree was rooted with p2ST26 plasmid from this study. Taxa in blue text are pST26 plasmids from this study. (Note. ST/CC- Multi locus sequence/clonal complex; ARG – Antibiotic resistance gene)



Extended Data Fig. 12. Minimum spanning tree (MST) of SH genomes from this study. The core genome multilocus sequence type (cgMLST) data generated by SISTR for 63 genomes from this study including SH-ancestor were used for MST construction. Colors were attributed to nodes according to the number cgMLST cluster levels found (I), antibiotic resistance profile (II), isolation source (III) and plasmid carried. (i) The susceptible SH-ancestor is part of cgMLST cluster 1 (n = 18 isolates). All nodes with distances equal or above 0 were linked. All links from the MST with a distance value above 4 were deleted.

Supplementary data

Supplementary data 1: Mutations found in *S*. Heidelberg isolates recovered from ceca and litter.

Supplementary data 2: Virulence genes present in SH-ancestor and the inverted region of the genomes of SH strains ic9b and og8-05a. The number of virulence genes found in each genome is written in the column headers.

Supplementary data 3: Alignment of IncI1-pST26 plasmids from this study using ProgressiveMauve. Complete plasmid DNA consensus sequence (n= 36) was determined by aligning whole genome FASTQ reads to a complete circular pST26 plasmids from this study. IncI1-pST26 (GenBank: CP016585) present in a *S*. Heidelberg strain available on NCBI was used as a reference genome for the alignment. Segments highlighted with dashed horizontal blue box denotes regions with relevant mutations.

Supplementary data 4: Contigs matching p2ST26 found in the cecal metagenome.

Supplementary data 5: Assembly report for bacterial genomes sequenced using short and long reads technology.

Supplementary data 1: Mutations found in S. Heidelberg isolates recovered from ceca and litter

