Title: ColE1 plasmids potentiate the fitness of *Salmonella* Heidelberg in poultry litter

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Heidelberg (*S*. Heidelberg) is a clinically-important serovar linked to food-borne illness, and commonly isolated from poultry. Investigations of a large, multistate outbreak in the USA in 2013 identified poultry litter as an important extra-intestinal environment that may have selected for specific *S*. Heidelberg strains. Poultry litter (PL) is a mixture of bedding materials and chicken excreta that contains chicken gastrointestinal (GI) bacteria, undigested feed, feathers, and other materials of host origin. In this study, we performed a series of controlled laboratory experiments which assessed the evolution of two *S*. Heidelberg strains (SH-2813 and SH-116) in PL previously used to raise 3 flocks of broiler chickens. The strains are closely related at the chromosome level, differing by only 69 single-nucleotide variants. Whole genome sequencing was performed on 86 isolates recovered after 0, 1, 7 and 14 days of evolution in PL. Only strains carrying an IncX1 (37kb), 2 ColE1 (4 and 6kb) and 1 ColpVC (2kb) plasmids survived more than 7 days in PL. Competition experiments showed that carriage of these plasmids was associated with increased overall fitness. This increased fitness was associated with an increased copy number of IncX1 and ColE1 plasmids. Further, all Col plasmid-bearing strains had similar mutations in 102 genes encoded on the chromosome and in 3 genes encoded on the IncX1 plasmid. Additionally, we observed a decrease in susceptibility to tobramycin, kanamycin, gentamicin, neomycin and fosfomycin for Col plasmid-bearing strains. Our study demonstrates how acquisition of multiple Col-like plasmids can change the evolutionary path of *S*. Heidelberg in poultry litter.

INTRODUCTION

The United States (U.S.) is the world's largest producer of poultry with over 8 billion broilers produced yearly (1). Maintaining a safe supply of poultry is increasingly important as demand

increases, production becomes more intensive and facilities become antibiotic free. Broilers (around 22,000 broilers per house) are typically raised in open plan barns on a deep floor of bedding materials (typically wood shavings, rice hulls or saw dust (1, 2). Poultry litter (PL) is a mixture of bedding materials and chicken excreta that contains chicken gastrointestinal (GI) bacteria, undigested feed, uric acid, feathers and other materials of host origin (3-6). As the cost of bedding materials increase, land nutrient management requirements become more stringent, and broiler production and turnover times decrease, farmers have chosen to increase the number of flocks raised on litter before cleanout. This practice is accepted in production environments, but the effect of litter reuse on microbial populations has not been well studied.

Available data suggest that litter re-use can result in significant changes in the microbiological, chemical and physical composition of litter (3, 7, 8). In addition, reused litter has been found to harbor fewer yeasts, molds, coliform bacteria and *Salmonella* compared to fresh litter (9, 10). However, fecal shedding of food borne pathogens such as *Salmonella* into poultry by colonized hosts can also be a source of new infection or re-inoculation. For instance, *Salmonella* in 131-day-old litter was shown to colonize 82% of day-old chicks after placement for 32 days (11) and *Salmonella enterica* subsp. *enterica* serovar Heidelberg (*S*. Heidelberg) has been shown to persist in recycled litter, even after litter treatment (12). *Salmonella* Heidelberg is a clinically-important serovar, linked to food-borne illness and commonly isolated from poultry. After *S*. Typhimurium, *S*. Heidelberg is the serovar of *Salmonella* most often associated with *Salmonella*-related deaths in the United States (13). In 2013, *S*. Heidelberg was responsible for a large multistate foodborne outbreak (634 cases) linked to consumption of chicken (14). Epidemiological and environmental investigations identified PL as an important extra-intestinal environment that may have selected for specific *S*. Heidelberg strains (14).

Salmonella Heidelberg is highly clonal and cannot be subtyped by conventional pulsed-field gel electrophoresis (PFGE) which poses an inherent difficulty when differentiating strains responsible for foodborne outbreaks from those associated with background sporadic cases (15-17). High quality core genome single nucleotide variant (hqSNV) analysis on whole genome DNA sequences (WGS) of *S*. Heidelberg has been recommended as a better approach to distinguish outbreak strains (15). However, core genome SNV is non-discriminatory between isolates harboring horizontally acquired antibiotic resistance genes (ARG) versus susceptible ones. Multidrug resistant *S*. Heidelberg isolates tend to cluster with susceptible isolates during outbreaks and differ by only 0 - 4 SNVs (16, 17). In a study by Edirmanasinghe et al. (17), the authors reported 65 out of 113 sequenced *S*. Heidelberg encoded the AmpC beta-lactamase (bla_{CMY-2}) gene on an incompatibility group I1 plasmid (IncI1) and were resistant to important 3rd generation cephalosporins. On the other hand, susceptible isolates (n=38) carried multiple variants of Col plasmids and encoded no known ARG. The function of these Col plasmids is not clear but they seem to be mobilized by co-resident conjugal plasmids, such as IncI1 and IncX, two plasmids frequently found among *S*. Heidelberg isolates (16-19).

The majority of *S*. Heidelberg isolates sequenced and available in the National Center for Biotechnology Information (NCBI) database were recovered from either food or clinical samples (18, 20-22), leaving a knowledge gap in our understanding of strains of environmental origin. The poultry litter microbiome may carry mobile genetic elements (MGE) including plasmids that can be horizontally transferred to *S*. Heidelberg upon introduction into PL. These newly acquired MGE may present a fitness advantage to permissive recipients, further enabling the persistence of *S*. Heidelberg in poultry environments.

In this study, we investigated the role of PL on the fitness of two *S*. Heidelberg strains with indistinguishable PFGE patterns, both recovered from chicken carcass in two states in USA. Both strains harbor an IncX1 plasmid (37 kb) but differ in the occurrence of Col plasmids. We performed a series of controlled laboratory experiments to assess the evolution of these strains in PL that was previously used to raise three flocks of broiler chickens. We demonstrate that the acquisition of multiple ColE1 plasmids in tandem with mutations in host chromosome and the IncX1 plasmid contributed to the persistence of *S.* Heidelberg in PL. Additionally, this improved fitness was associated with a decreased susceptibility to tobramycin, kanamycin, neomycin and fosfomycin. This study elucidates for the first time how acquisition of multiple Col plasmids from PL alters the evolutionary trajectory of *Salmonella* Heidelberg.

MATERIALS AND METHODS

Poultry Litter for microcosm evolution studies. Poultry litter (PL) for microcosm studies was provided by the University of Georgia poultry research center in Athens, GA, USA. The PL was previously used to raise 3 flocks of broiler chickens under simulated commercial poultry production conditions. The litter was collected ca. 2 weeks after the third flock of birds were removed. The PL bedding material was composed of pine shavings.

Bacterial strains and preconditioning. *S.* Heidelberg strains (SH-2813 and SH-116) used in this study were originally recovered from broiler chicken carcass rinsates. The genotypic/phenotypic characteristics of these strains are provided in Table 1. The strains were preconditioned in poultry litter extract (PLE) media prepared as described in supplementary material or in Brain Heart Infusion broth (BHIB; BD Difco, Sparks, MD) before inoculation into PL. For each strain, 5 single colonies from Sheep Blood Agar (SBA) (Remel Inc, San Diego, CA) were transferred to 10 ml of PLE and BHIB, and incubated at 37 ^{o}C for 24 h. Serial batch

culturing was carried out for ca. 50 generations in PLE and BHIB by transferring 100 µl of culture to 10 ml of fresh PLE and BHIB (100-fold dilution) to yield ca. 10 gen. d^{-1}. After 5 days, the population was used as inoculum for evolution experiments in PL microcosms.

Experimental evolution in PL. Poultry litter was collected in a 5-gallon bucket and thoroughly mixed before 30 g aliquots were placed into 48 250 ml Nalgene wide jars and 1X PBS (3-4 ml) was added to achieve 30 % moisture. Microcosms were incubated for 72 h at 37°C before being individually inoculated with ca. 10^6 colonies forming units (CFU) of either SH-2813 or SH-116 preconditioned previously in PLE or BHIB. These 4 groups/treatments were as follows: SH-2813 + BHIB, SH-2813 + PLE, SH-116 +BHIB and SH-116 +PLE. Inoculated microcosms were thoroughly mixed and then incubated for 14 days at 37 ^{o}C (Supplementary Fig. 1). The control microcosm remained un-inoculated. Three microcosms per group were destructively sampled at days 0, 1, 7 and 14.

Poultry litter microcosm sampling. Poultry litter samples (25 g) were mixed with 150 ml 1X PBS and 5-10 ml glass beads (Fisher Sci: S800242) then homogenized for 10 min at 450 rpm in a hand wrist shaker (Boekel Scientific, Feasterville, PA). The PL mixtures were allowed to settle for 5 min to allow settling of large debris/pine shavings and then passed through cheesecloth and hand pressed to recover eluates which were used for downstream microbiological and chemical analysis. The moisture content of each PL sample was determined gravimetrically by drying ca. 1 g of PL at 107 °C for 24 h.

Microbiological methods. Recovered PL eluate was serially diluted and plated onto XLT-4 and Brilliant Green Sulfa (BGS). For enrichment and recovery of injured/viable *Salmonella* spp., one ml eluate was transferred to 20 ml of 1 % Buffered Peptone Water (BPW) and incubated 24 h before being plated onto XLT-4 agar. All agar plates and broth tubes were incubated at 37 ^{o}C for

24 – 48 h before colony forming units were enumerated. Reported counts were log-normalized and reported per gram PL dry weight. At least three single colonies representing three unique clones of *Salmonella* from each microcosm were randomly chosen from XLT-4 plate dilutions and archived in 30 % Luria Bertani (LB) glycerol at -80 ^{o}C.

DNA sequencing and Bioinformatics. We sequenced 86 evolved *S*. Heidelberg isolates encompassing clones recovered from the four treatments. Isolates were selected randomly from the highest and lowest dilution of XLT-4 plates; and when populations decreased below LOQ, isolates were selected after 24 h enrichment in BPW.

Genomic DNA (gDNA) was extracted from pure isolate cultures using FastDNA Spin Kit (Mp Biomedicals, Solon, Ohio) and quantified with a Qubit Fluorometer (ThermoFisher Scientific). Whole genome sequencing (WGS) libraries were prepared using MiSeq Nextera XT library preparation kit (Illumina, Inc., San Diego, CA). Sequencing was performed on the Illumina MiSeq platform with 250-bp paired end reads using the MiSeq reagent V2 (500 cycles). All isolates were sequenced for an average coverage of 100X. Sequence reads were assembled de novo into contigs using SPAdes (23). The quality of assembled genomes was accessed using Quality Assessment Tool for Genome Assemblies (QUAST). (For detailed information on assembly, coverage and quality of sequenced genomes see supplementary information).

The original strains of SH-2813 and SH-116 are referred to as ancestral strains, while isolates from PL evolution experiments are referred to as evolved strains. Assembled contigs were submitted to the Center for Genomic Epidemiology's multilocus sequence type (MLST) and PlasmidFinder modules to determine the whole-genome sequence types (ST) and existing plasmid replicon types. Prophages were identified using PHAST (24) and sequences for intact phages were extracted, and annotated with Prokka (25). The Comprehensive Antibiotic

Resistance Database (CARD) was used for the identification of resistance genes present on plasmid and chromosome (26).

Plasmids were classified into MOB families using methods described previously (27). To investigate the topology of contigs identified with plasmid replicon, we used a custom script to inspect the path in the SPAdes FASTG assembly graph file that corresponded to the plasmid contig identified in the FASTA file; the contigs.paths file was used to determine correspondence between FASTA contigs and FASTG edges. Plasmid contigs were assigned as 'complete circular'; 'complete non-circular', i.e. linear but not connected to other contigs; and 'non-complete', i.e. linear and connected to other contigs. (see Supplementary Methods for detailed information). Complete circular plasmids were annotated using Prokka (25) and mapped with Snapgene v 4.1.2. When coding DNA sequence (CDS) was annotated as hypothetical/uncharacterized, we performed a PSI-BLAST search with predicted protein sequences run to ≤3 iterations. Plasmids were analyzed by comparative analysis using ProgressiveMAUVE (28) and CDS predicted in regions with marked differences were aligned with MUSCLE (29). Phylogenetic tree was produced from aligned conserved relaxase or mobilization protein sequences using the maximum likelihood method implemented in RAxML v 8 (30) - with a rapid bootstrapping run to generate 1000 replicates.

Single Nucleotide Variants (SNVs) were determined by mapping raw sequence reads to the chromosome and plasmid genome of *S*. Heidelberg strain AMR588-04-00435 available in NCBI under accession numbers CP016573 and CP016575, respectively, using Burrows-Wheeler Aligner (BWA) (31). SAM file sorting, and removal of PCR duplicates was done using SAMtools (32). Genome Analysis ToolKit (33) with a minimum mapping quality of 30 and a

minimum base quality of 30 was used for SNV calling. A SNV based phylogenetic tree was generated using the maximum likelihood method implemented in RAxML v 8.

Growth Assay, plasmid copy number determination and gene expression. Plasmid copy number (PCN) and differential expression of selected transcripts was determined by performing a growth assay on ancestral and evolved strains. Three populations were initiated from three single colonies from strains SH-2813$_{anc}$, SH-2813$_{evol}$, SH-116$_{anc}$, and SH-116$_{evol}$. An individual colony was transferred to 20 ml of Mueller Hinton Broth (MHB) in 50 ml centrifuge tube and incubated overnight at 37 ^{o}C. Following overnight growth, 6 technical replicate populations (18 total per strain) were established from each culture by transferring 3 ml to 50 ml centrifuge tubes containing 17 ml MHB. Cultures were incubated without shaking at 37 ^{o}C for 24 h. Optical density (OD_{600nm}) was periodically measured in the 3 populations with a Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific). Bacteria concentration was determined for 2 populations at 0.5, 4 and 24 h via serial dilution plating on R2A agar. Additionally, bacteria culture (ca. 20 ml) was filtered onto 0.2 µm pore size polycarbonate filters (MilliporeSigma, Burlington, MA) which were transferred to Lysing Matrix E and B tubes (MP Biomedicals, Solon, Ohio) for genomic DNA and RNA isolation respectively; and saved at 80 ^{o}C until use. For a complete description of PCN and gene expression determination, see Supplementary Methods.

Competitive fitness and conjugation assay. The fitness of three strains carrying the 3 Col plasmids (SH-2813$_{evol}$, SH-116$_{anc}$, and SH-116$_{evol}$) was determined relative to SH-2813$_{nal}$ nalidixic resistance with no Col plasmid. The SH-2813$_{nal}$ strain is isogenic to SH-2813$_{anc}$ but was made resistant to 200 ppm nalidixic acid by spontaneous mutation in *gyrA*. Five colonies from SBA for each strain were incubated in 9 ml of fresh MHB at 37 ^{o}C for 20 h. Thereafter, cultures

of the strains were mixed at a ratio of 1:1 (~500 µl of strain under study to 500 µl of SH-2813$_{nal)}$. We assumed that each strain had the same initial concentration because 24 h population were not significantly different (Table 2). Mixtures were diluted 10,000-fold in 50 ml centrifuge tubes (4 technical replicates per mixture) containing 20 ml of MHB and competed for 24 h at 37 ^{o}C without shaking. The final population was measured by serial plating onto R2A agar supplemented with and without 32 ppm of nal. The fitness of the strains carrying Col plasmids relative to the SH-2813-nal strain was determined as described by ref (34). For details see Supplementary Methods.

To pool the information from the competition assay with conjugation assay we employed the same data set to determine the conjugation rate constant for the three Col plasmid types present during the competition assay. Conjugation rate constants were determined as described by Loftie-Eaton et al (35). For details see Supplementary Methods.

Antibiotic Susceptibility Testing. We performed antimicrobial susceptibility testing (AST) on selected evolved strains following National Antimicrobial Resistance Monitoring System (NARMS) protocols for Gram-Negative bacteria (36) and Kirby-Bauer disc diffusion assay (37). For more details on the disc diffusion protocol used, see Supplementary Methods.

For categorizing susceptibility, breakpoints were adopted from NARMs. There is no standardization by Clinical and Laboratory Standards Institute (38) for tobramycin, kanamycin, neomycin, netilmicin, and fosfomycin by disc diffusion for *Salmonella* Heidelberg. Therefore, results were interpreted with diameter difference in zone of inhibition between isolates.

Plasmid-specific genes prevalence in poultry litter. To assess the prevalence of Col plasmids in PL, we determined the concentration of plasmid-specific genes in various litter collected for another on-going experiment in our laboratory. These litter were sourced from 7 poultry farms in

4 states in USA, practicing either conventional or backyard method of raising chicken flocks. DNA was extracted in triplicates from each PL as described previously for PL DNA extraction. We performed qPCR on 2 µl of purified DNA extract as described previously in qPCR assays, with primer sets specific to each plasmid of interest (Supplementary Table 9).

Statistical analysis. Analyses were performed using R (version 3.4.1). Survival difference between strains preconditioned in PLE versus BHIB was done using a profile analysis followed by a multivariate analysis of variance (MANOVA). Comparisons among strains or treatment were performed using Wilcoxon signed-rank Test. A multiple linear regression was used to model the relationship between IncX1 and Col plasmid copy numbers derived from whole genome sequencing coverage.

Ethics statement. The authors did not physically interact with chickens raised on the poultry litter used in this study, therefore, we were exempt from university guidelines and USDA/NIH regulations regarding animal use. Institutional Animal Care and Use Committee (IACUC) - UGA Office of Animal Care and Use.

Data availability. All sequencing data are available under NCBI Sequence Read Archive (SRA) BioProject: PRJNA448609

RESULTS

Experimental evolution system. The survival of two *S*. Heidelberg strains (SH-2813 and SH-116) in previously used PL was evaluated over a period of 14 days. The strains which were recovered from chicken carcass from two states in the USA were closely related at the chromosomal level, differing by only 69 single-nucleotide variants (SNVs). In addition, both strains carried the IncX1 plasmid and SH-116 carried 2 ColE1-like plasmids (4 and 6 kb) and a cryptic ColpVC plasmid (2 kb) (Table 1).

Table 1. Characteristics of the strains used in this study

Strain ID	Serotype	Plasmid Profile (size)	*Resistance profile	#Antimicrobial resistance genes	Unique Single Nucleotide Variants (SNV)
SH-2813	ST15	IncX1 (37kb)	Ery, Tyl,	AAC (6')-Iy, FosA7	69
SH-116	ST15	IncX1 (37 kb), ColE1(6 kb) ColE1 (4 kb) ColpVC (2 kb)	Ery, Tyl, Pen	AAC (6')-Iy, FosA7	49

Note. - Ery = Erythromycin; Pen = Penicillin; Tyl= Tylosin

***** Susceptible to all 14 antibiotics on the NARMs Gram-negative AST panel

Present on chromosome

The concentration of the two *S*. Heidelberg strains preconditioned in either PLE or BHIB decreased by several orders of magnitude in all microcosms and their survival dynamics differed by preconditioning media and strain-type (Fig. 1a). **Figure 1. Survival curve and mutation based phylogeny of evolved *S*. Heidelberg strains**. (**a**) *S*. Heidelberg concentration from poultry litter microcosms reported in colony forming units (CFU) and normalized by litter dry weight. (**b** and **c**) Top: Single nucleotide variants (SNV) based phylogenetic tree for SH-2813 and SH-116 evolved strains, Bottom: Corresponding Venn diagram comparing number of SNV shared across time points. Clades highlighted in blue shadows denotes clones carrying 3 Col plasmids. Node tips highlighted in green rectangular boxes represents isolates recovered following overnight enrichment in buffered peptone water. Trees were rooted with SH-2813 and SH-116 ancestors. (d) Venn diagram comparing SNV shared between Col plasmid-carrying SH-2813 and SH-116 evolved strains. The concentration of SH-2813-PLE, decreased by 4-log within 1 day of inoculation into PL but rebounded 1.5-log after 7 days. In contrast, the concentration of

SH-2813-BHIB was below the limit of quantification (LOQ) within 1 day of inoculation into PL and increased ca. 4-log by day 7. The final concentration of SH-2813-PLE and SH-2813-BHIB in PL was log 2.5 ± 0.03 CFU g^{-1}. On the other hand, the concentration of SH-116-PLE decreased gradually for 14 days while SH-116-BHIB was below LOQ within 1 day of inoculation into PL and increased by ca. 3-log at day 7. The final concentration for SH-116 was below our LOQ for direct culture counts but the strain was isolated at varying levels following enrichment in buffered peptone water (BPW) (Supplementary Table 1).