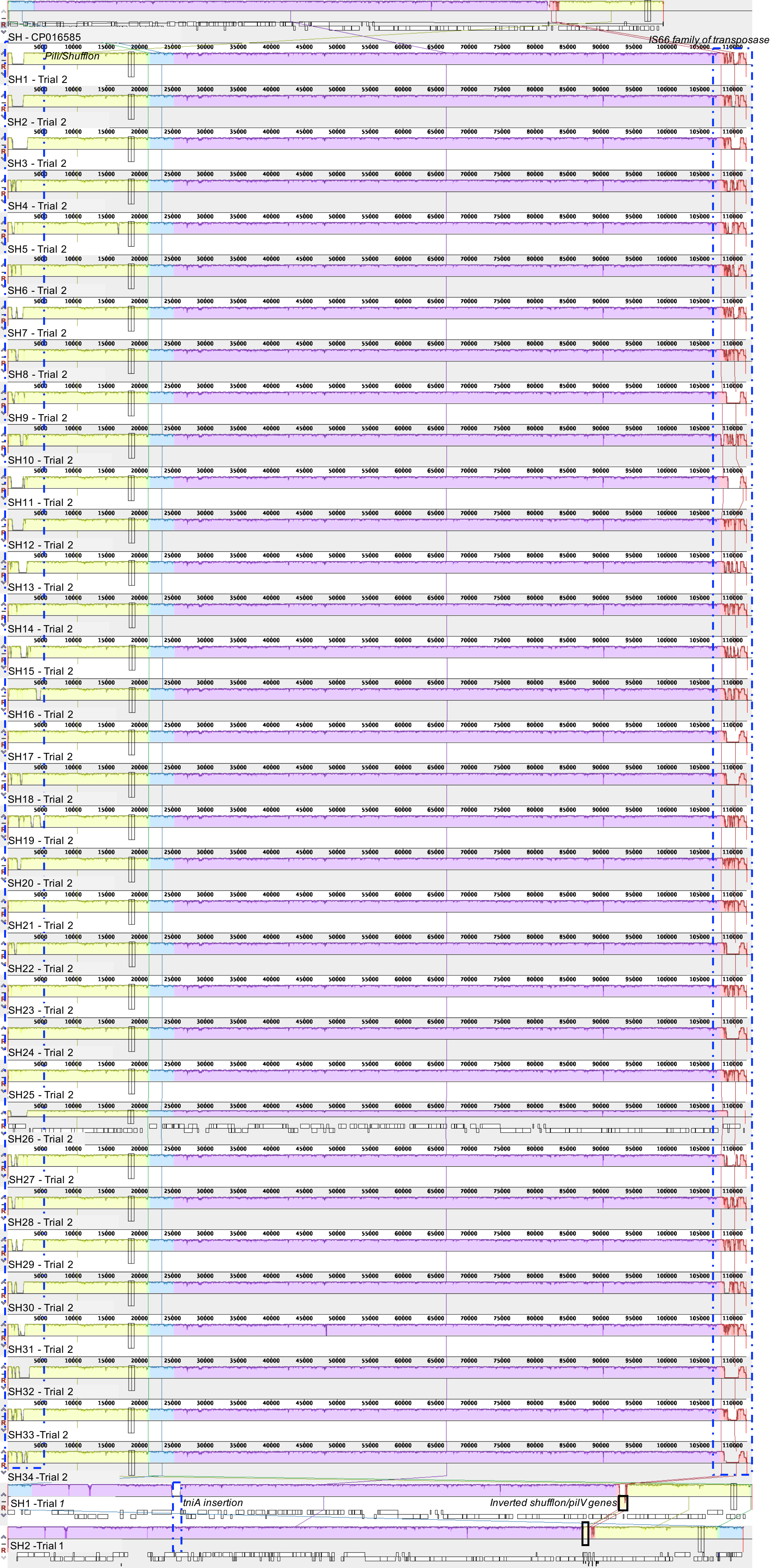
Loci*	Location†	Product	Mutation Change‡	Mutation type§	No of Isolates‖
95760	CDS	hypothetical protein	Pro23Thr	NS	1
130492	CDS	lipopolysaccharide 1,3-galactosyltransferase (WaaB)	Ile2Asn	NS	1
413914	CDS	putative surface-exposed virulence protein (BigA)	N/A	Ins	1
414022	CDS	putative surface-exposed virulence protein (BigA)	Val157Val	S	4
472700	CDS	16S ribosomal RNA (rRNA)	N/A	N/A	1
473535	Intergenic	upstream tRNA; downstream RNA	N/A	N/A	8
473536	Intergenic	upstream tRNA; downstream RNA	N/A	N/A	8
501858	CDS	hypothetical protein	N/A	Del	1
501860	CDS	hypothetical protein	N/A	Del	1
636934	CDS	hypothetical protein	N/A	Ins	2
693268	CDS	hypothetical protein (Kch)	Val58Met	NS	1
927855	CDS	putative regulatory protein (LysR)	Ile163Ile	S	1
1057273	CDS	N-acetylglucosaminyl transferase (MurG)	Ser322Ala	NS	1
1080335	CDS	aromatic amino acid transport protein (AroP)	Ser257Leu	NS	1
1080340	CDS	aromatic amino acid transport protein (AroP)	Leu255Leu	S	1
1208313	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	21 and SH-2813nal^{R}
1209197	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	14
1209203	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	13
1223192	Intergenic	upstream tRNA; downstream (TssA)	N/A	N/A	1
1339554	CDS	fimbriae usher protein (StbC)	N/A	Del	1
1351680	CDS	multidrug efflux system, outer membrane factor (OprM)	Thr120Pro	NS	1
1670308	CDS	altronate dehydratase (UxaA)	Leu21Leu	S	1
1744150	CDS	putative glycosyltransferase (WbbK)	Leu246Leu	S	1
1766719	Intergenic	upstream hypothetical protein; downstream hypothetical protein	N/A	N/A	1
2092586	Intergenic	upstream hypothetical protein; downstream hypothetical protein	N/A	N/A	1
2137198	CDS	glyoxylate/hydroxypyruvate reductase A (GhrA)	N/A	Del	1
2250686	CDS	putative outer membrane lipoprotein (RzoR)	Ser78Phe	NS	1
2260332	CDS	putative regulatory protein (CitB)	Arg110Trp	NS	1
2262081	Intergenic	upstream hypothetical protein; downstream hypothetical protein	N/A	N/A	1
2392728	Intergenic	upstream PykF; upstream SsaB	N/A	N/A	1
2415691	CDS	multidrug efflux transporter (MdtK/NorM)	Ala199Val	NS	1
2660396	Intergenic	downstream hypothetical protein; downstream YdbK	N/A	N/A	1
2961953	CDS	flagellin (FliC)	Gln129His	NS	70 and SH-2813nal^{R}
3289224	CDS	DNA gyrase subunit A (GyrA)	Ser83Tyr	NS	70 and SH-2813nal^{R}
3341410	CDS	NADH-ubiquinone oxidoreductase chain H (NuoH)	Val269Val	S	1
3356426	CDS	hexitol phosphatase (HxpA)	N/A	Del	1
3428428	Intergenic	upstream AlpA; downstream PgtE	N/A	Ins	1
3428439	Intergenic	upstream AlpA; downstream PgtE	N/A	Ins	1
3428448	Intergenic	upstream AlpA; downstream PgtE	N/A	Ins	1