A profile analysis followed by MANOVA on the observed bacteria concentration revealed that preconditioning had a significant effect on SH-2813 concentrations at day 0 and day 1 (*p*-value< 0.004). For SH-116, this effect was only significant at day 1 (*p*-value< 0.0001). We also monitored the pH, moisture, nutrients, cations and resident aerobic bacteria present in PL during the 14-day experiment, however we could not draw significant conclusions from analyzed results (Supplementary Table 1 and 2)

IncX1 and Col plasmids are associated with *S*. Heidelberg persistence in PL. To investigate the genetics of *S*. Heidelberg persistence in PL, we isolated and performed WGS on 87 isolates obtained after 0, 1, 7 and 14 days in PL (Supplementary Table 3). All SH-2813_{evol} strains (n= 27) obtained from day 7 and 14 microcosms carried 3 Col plasmids in addition to an IncX1 plasmid, and differed from day 0 and 1 isolates by 76 -79 unique SNV on the chromosome (Fig. 1b). It is noteworthy that SH-2813 isolates recovered following enrichment in BPW (n = 6) were the only day 1 strains carrying Col plasmids. For SH-116, IncX1 and Col plasmids were present in all sequenced strains (n = 38) regardless of time of incubation in PL and isolates differed by only 0 - 4 unique SNV on the chromosome (Fig. 1c). The majority (276/306) of the plasmid contigs represented complete circular plasmids (Supplementary Table 4). The IncX1 belonged to the

MOB_Q family of relaxases and was present in all sequenced genomes. The ColE1 plasmids taken up by SH-2813_{evol} belonged to the MOBp family and showed a conserved size of 6189 (ColE1-6kb) and 4784 (ColE1-4kb) bp respectively. The cryptic ColpVC was 2223 bp (ColpVC-2kb). For all complete IncX1 plasmids, there was a 21-base pair (bp) difference in strains carrying the Col plasmids (37, 824 bp) compared to strains with no Col plasmids (37, 845 bp).

Direct-repeat deletion is associated with IncX1 copy number increase. To determine the reason for the 21-bp size difference in the IncX1 plasmids present in *S*. Heidelberg populations carrying Col plasmids, all complete circular IncX1 plasmid sequences were subjected to whole genome sequence alignment and SNV identification. The 22-bp direct-repeat deletion was located at the origin of replication (*ori*) adjacent to the replication protein (RepB) and an additional 1-bp (G-> GA) insertion occurred in the type IV secretion protein (T4SS) VirB8 (Fig. 2a and Supplementary Table 5). **Figure 2. Deletion of 22-bp iteron of IncX1 reduces *rep* expression**. (**a**) Annotated IncX1 plasmid map of SH-2813_{anc} and SH-116_{anc}. Regions that acquired mutations in evolved strains carrying Col plasmids are highlighted in rectangular boxes (**b**) Multiple alignment of the IncX1 iteron-based direct repeat (DR) origin of selected strains from this study, and 22 *S*. Heidelberg genomes available on NCBI. (**c**) Differences in expression of IncX1 genes in evolved SH-2813 and SH-116 compared to SH-2813_{anc} and SH-116_{anc} (log_2 scale, n = 3, **p*.value =0.086 ;Wilcoxon signed-rank Test). The error bars represent the standard error of the mean.

The 22-bp deletion reduced the number of direct repeats from 3 to 2, making it a truncated *ori* (Fig. 2b). Direct repeat iterons of theta plasmids are known to bind Rep proteins and have been shown to participate in negative control of their initiation (39). Deleting or adding more copies of the iteron have been shown to increase or decrease plasmid copy number (PCN) (40).

Therefore, we hypothesized that the direct repeat deletion in IncX1 of SH-2813$_{evol}$ would result in higher copies compared to SH-2813$_{anc}$ since the 22-bp is absent in the ancestral strain while no change would be expected in IncX1 copies for SH-116$_{evol}$. To test this hypothesis, we determined the copy numbers of IncX1 and Col plasmids present in ancestor (SH-2813$_{anc}$ and SH-116$_{anc}$) and 2 evolved strains (SH-2813$_{evol}$ and SH-116$_{evol}$ respectively). We assumed that these evolved strains represent the "fittest" SH-2813 and SH-116 strains given their persistence in PL microcosms for 14 days.

As expected, the copy number of IncX1 for SH-2813$_{evol}$ (~7 copies/cell; s.d.=1.8) was higher than that of SH-2813$_{anc}$ (~4 copies/cell; s.d.=0.3) at 0.5 h (*p*. value = 0.086; Wilcoxon rank-signed test). In contrast, SH-116 did not exhibit any significant difference in IncX1 copies between SH-116$_{anc}$ (~5 copies/cell; s.d.=1.7) and SH-116$_{evol}$ (~6 copies/cell; s.d.=0.8) (*p*. value = 0.773) (Table 2).

Table 2. Plasmid copy numbers of ancestral and evolved strains.

				Plasmid Copy Number/Cell (S.D)			
Strain	Time (h)	Optical Density (S.D)	Bacteria concentration (Log CFU/ml)	IncX1	ColE1-6kb	ColE1-4kb	ColpVC
SH-2813$_{anc}$	0.5	0.015±0.00	7.98±0.0	4.17±0.3	N/A	N/A	N/A
	4	0.040±0.00	8.60±0.2	0.47±0.5	N/A	N/A	N/A
	24	0.070±0.00	8.90±0.1	0.97±0.9	N/A	N/A	N/A
SH-2813$_{evol}$	0.5	0.016±0.00	7.94±0.0	6.92±1.8	10.71±4	12.4±5.5	110.77±45
	4	0.056±0.00	11.16±2.1	0.59±0.3	3.28±0.6	2.77±1.0	86.21±25
	24	0.080±0.00	8.57±0.0	0.64±0.1	5.25±0.7	4.2±0.6	115.32±70
SH-116$_{anc}$	0.5	0.014±0.00	7.83±0.1	4.55±1.7	0.27±0.3	2.54±1.9	82.39±8
	4	0.037±0.00	8.56±0.1	0.80±1.3	0.14±0.0	0.54±0.7	54.01±26

	24	0.093±0.00	9.07±0.0	2.33±9.3	2.23±0.0	0.92±1.3	39.94±28
SH-116$_{evol}$	0.5	0.017±0.00	7.91±0.0	5.61±0.8	9.44±3.5	10±4.4	90.47±45
	4	0.065±0.00	8.73±0.2	0.60±0.8	3.83±1.7	2.9±1.3	93.83±11
	24	0.069±0.00	8.69±0.1	0.35±0.4	4.39±1.0	3.5±0.3	72.56±20

Note. – N/A not applicable

Gene expression of IncX1 replication protein and stabilization genes. We monitored the expression of the *repB* replication gene and *relBE* genes which encode a type II toxin-antitoxin (TA) system associated with the IncX1 plasmid. The *relBE* genes were selected since they are adjacent to the *ori* (Fig. 2a) suggesting they may share the same promoter as *repB*; and due to their implicated role in plasmid stabilization (41, 42). Gene expression results in SH-2813$_{evol}$ revealed a marked 4.5-fold reduction in *repB* expression and 2.2-fold reduction in *relB* expression at 4 h relative to SH-2813$_{anc}$; while the expression levels of toxin *relE* remained unchanged in both ancestral and evolved SH-2813 (Fig. 2c).

SH-116 strains lacks the extra 22-bp direct repeat present on IncX1, thus no change in Rep expression was expected. However, the same pattern of expression was observed for these transcripts for SH-116$_{evol}$ — reductions in *repB* and *relB* and no change in *relE* when compared to SH-116$_{anc}$ (Fig. 2c). We hypothesize that the reduction in expression of Rep for evolved strains was a result of selecting for high copy ColE1 plasmids in PL. In fact, SH-116$_{evol}$ carried ColE1 plasmids with higher copies (ColE1-6kb =9±3.5, ColE1-4kb = 10±4.4, ColpVC-2kb = 90±45) than SH-116$_{anc}$ (ColE1-6kb =0.3±0.3 ColE1-4kb = 3±1.9, ColpVC-2kb = 82±7.9) (Table 2). This difference was further corroborated by comparing PCN determined from WGS assembly coverage, showing that all SH-116$_{evol}$ isolates from day 7 and 14 on average had higher ColE1 plasmid copies than SH-116$_{anc}$ (Supplementary Table 6).

Further, multiple regression analysis of IncX1 and Col plasmid copies showed that IncX1

was a statistically significant predictor of Col plasmid copies in a cell (F (3, 68) =13.82, *p*.value < 0.000, R^2 =0.39). SH-116 strains were predicted to have ca. six ColE1-6kb plasmid copies for every IncX1, whereas, SH-2813 was predicted to have 3 copies per IncX1 (Fig. 3). **Figure 3. Correlation between IncX1 and ColE1-6kb plasmid copies.** Plasmid copy number (PCN) was determined from whole genome sequencing de-novo assembly coverage. The presence of this high copy ColE1 plasmids correlated with faster growth rates in MHB. The optical density of SH-2813$_{evol}$ and SH-116$_{evol}$ was 1.4 and 1.7-fold higher than that of SH-2813$_{anc}$ and SH-116$_{anc}$, respectively, at 4 h (Table 2).

ColE1 plasmids differed in mobilization genes and non-coding RNA. ProgressiveMauve (28) alignments showed that ColE1 plasmids present in sequenced strains were highly similar and only differed in nucleotide regions coding for mob genes (Fig. 4; Supplementary Fig. 2b and c).

Figure 4. Col-like plasmid maps and conserved mobilization genes. (**a**, **b** and **c**) Annotated Col plasmid maps of (**a**) ColE1-6kb (**b**) ColE1-4kb and (**c**) ColpVC from SH-116$_{anc}$. (**d and e**) Maximum likelihood based tree of aligned conserved protein sequences of (**d**) relaxase (MbeA) and (**e**) MbeC genes of Col plasmid-carrying strains. Clades highlighted in blue and red shadow represent ColE1-4kb and ColE1-6kb plasmids, respectively. Trees were rooted with MbeA and MbeC sequences of *E. coli* (Accession number: NC_001371).

We identified 5-6 open reading frames (ORF) encoding 2-3 mobilization (mob) genes, a putative type IV TA system (Abi), a putative aminoglycoside 6-N-acetyltransferase (aac(6')-Ib) and a hypothetical protein for ColE1-6kb (Fig. 4a). The ColE1-4kb plasmids had 4-6 ORFs encoding a protein named macrophage stimulating factor (MSF), a hypothetical protein and 1-4 mob genes (Fig. 3b). Both ColE1 plasmids harbored a non-coding RNAI and RNAII, while ColE1-6kb carried an additional non-coding repressor of primer (Rop) protein known to regulate

replication and ColE1 plasmid copy numbers. The ColpVC-2kb plasmids had 2-3 ORFs, encoding 1-2 replication proteins (Rep) and a hypothetical protein (Fig. 4c). The RNAI present in the ColE1 plasmids had ca. 62 % DNA sequence homology (data not shown).

These mob CDS were identified as MbeA and MbeC and were present in different sizes and arrangement. In some cases, the *mbeA* gene overlapped ORF annotated as *mbeB and mbeD* (Fig. 4; Supplementary Fig. 3), suggesting MbeB and MbeD may be part of the mob region in some but not all ColE1 plasmids. Multiple alignments of MbeA relaxase protein sequences showed high conservation in the N-terminal region and high variation in their C-terminal (data not shown). According to the phylogenetic tree obtained for these conserved amino acids, MbeA proteins belonging to ColE1-4kb plasmids clustered in a unique clade, whereas the ColE1-6kb clustered next to the signature *E. coli* ColE1 plasmid (NC_001371) (Fig. 4d). Accordingly, *mbeC* genes from ColE1-6kb and ColE1-4kb were represented by separate clades on phylogenetic tree (Fig. 4e).

Col plasmids did not impose a fitness cost on host cell. Although acquisition of Col plasmids could result in faster growth in host cells it may also translate to a fitness cost if their regulation is not equally constrained. To establish if acquisition of Col plasmids imposed a fitness cost on *S.* Heidelberg, we competed 2 evolved (SH-2813$_{evol}$ and SH-116$_{evol}$) and 1 ancestral (SH- 116$_{anc}$) strain carrying three Col plasmids against a Col plasmid-free isogenic SH-2813 harboring a *gyrA* mutation for nalidixic acid resistance (SH-2813$_{nal}$). The fitness of each strain was determined relative to SH-2813$_{nal}$ as described by Millan et al (34). Results show that Col plasmid carrying strains had a higher relative fitness of 1.27 (s.d. =0.12, n =3) compared to SH-2813$_{nal}$, and differences were observed between strains (Fig. 5a). **Figure 5. Evolution of fitness and Conjugation rate (a)** Fitness of Col plasmid-carrying strains of SH-2813 and SH-116 relative to

the Col plasmid-free ancestor of SH-2813$_{nal.}$ Bars represent means of four technical replicates for each population. The horizontal dashed red line represents the fitness of SH-2813$_{nal}$. (b) Conjugation rates of Col plasmids from Col plasmid-carrying strains into SH-2813$_{nal}$ ancestor. Conjugation rates was estimated using data from fitness experiment. Bars represent means of four technical replicates for each population.

The transfer rate for ColE1-4kb plasmid from SH-116$_{evol}$ to SH-2813$_{nal}$ was below the detection limit of our assay. This difference in fitness between strains can be may be attributed to a higher conjugation rate observed for ColE1-6kb and ColpVC for SH-116$_{evol}$ (Fig. 5b).

Antimicrobial resistance associated with the acquisition of Col plasmids. The ColE1-6kb plasmid in the current study carries a putative aac (6')-Ib gene that is 56 % (*e.value* = 1<e-13) identical to the aac (6')-Ib gene present in *Klebsiella pneumoniae* Sul1-type integron (GenBank accession number: AAR18814.1) and 34 % (*e..value* = 6<e-28) identical to the one present in *Thiomona* sp. X19 (GenBank accession number SCC93141.1); suggesting it may play a role in aminoglycoside resistance (Fig. 6a and b). **Figure 6. Acquisition of ColE1 plasmids was associated with decreased susceptibility to aminoglycosides and fosfomycin.** (**a**) Multiple alignment of conserved N-terminal residues for aminoglycoside acetyltransferase (AAC-6') genes encoded in the *S*. Heidelberg (SH-2813) chromosome and ColE1-6kb plasmid from this study, and AAC-6' genes with known resistance phenotype. (**b**) Corresponding neighbor-joining tree of aligned AAC-6' protein sequences. (**c**) Inhibition zone diameter distributions for evolved strains and selected antimicrobial drugs.

Further, *S*. Heidelberg ancestral strains each carried chromosomally encoded aminoglycoside (aac (6')-Iy) and fosfomycin (fosA7) genes (43) that have been demonstrated to confer resistance to tobramycin, netilmicin, neomycin, amikacin, kanamycin and fosfomycin

(44-46). To assess the correlation between AMR and the acquisition of Col plasmids, we performed antibiotic susceptibility testing (AST) on evolved *S*. Heidelberg strains (n=42) on the NARMs antimicrobials (36). In addition, we used disc-diffusion assay (37) to evaluate susceptibility to tobramycin, netilmicin, gentamicin, neomycin, kanamycin and fosfomycin.

The majority of the isolates tested (97.6 %) were susceptible to the 14 drugs on NARMs AST panel (Supplementary Table 7) However, 12 of 16 (75 %) SH-2813$_{evol}$ isolates recovered from PL on day 7 and day 14 displayed an increase in MIC for gentamicin. The average zone of inhibition (mm) for kanamycin, tobramycin and fosfomycin for SH-2813$_{anc}$ strains was larger than the zones measured for SH-2813$_{evol}$ strains (Wilcoxon signed-rank test; p-value < 0.0001) (Fig. 6c). In contrast, SH-116 showed no significant difference in zone of inhibition between SH-116$_{anc}$ and SH-116$_{evol}$ strains for any of the antibiotics tested (Wilcoxon signed-rank test; p-value > 0.01).

Col plasmid-encoded genes are prevalent in PL. To extrapolate beyond our microcosm evolution experiment, bioinformatics and quantitative PCR were used to assess the pattern of distribution of these Col plasmids across genomes and in PL. DNA sequences for complete Col plasmids from this study were subjected to BLAST searches against genomes that have been deposited in GenBank. No BLAST hit matched the complete sequence of ColE1-6kb plasmids, but the majority of hits covered ca. 55 % of the plasmid genome. In most cases, these hits corresponded to mobilization genes.

For ColE1-4kb, the closest hit was 99 % identical to the colicigenic plasmid of *Salmonella enterica* serovar Berta (pBERT) and covered the entire genome. All other plasmid hits covered <82 % of the ColE1-4kb genome and were represented in various species of Gram-negative bacteria. The ColpVC-2kb was >99 % identical to plasmids from 5 *Salmonella* sp, including

three *S.* Heidelberg strains isolated from Chicken in Canada (data not shown). These results suggested that Col plasmids of poultry origin are not well represented in GenBank.

To address this possibility, we quantified Col plasmids in litter from conventional and backyard poultry farms located in 4 states in USA. The Col plasmid genes were detected in all litter types except 1 backyard litter, whereas the Rep protein for IncX1 was detected in 4 of 7 of the litter types, suggesting that these genes are present in PL (Fig. 7). **Figure 7. Concentration of plasmid-specific genes in poultry litter of different types.** Poultry litter (PL) was collected as grab samples from multiple locations on the farm and composited in 1-gallon Whirl-Pak bags. qPCR was performed on DNA extracted from 250 mg of PL in triplicates. The error bars represent the standard error of the mean. Con =Conventional farming, Back = Backyard farming, TSC =Target sequence copies

DISCUSSION

Col plasmids and IncX1 contributed to the fitness of *S.* Heidelberg

We show for the first time the evolutionary trajectory of *Salmonella enterica* serovar Heidelberg in PL. We chose *S.* Heidelberg for its increasing role in food-borne outbreaks and its carriage of Col-like plasmids (14, 16, 17, 22, 47). In our study, only *S.* Heidelberg strains carrying 2 ColE1 and a cryptic ColpVC plasmids survived more than 7 days in used PL. The ColE1-6kb relaxases (MbeA) shared >77 % homology with the relaxase of ColE1-4kb but was more closely related to the prototype ColE1 (>96 %) plasmid of *E. coli*. When the ColE1 plasmids sequences from this study were compared to those available in NCBI, we found that these plasmids were underrepresented in this database. The observed low frequency may be a consequence of the bias toward sequencing strains of clinical origin, where plasmids carrying known antibiotic resistance genes are of primary interest.

After screening multiple PL types with qPCR, we detected Col plasmid genes at high levels in 6 of 7 litter types. Our data further suggest that the PL environment may impose selective pressure that promotes the acquisition of these Col plasmids.

The ColE1-6kb plasmids were present in higher copy numbers than ColE1-4kb and carried 2 putative genes encoding a type-IV TA system and an aminoglycoside-acetyltransferase. The TA system was linked by STRINGDB (48) to the widespread abortive bacterial infection (Abi) system belonging to the cluster of orthologous group COG1106 (domain of ATPases) and the closest homologs were those present in Pseudomonadaceae and Lactobacillaceae. In some bacteria, this protein is annotated as ATP-binding protein or abortive infection protein. Abi systems induce phage resistance to prophages of type 936 in *Lactococcus* and enhance plasmid maintenance/stabilization via bacteriostatic toxicity (49). Consequently, cells carrying plasmids encoding the Abi gene will be immune to phage attack or replication, thereby providing protection for their clonal population. Their role in *Salmonella* remains questionable; however, their high expression under laboratory growth conditions in our study (Supplementary Fig. 4) suggests that they are functional and may have contributed to the persistence of *S*. Heidelberg in PL.

We do not know the potential functions of the accessory genes on ColE1-4kb, but their compatibility with ColE1-6kb may stem from differences in their RNAI sequences (50) and their mob region. The ColE1 plasmid family represents plasmids that are only transmissible in the presence of an additional plasmid with conjugative functions and usually carry a mob region encoding specific relaxase and accessory mob genes (*mbeB*, *mbeD mbeC*). The relaxase component is crucial for both initiation and transfer of DNA and is highly conserved (51, 52). In addition, it has been predicted that the C-terminal region of MbeA binds ssDNA, while the N-

terminal fragment contacts MbeC (53, 54). The observed differences in C and N- terminal of MbeA and MbeC in this study suggests that such modifications may be critical for successful co-existence of multiple ColE1 plasmids in one host cell.