3428454	Intergenic	upstream AlpA; downstream PgtE	N/A	N/A	1
3428456	Intergenic	upstream AlpA; downstream PgtE	N/A	Del	1
3428481	Intergenic	upstream AlpA; downstream PgtE	N/A	Del	19
3428490	Intergenic	upstream AlpA; downstream PgtE	N/A	N/A	19
3619800	CDS	transcriptional activator of cad operon (CadC)	N/A	Del	1
3672875	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672877	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672881	CDS	23S ribosomal RNA (rRNA)	N/A	Ins	2
3672883	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672885	CDS	23S ribosomal RNA (rRNA)	N/A	Del	2
3672893	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672899	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672900	CDS	23S ribosomal RNA (rRNA)	N/A	Ins	2
3672905	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672906	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672912	CDS	23S ribosomal RNA (rRNA)	N/A	Del	2
3672922	CDS	23S ribosomal RNA (rRNA)	N/A	Del	2
3672935	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672937	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672939	CDS	23S ribosomal RNA (rRNA)	N/A	Del	2
3672948	CDS	23S ribosomal RNA (rRNA)	N/A	Del	2
3672953	CDS	23S ribosomal RNA (rRNA)	N/A	Ins	2
3672956	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	2
3672958	CDS	23S ribosomal RNA (rRNA)	N/A	Ins	2
3672963	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	1
3672966	CDS	23S ribosomal RNA (rRNA)	N/A	Ins	1
3672969	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	1
3672970	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	1
3672973	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	1
3674946	CDS	16S ribosomal RNA (rRNA)	N/A	N/A	3
3674946	CDS	16S ribosomal RNA (rRNA)	N/A	N/A	4
3742216	CDS	putative inner membrane protein	Phe224Ser	NS	1
3744202	CDS	putative inner membrane protein	Glu267Glu	S	1
3930073	CDS	sulfite reductase [NADPH] hemoproteinbeta-component (CysI)	Ile352Ser	NS	70 and SH-2813nal^{R}
4002993	CDS	exodeoxyribonuclease V gamma chain (RecC)	Gln321Gln	S	1
4158109	CDS	hypothetical protein	Phe34Leu	NS	1
4251311	Intergenic	upstream YqjA; downstream SstT	N/A	Del	1
4369883	CDS	oxaloacetate decarboxylase Na (+) pump, alphachain (OadA)	Ala48Val	NS	1
4418343	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	1
4418421	Intergenic	upstream 23S ribosomal RNA; downstream tRNA	N/A	N/A	2
4418422	Intergenic	upstream 23S ribosomal RNA; downstream tRNA	N/A	N/A	2

4696844	CDS	23S ribosomal RNA (rRNA)	N/A	N/A	38 and SH-2813nal^{R}
4698491	CDS	upstream 23S ribosomal RNA; downstream tRNA	N/A	N/A	68 and SH-2813nal^{R}
4699877	CDS	16S ribosomal RNA (rRNA)	N/A	N/A	2

***** SH-ancestor was used as the reference genome (4,788,855 bp).
† CDS- Coding DNA sequence
‡ N/A – Not applicable
§ S – synonymous, NS – non-synonymous, Ins – insertion, Del – deletion, N/A – Not applicable
‖The number of evolved SH isolates carrying the same mutation. Six mutations are present in SH-2813nal^{R} but absent in SH-ancestor.