The Incompatibility factor X1 plasmid harbored by the *S*. Heidelberg strains used in our study more than likely served a conjugative role. IncX1 is a low copy plasmid and carries required mating pair formation system for horizontal gene transfer (HGT) including type IV secretion systems (T4SS), coupling proteins and a relaxase (55, 56) (Fig. 2a). This plasmid was present in all strains recovered which infers that they are stably maintained and critical to the survival *S*. Heidelberg strains carrying them.

Stability of conjugative plasmids has been attributed to high conjugation rates and plasmid stabilization proteins (57, 58) . The Indel present in VirB8 resulted in a frame-shift mutation in this protein and subsequent changes in DNA regions coding for T4SS proteins. Indeed, this mutation resulted in the fusion of a smaller VirB8 (4.4 kDa) encoding gene with a bigger one (23.4 kDa) (Fig. 2a). In addition, differences were observed in sequences coding for the conjugal transfer protein (TraG), an important protein for bacterial conjugation (59). Interestingly, one strain without TraG (SH-33A0.5-116-Day7-PLE) was isolated but we do not know the conjugal implication of this deletion (Supplementary Fig. 5)

Following the acquisition of Col plasmids, we observed a significant reduction in the expression of replication protein (Rep) protein and a type II toxin-antitoxin (TA) gene. This reduction in *rep* expression suggests that *rep* expression may be linked to the cost of taking up new MGE. Reducing *rep* expression will subsequently decrease the copies of IncX1 available for ColE1 plasmids mobilization — thus reducing the burden associated with maintaining multiple plasmids. Also, regression analysis of IncX1 and Col copies showed that IncX1 was a good

predictor of ColE1-6kb copies per cell — evolved strains carrying high IncX1 copies also carried high ColE1-6kb copies (Fig. 3; Supplementary Table 6). This result suggests there might be an equilibrium between the number of “helper plasmids” to mobilizable plasmids residing in a cell.

Bacteria (TA) modules are genetic elements composed of a toxin protein component that disrupts bacterial growth by interfering with vital cell processes and an antitoxin that impairs the functionality of the toxin until this inhibition is abolished in response to cellular signaling. Type II TA system carried on plasmids are important players in post-segregational killing — plasmid-less cells are killed by the toxin, thereby establishing the dominance of plasmid-bearing cells in a population (41, 42, 60).

At late logarithm and stationary phase growth in Mueller Hinton Broth, the *relB* gene (antitoxin) of IncX1 was significantly downregulated in evolved strains compared to ancestral strains, and no change was observed in *relE* (toxin) expression (Fig. 2c). This continuous expression of *relE* will ensure that only IncX1 carrying cells are present in the population. Therefore, disrupting the conjugative and stability genes of conjugative plasmids may represent a critical point for limiting their persistence in the environment. For instance, a combination treatment with linoleic and phenothiazine reduced the conjugation efficiency of six conjugative plasmids by 3 -fold, including the IncX plasmid of RP4 (61).

Furthermore, the IncX1 present in Col plasmid-bearing strains of *S*. Heidelberg carried a 22-bp direct repeat deletion at the origin of replication (*ori*), an Indel in VirB8 (G-> GA) and a non-synonymous substitution in a hypothetical protein (I111T). The Rep protein of IncX1 belongs to the family of plasmids that use the theta mode of replication similar to the F, P1, R6K and ColE1-like plasmids. The Rep protein interacts with iteron sequences in order to control the copy number of plasmids in a cell (39, 62).

Briefly, the Rep-iteron complexes stimulate the initiation of replication at low copy until the regular copy number is achieved. However, when the copy number is increased beyond the normal levels, the Rep-iteron concentration is high enough to bind almost all protein bound iterons. This direct interaction between the plasmid molecules produces the coupling or handcuffing of the origins, making them unavailable for the initiation of replication. Moreover, these repeated sequences are important incompatibility determinants for iteron-based plasmids.

Strains with this deletion had higher copies of IncX1 than ancestral strain carrying the extra 22-bp direct repeat. This result supports other studies showing that the deletion of iterons increased plasmid copy number of theta plasmids (reviewed in (63)). For example, Millan et al (64) showed that mutations acquired in the OriV of a synthetic ColE1-like plasmid (pBGT) carrying bla_{TEM-1} β-lactamase gene increased plasmid copy number and subsequently increased ceftazidime resistance several folds.

To expand beyond the *S*. Heidelberg strains used in our study, we performed an alignment of the *ori* of closed "circular" IncX1 plasmids of ca. 37kb present in *S*. Heidelberg strains available in NCBI. We observed that all the strains analyzed carried only two iterons (Fig. 2b), which implies that this deletion is overrepresented in sequenced *S*. Heidelberg carrying IncX1 plasmids. The majority of these strains were from clinical settings (Supplementary Table 8) which suggests they are being recovered more often and may be associated with an increase in fitness of those strains as was also observed in our study. Together, our results support the idea that iterons regulate IncX1 plasmid replication and their compatibility with other plasmids.

Mutations acquired on plasmids and chromosomes after an HGT event have been shown to compensate for the fitness cost associated with the acquisition of a new (MGE) (35, 64-66). Here, strains carrying Col plasmids had a total of 4 plasmids and persisted for up to 14 days,

which is a “plasmid paradox,” where the acquisition and maintenance of multiple plasmids over the long term is supposed to impose a burden on their host (57, 58).

This principle has been elucidated upon, where positive epistasis minimized the cost associated with carrying small plasmids of *Pseudomonas aeruginosa* over a long-time scale (34). Recently, a study showed that the acquisition of three ColE1 plasmids in naïve *H. influenzae* hosts did not impose a fitness cost as long as the bacterium compensated for the first plasmid (67). The presence of one plasmid favored the presence of a second plasmid and the presence of this second plasmid or third plasmid did not impose an additional significant biological cost, even though these plasmids produced a cost when they are alone in the cell .

In this study, Col plasmid-bearing strains had increased fitness compared to a strain without a Col plasmid and shared 102 single nucleotide variants (SNV) in common (Fig. 1d). It is unlikely that all observed SNV are to compensate for Col plasmid acquisition, as some will be compensatory for other MGE such as bacteriophages (68-70). For example, the prophage Gifsy-2 was found intact in 97% of *S*. Heidelberg isolates sequenced in our study, suggesting the PL environment may have selected them for increased fitness (Supplementary Fig. 6). Further, the ubiquity of Gifsy-2 in *S*. Heidelberg strains has been reported, which infers they are important for *S*. Heidelberg survival (13). We observed a mutation in the replication protein (RepO) of Gifsy-2 denoting these events are linked (Supplementary Table 5). Taken together, it is plausible that mutations observed in this study are associated with HGT events involving both ColE1 plasmids and prophages; in addition to mutations selected for persistence in PL.

Plasmid and chromosome encoded genes mediated antibiotic resistance in *S*. Heidelberg

Another significant observation from this study is the decrease in susceptibility of Col plasmid-bearing strains towards kanamycin, tobramycin and fosfomycin. We do not know what

these reductions in inhibition zones mean in terms of MIC as there are no current CLSI standards (38) for *S.* Heidelberg. In addition, it has been shown that simple back- calculations from inhibition zones to MIC can be confounded by several covariates such as serotype, host species, and year of isolation (71).

Kanamycin and tobramycin are not used for treating *Salmonella* infections due to their resistance in-vivo, although tobramycin in combination with fosfomycin is used to treat people with cystic fibrosis caused by *Pseudomonas aeruginosa* (72). Since no drugs were added to the litter used in this study (Personal Communication- Dr. Casey Ritz, University of Georgia), experimental selection for these resistance phenotypes is unlikely. Although alternative explanations including Col plasmid selection or population factors like competitive exclusion and the presence of "cheaters" (73-75) may account for this result, more research is needed to test these possibilities.

Despite the fact that the acquisition of Col plasmids coincided with the observed change in susceptibility, we cannot say for sure if these plasmids played any role. The putative aminoglycoside acetyltransferase (aac-(6')) gene carried on ColE1-6kb represents a fragmented aac-6' gene and shares significant homology with the N-terminal of aac(6')-Ib of *K. pneumoniae*. Its small size (81 amino acid residues) compared to other known aac genes (Fig. 5a), and the absence of homologs in all antibiotic resistance genes (ARG) databases queried suggests it may be an uncharacterized ARG. Importantly, its presence on ColE1 will result in more copies of these gene compared to the aac-(6') gene carried as a single copy on the chromosome. More studies will be required to determine if these two aac-(6') genes confer decreased susceptibility towards the aforementioned aminoglycosides.

Regarding fosfomycin, Rehmann et al (43) determined that *S*. Heidelberg strains with an inhibition zone of 26 mm were resistant to >32 µg/ml of fosfomycin, which suggests that evolved *S*. Heidelberg strains carrying Col plasmids in our study may be resistant to fosfomycin. The fosA7 gene is present in many *S*. Heidelberg genomes and resistance to fosfomycin has been demonstrated in a few (43). In addition, *S*. Heidelberg harbors other chromosomal genes associated with fosfomycin resistance including *glpT* and *uhpT* (76).

In this study, we did not observe any mutation in these genes, thus cannot correlate fosfomycin resistance to a specific ARG. This result shows the intrinsic bias that could be associated with estimating resistance phenotype from only known ARG that are available in curated databases. More concerning, is the spontaneous mutant (SH-17BBPW-2813) that emerged with reduced susceptibility to 3 classes of antibiotics following overnight enrichment in buffered peptone water (BPW) (Supplementary Table 7). One explanation is that BPW enriched for the limited strains present in PL or it created an environment where only the fittest strain emerged. Therefore, we argue that multiple chromosomal mutations in addition to Col plasmids are responsible for the reduced susceptibility displayed by evolved *S*. Heidelberg isolates, including a non-synonymous substitution (T153A) in efflux mediated multi-drug resistance gene *acrA* (77, 78).

Results from these PL evolution experiments suggest that IncX1 and ColE1 plasmids contributed to the fitness of *S*. Heidelberg in this PL. However, if Col plasmids are a true horizontally acquired fitness factor for *S*. Heidelberg survival in PL, one would expect SH-116 to survive longer than SH-2813 since the ancestral populations carry Col plasmids. This was not the case in our study (Fig. 1a). SH-116 strains were only detected in 1 of 6 microcosms by direct count and in 3 of 6 after enrichment in buffered peptone water (BPW) after 14 days incubation in

PL. In contrast, SH-2813 was recovered by direct count in all 6 microcosms (Supplementary Table 1).