Virulence Factor Class	Virulence Factors	Related Genes	Ancestral=168	Ic9B=58	Og8-05a=102
Fimbrial Adherence Determinants	Agf/Csg	*csgA*			
		csgB			
		csgC			
		csgD			
		csgE			
		csgF			
		csgG			
	Bcf	*bcfA*			
		bcfB			
		bcfC			
		bcfD			
		bcfE			
		bcfF			
		bcfG			
	Fim	*fimA*			
		fimC			
		fimD			
		fimF			
		fimH			
		fimI			
		fimW			
		fimY			
		fimZ			
	Lpf	*lpfA*			
		lpfB			
		lpfC			
		lpfD			
		lpfE			
	Stb	*stbA*			
		stbB			
		stbC			
		stbD			
		stbE			
	Stc	*stcA*			
		stcB			
		stcC			
		stcD			
	Std	*stdA*			
		stdB			
		stdC			
	Ste	*steA*			
		steB			
		steC			
		steD			
		steE			
		steF			
	Stf	*stfA*			
		stfC			
		stfD			
		stfE			
		stfF			
		stfG			
	Sth	*sthA*			
		sthB			
		sthC			
		sthD			
		sthE			
	Sti	*stiA*			
		stiB			
		stiC			
		stiH			
	Stj	*Undetermined*			
		Undetermined			
		Undetermined			
		stjB			
		stjC			
	Stk	*stkA*			
		stkB			
		stkC			
		stkD			
		stkE			
		stkF			
		stkG			
	Tcf	*tcfA*			
		tcfB			
		tcfC			
		tcfD			
Macrophage Inducible	Mig-14	*mig-14*			
Magnesium Uptake	Mg2+ transport	*mgtB*			
		mgtC			
Nonfimbrial Adherence Determinants	MisL	*misL*			
	RatB	*ratB*			
	ShdA	*shdA*			
	SinH	*sinH*			
Regulation	PhoPQ	*phoP*			
		phoQ			
Secretion System	TTSS (SPI-1 encode)	*hilA*			
		hilC			
		hilD			
		iacP			
		iagB			
		invA			
		invB			
		invC			
		invE			
		invF			
		invG			
		invH			
		invI			
		invJ			
		orgA			
		orgB			
		orgC			
		prgH			
		prgI			
		prgJ			
		prgK			
		sicA			
		sicP			
		sipD			
		spaO			
		spaP			
		spaQ			
		spaR			
		spaS			
		sprB			
	TTSS (SPI-2 encode)	*ssaC*			
		ssaD			
		ssaE			
		ssaG			
		ssaH			
		ssaI			
		ssaJ			
		ssaK			
		ssaL			
		ssaM			
		ssaN			
		ssaO			
		ssaP			
		ssaQ			
		ssaR			
		ssaS			
		ssaT			
		ssaU			
		ssaV			
		sscA			
		sscB			
		sseA			
		sseB			
		sseC			
		sseD			
		sseE			
		ssrA			
		ssrB			
	TTSS effectors translocated via both s	*slrP*			
	TTSS-1 translocated effectors	*avrA*			
		sipA			
		sipB			
		sipC			
		sopA			
		sopB/sigD			
		sopD			
		sopE2			
		sopE			
		spiP			
	TTSS-2 translocated effectors	*pipB2*			
		pipB			
		sifA			
		sifB			
		sopD2			
		spiC/ssaB			
		sseF			
		sseG			
		sseJ			
		sseK2			
		sseL			
		sspH2			
Stress Adaptation	SodCI	*sodCI*			


SH - CP016585
IS66 family of transposase
Pili/Shufflon
SH1 - Trial 2
SH2 - Trial 2
SH3 - Trial 2
SH4 - Trial 2
SH5 - Trial 2
SH6 - Trial 2
SH7 - Trial 2
SH8 - Trial 2
SH9 - Trial 2
SH10 - Trial 2
SH11 - Trial 2
SH12 - Trial 2
SH13 - Trial 2
SH14 - Trial 2
SH15 - Trial 2
SH16 - Trial 2
SH17 - Trial 2
SH18 - Trial 2
SH19 - Trial 2
SH20 - Trial 2
SH21 - Trial 2
SH22 - Trial 2
SH23 - Trial 2
SH24 - Trial 2
SH25 - Trial 2
SH26 - Trial 2
SH27 - Trial 2
SH28 - Trial 2
SH29 - Trial 2
SH30 - Trial 2
SH31 - Trial 2
SH32 - Trial 2
SH33 -Trial 2
SH34 -Trial 2
SH1 -Trial 1
tniA insertion
Inverted shufflon/pilV genes
SH2 -Trial 1