The dynamics observed in our experiment can be modeled according to Watve et al (79) Rock-Paper-Scissors (RPS) model of sociobiological control of plasmid copy number. In our scenario, SH-116 cells with lower copy ColE1 plasmids were displaced by those with higher copy ColE1 plasmids mobilized from PL microbiota until the point at which the intrinsic survival rate of *S*. Heidelberg was greatly reduced (Fig. 1a). In contrast, a stable co-existence of SH-2813 of low, intermediate and high copy ColE1 plasmids was observed in the SH-2813 population, since they are invading strains lacking Col plasmids to begin with.

This observation was corroborated by qPCR on extracted DNA from PL with Col plasmid gene primer sets (Supplementary Table 9) . At day 14, the concentrations of targeted genes were several orders of magnitude higher in SH-116 microcosms than SH-2813 (Supplementary Fig. 6), which supports the notion that the original SH-116 strains may have been displaced by those carrying higher copy ColE1 plasmids.

In this work, we demonstrate that ColE1 plasmids acquired from PL contributed to the persistence of *Salmonella enterica* serovar Heidelberg, however the identity of the bacterial donors of these plasmids remains unknown. Future studies from our laboratory will attempt to provide answers.

ACKNOWLEDGEMENTS

We are grateful to Dr. Casey Ritz for providing us with the poultry litter used in this study and for Caroline Plymel for her help with sample analysis. We also appreciate Steven Lankin and Mary Katherine Crews for manuscript review. Any opinions expressed in this paper are those of the authors and do not necessarily reflect the official positions and policies of the USDA and any

mention of products or trade names does not constitute recommendation for use. The authors declare no competing commercial interests in relation to the submitted work.

Supplementary Figures

Figure S1. Experimental design and microcosm sampling.

Figure S2. ProgressiveMauve alignment of (**a**) IncX1 (**b**) ColE1-6kb and (**c**) ColE1-4kb plasmids from selected strains.

Figure S3. Circular maps of (**a and b**) ColE1-6kb (**c and d**) ColE1-4kb and (**d and e**) ColpVC plasmids carried by evolved strains used in competition and conjugation experiments.

Figure S4. Gene expression of selected Col plasmid genes determined by qRT-PCR for (**a**) SH-116_{anc} (**b**) SH-116_{evol} and (**c**) SH-2813_{evol} strains.

Figure S5. Circular map of IncX1 plasmid of strain SH-33A0.5-116-Day7-PLE.

Figure S6. Intact prophages in sequenced genomes of *S*. Heidelberg.

Figure S7. Concentration of selected Col plasmid genes determined using qPCR for (**a**) SH-2813-PLE and (**b**) SH-116-PLE poultry litter microcosms.

Supplementary Tables

Table S1. Bacteria concentration in poultry litter microcosms.

Table S2. Physicochemical properties of poultry litter microcosms.

Table S3. Metadata on isolates sequenced.

Table S4. Identified plasmid topology.

Table S5. Single nucleotide variants shared among Col-plasmid bearing strains.

Table S6. Plasmid copy number determined by whole genome sequencing coverage.

Table S7. Antibiotic susceptibility test results.

Table S8. Metadata on NCBI *S*. Heidelberg strains carrying IncX1 plasmid.

Table S9. Primers used in this study.

Supplementary Files

Supplementary File 1. Quality assessment and coverage of sequenced SH-2813 strains.

Supplementary File 2. Quality assessment and coverage of sequenced SH-116 strains.

Supplementary File 3. Disc diffusion inhibition zone measurements.

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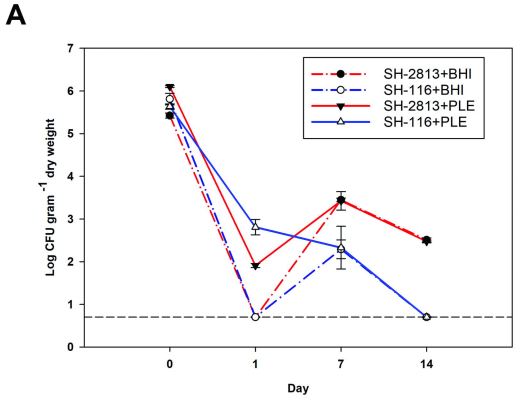
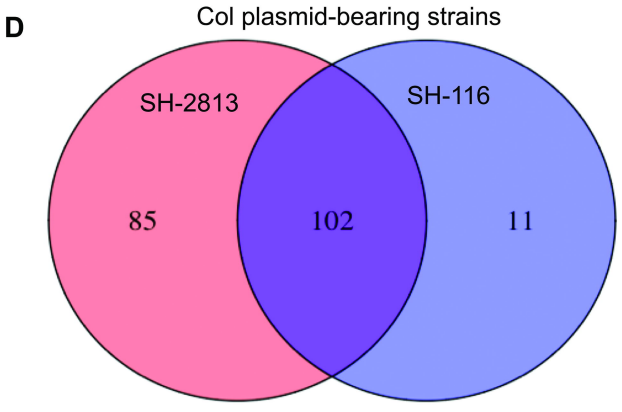
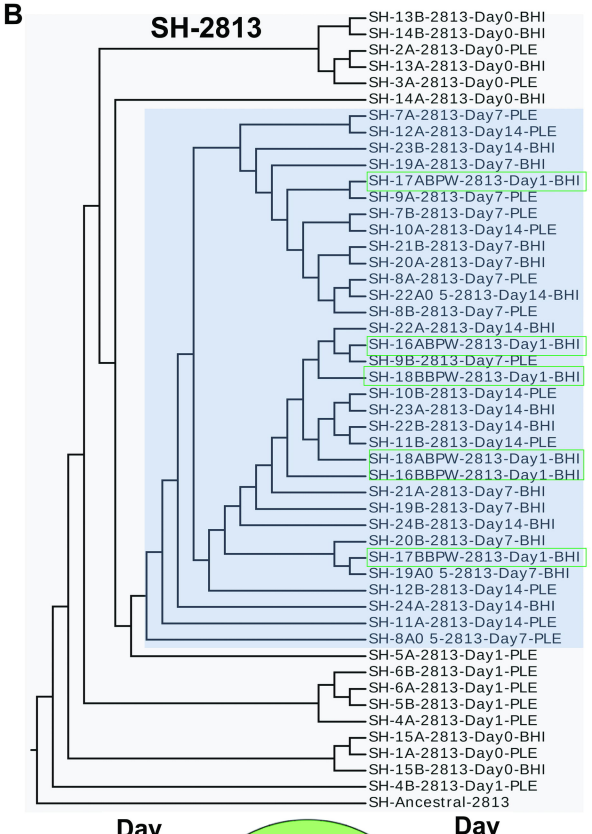

A
SH-2813+BHI
SH-116+BHI
SH-2813+PLE
SH-116+PLE
Log CFU gram -1 dry weight
Day
0
1
7
14


D
Col plasmid-bearing strains
SH-2813
SH-116
85
102
11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SH-14B-2813-Day0-BHI
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SH-13A-2813-Day0-BHI
SH-3A-2813-Day0-PLE
SH-14A-2813-Day0-BHI
SH-7A-2813-Day7-PLE
SH-12A-2813-Day14-PLE
SH-23B-2813-Day14-BHI
SH-19A-2813-Day7-BHI
SH-17ABPW-2813-Day1-BHI
SH-9A-2813-Day7-PLE
SH-7B-2813-Day7-PLE
SH-10A-2813-Day14-PLE
SH-21B-2813-Day7-BHI
SH-20A-2813-Day7-BHI
SH-8A-2813-Day7-PLE
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SH-8B-2813-Day7-PLE
SH-22A-2813-Day14-BHI
SH-16ABPW-2813-Day1-BHI
SH-9B-2813-Day7-PLE
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SH-19B-2813-Day7-BHI
SH-24B-2813-Day14-BHI
SH-20B-2813-Day7-BHI
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SH-24A-2813-Day14-BHI
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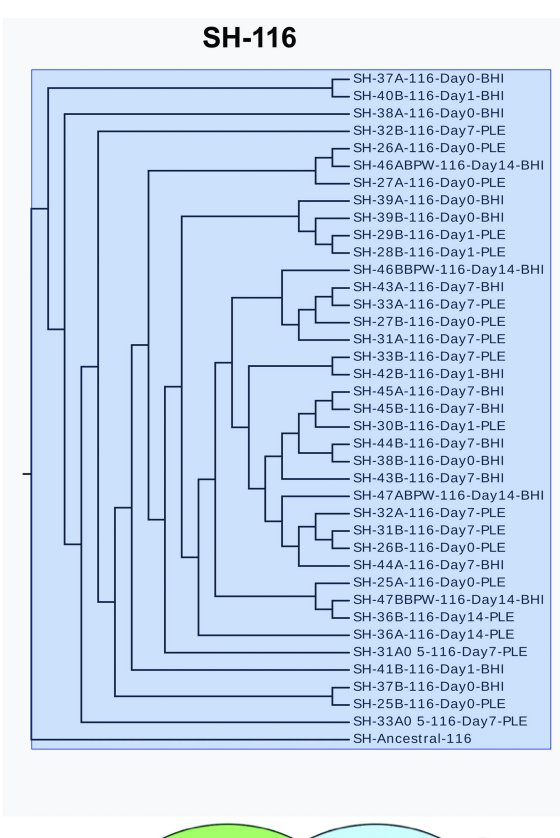
SH-Ancestral-2813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SH-26A-116-Day0-PLE
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SH-32A-116-Day7-PLE
SH-31B-116-Day7-PLE
SH-26B-116-Day0-PLE
SH-44A-116-Day7-BHI
SH-25A-116-Day0-PLE
SH-47BBPW-116-Day14-BHI
SH-36B-116-Day14-PLE
SH-36A-116-Day14-PLE
SH-31A0 5-116-Day7-PLE
SH-41B-116-Day1-BHI
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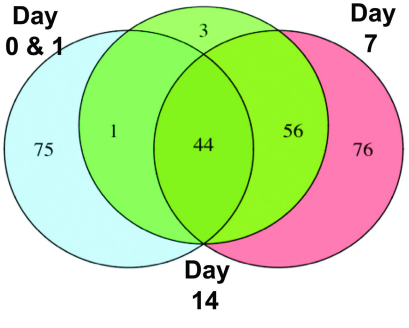
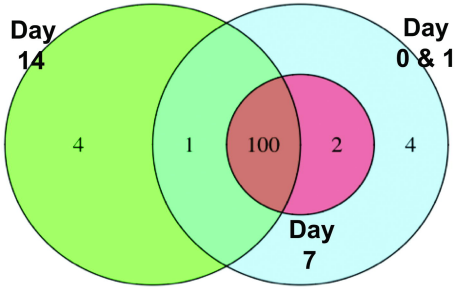
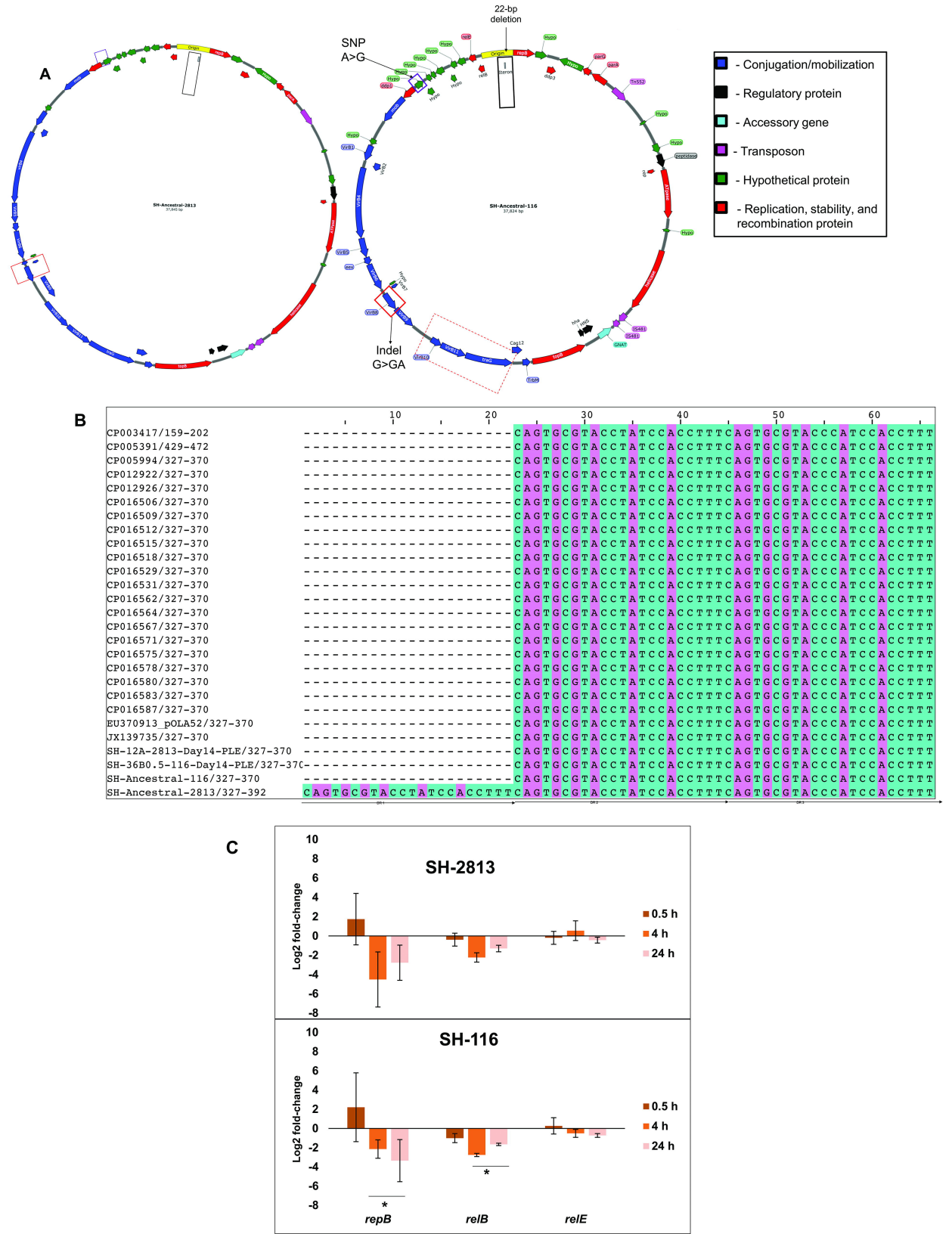
SH-Ancestral-116


Day
0 & 1
Day
7
Day
14
75
3
1
44
56
76


Day
14
Day
0 & 1
Day
7
4
1
100
2
4


A
SH-Ancestral-2813
37,845 bp
SH-Ancestral-116
37,824 bp
SNP
A>G
22-bp
deletion
Indel
G>GA
- Conjugation/mobilization
- Regulatory protein
- Accessory gene
- Transposon
- Hypothetical protein
- Replication, stability, and recombination protein
B
CP003417/159-202
CP005391/429-472
CP005994/327-370
CP012922/327-370
CP012926/327-370
CP016506/327-370
CP016509/327-370
CP016512/327-370
CP016515/327-370
CP016518/327-370
CP016529/327-370
CP016531/327-370
CP016562/327-370
CP016564/327-370
CP016567/327-370
CP016571/327-370
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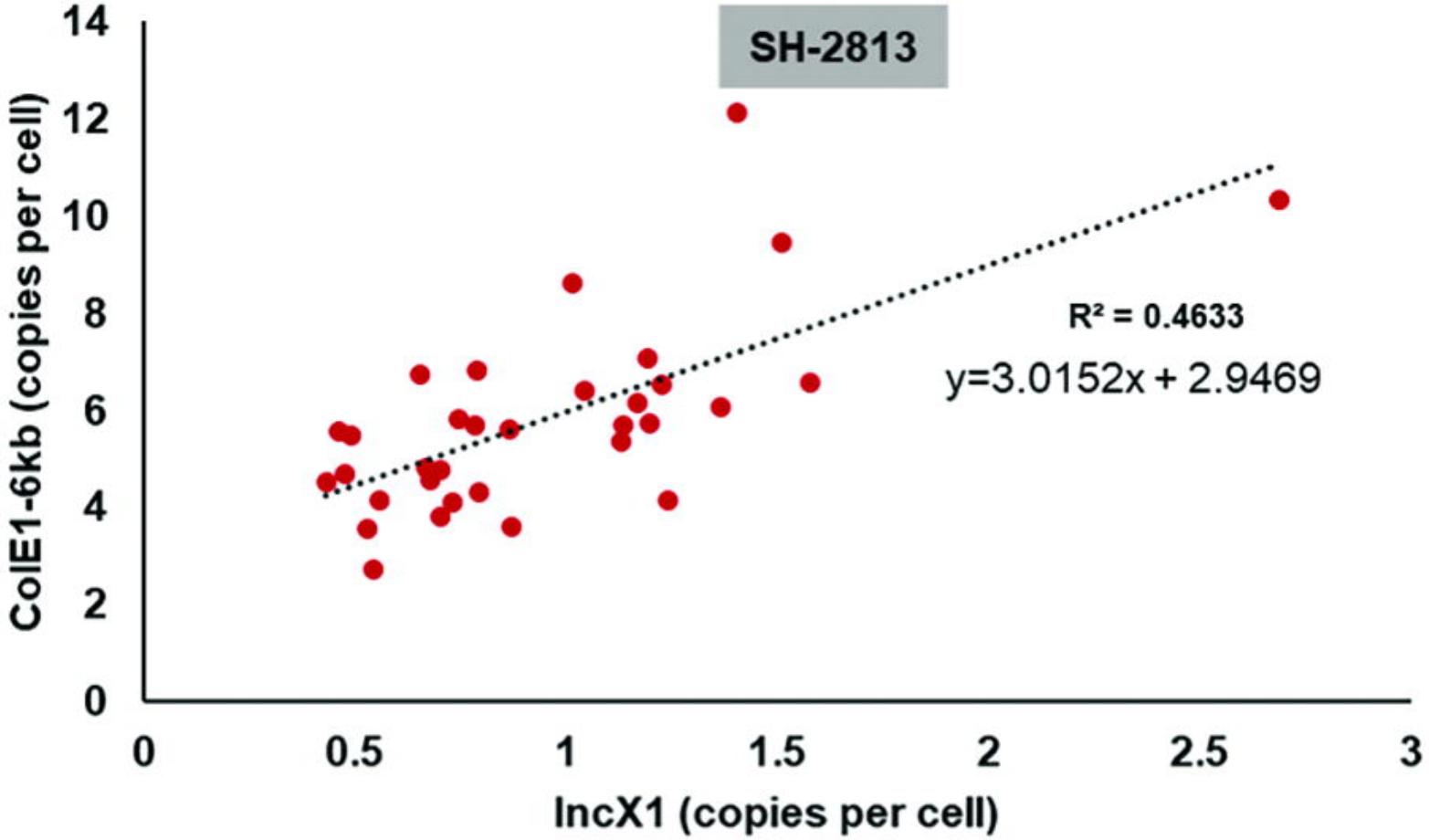
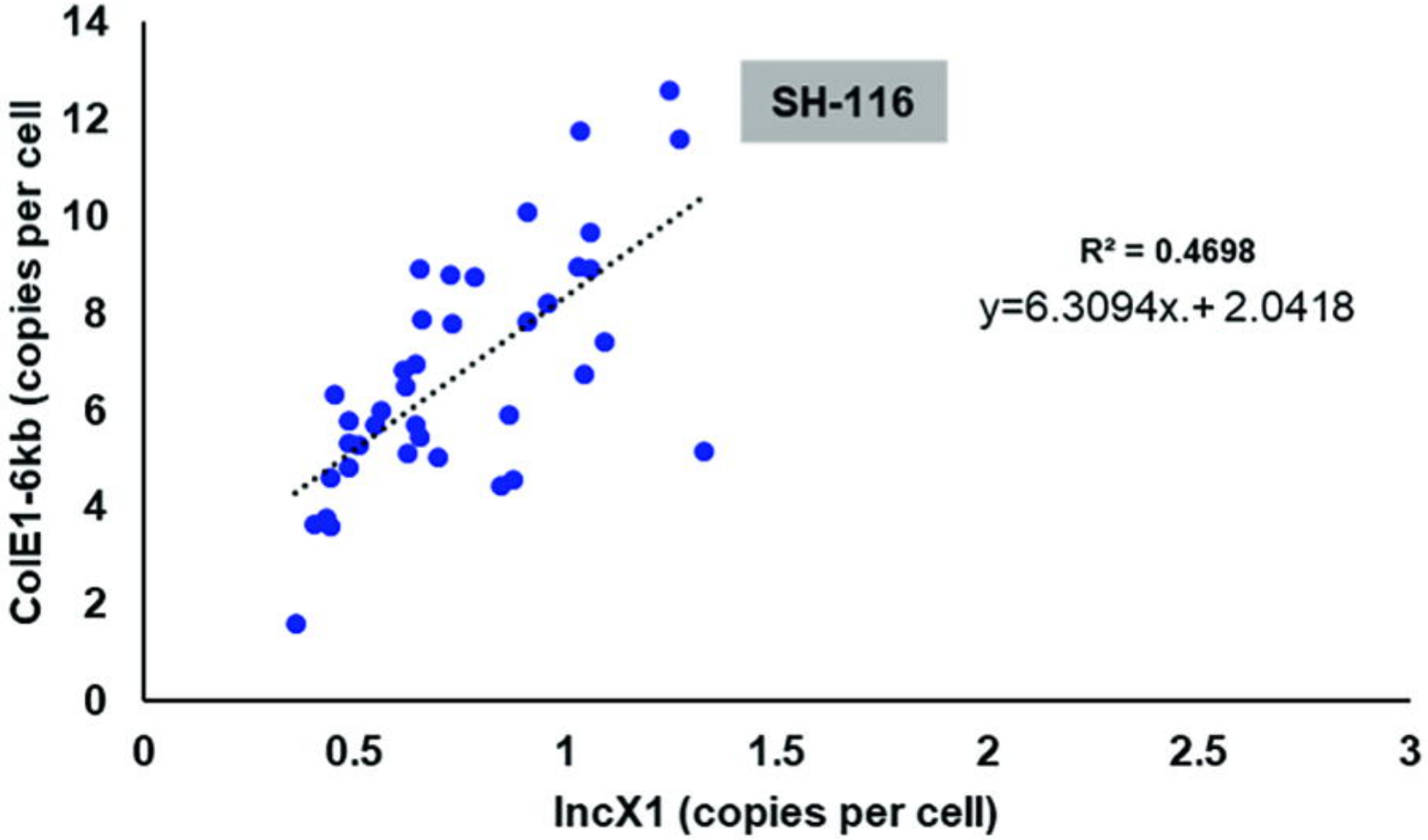
CP016578/327-370
CP016580/327-370
CP016583/327-370
CP016587/327-370
EU370913_pOLA52/327-370
JX139735/327-370
SH-12A-2813-Day14-PLE/327-370
SH-36B0.5-116-Day14-PLE/327-370
SH-Ancestral-116/327-370
SH-Ancestral-2813/327-392
C
SH-2813
SH-116
Log2 fold-change
0.5 h
4 h
24 h
repB
relB
relE