Supplementary data 4: Contigs matching p2ST26 found in the cecal metagenome

Protein ID	IC-FL2	IC-FL1	Number of contigs*
YhdJ	no	yes	1
AadA	no	yes	1
Aminoglycoside N(3)-acetyltransferase	no	yes	1
KlcA	no	yes	2
ParA	no	yes	1
Conjugal transfer protein	no	yes	3
Doc toxin	no	yes	3
Eaa	no	yes	1
Hypothetical	yes	yes	9
Hypothetical	no	yes	1
Hypothetical	no	yes	1
Hypothetical	no	yes	1
Hypothetical	yes	yes	6
Hypothetical	no	yes	3
Hypothetical	no	yes	1
Hypothetical	no	yes	2
Hypothetical	no	yes	1
DotA/TraY	no	yes	2
Replication initiation protein	yes	yes	2
DNA primase	no	yes	4
Hypothetical protein	no	yes	20
Pili assembly chaperone	no	yes	3
ParB/RepB/Spo0j	yes	yes	3
YubH	no	yes	1
Hypothetical	no	yes	2
Hypothetical	no	yes	2
Hypothetical	no	yes	1
Hypothetical	no	yes	1
MobC	no	yes	2
plasmid partitioning/stability family protein	no	yes	1
TraF	no	yes	2
TraE	no	yes	1
ArdA	no	yes	1
Rpn	yes	yes	4
3'-5' exonuclease	no	yes	1
ProQ/FINO	no	yes	1
Colicin	no	yes	1
Colicin transporter	no	yes	1
GroES	yes	yes	8
TraX	no	yes	1
TraQ	no	yes	1
TraP	no	yes	1
TraO	no	yes	1
TraN	no	yes	1
DotI/IcmL/TraM	no	yes	2
PilQ	yes	yes	2
PilR	no	yes	1
PilN	no	yes	2
TraB	no	yes	1
PilV	no	yes	2
PilS	no	yes	1
PilI	no	yes	2
PilK	no	yes	3
PilL	no	yes	1
PilM	no	yes	1
TraA	no	yes	1

TraC	no	yes	2
TraG	no	yes	1
TraH	no	yes	1
TraI	no	yes	1
TraJ	no	yes	2
TraR	no	yes	1
TraS	no	yes	2
TraU	no	yes	2
TraW	no	yes	1
PilT	no	yes	1
PilP	no	yes	1
Integron integrase IntI1	no	yes	1
Mercuric ion reductase	yes	yes	2
MerR	no	yes	4
MerD	yes	yes	48
21PP	no	yes	1
MerT	no	yes	1
TnAs3	yes	yes	3
IS66 transposase	yes	yes	11
IS66 insertion sequence element	yes	yes	14
Helix-turn-helix domain-containing protein	yes	yes	3
Mobile element protein*	yes	no	2
MerP	yes	yes	2
DMT	no	yes	1
Plasmid conjugative transfer endonuclease	no	yes	1
PsiA	no	yes	2
PsiB	yes	yes	2
Phd	no	yes	2
StbA	no	yes	1
Relaxase/helicase	no	yes	1
Relaxase	no	yes	2
Relaxasome component	no	yes	1
Resolvase	yes	yes	3
Shufflon-specific DNA recombinase	no	yes	2
Single-stranded DNA-binding protein	yes	yes	4
Surface exclusion protein	no	yes	2
TetR	no	yes	1
TetA	no	yes	1
Urf2	no	yes	1
TniA	no	yes	2
InsC	yes	yes	3
InsD	yes	yes	4
Type-IV secretion leader peptidase/N-methyltransferase	no	yes	1
YkfF	yes	yes	2
YadA	no	yes	3
YDFB	no	yes	1

*Total number of contigs found encoding the gene in the cecal metagenome samples