SH-2813
ColE1-6kb (copies per cell)
IncX1 (copies per cell)
R² = 0.4633
y=3.0152x + 2.9469
0
2
4
6
8
10
12
14
0
0.5
1
1.5
2
2.5
3


SH-116
ColE1-6kb (copies per cell
IncX1 (copies per cell)
R² = 0.4698
y=6.3094x.+ 2.0418
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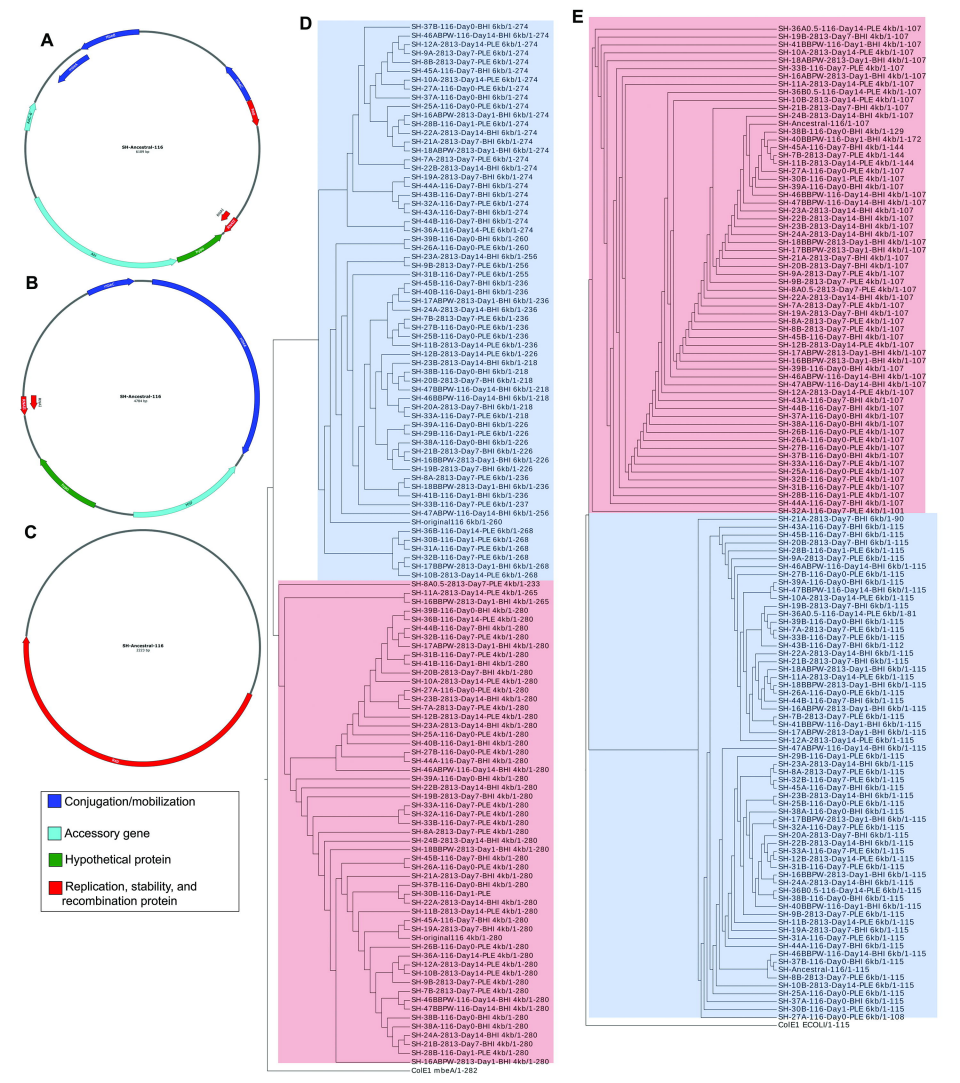
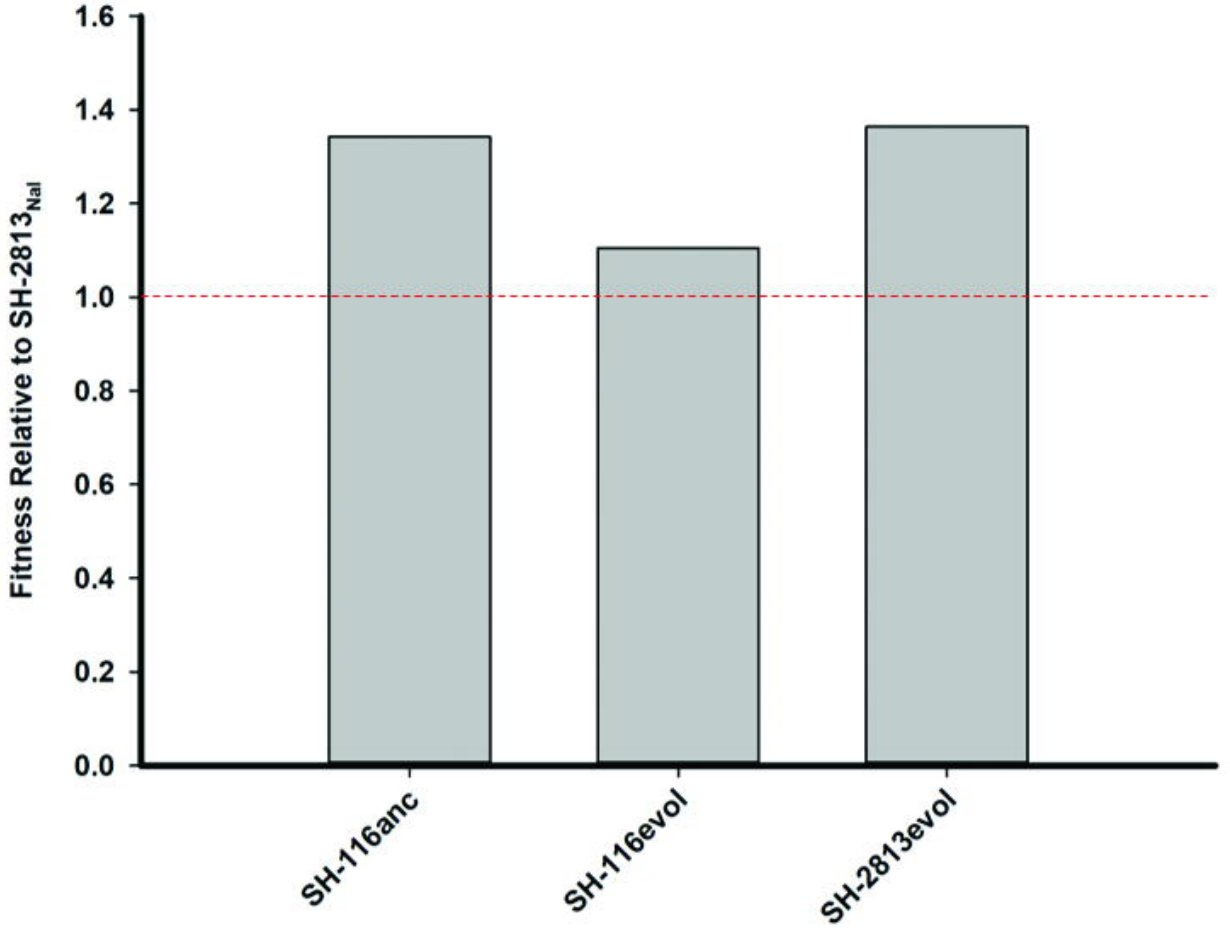
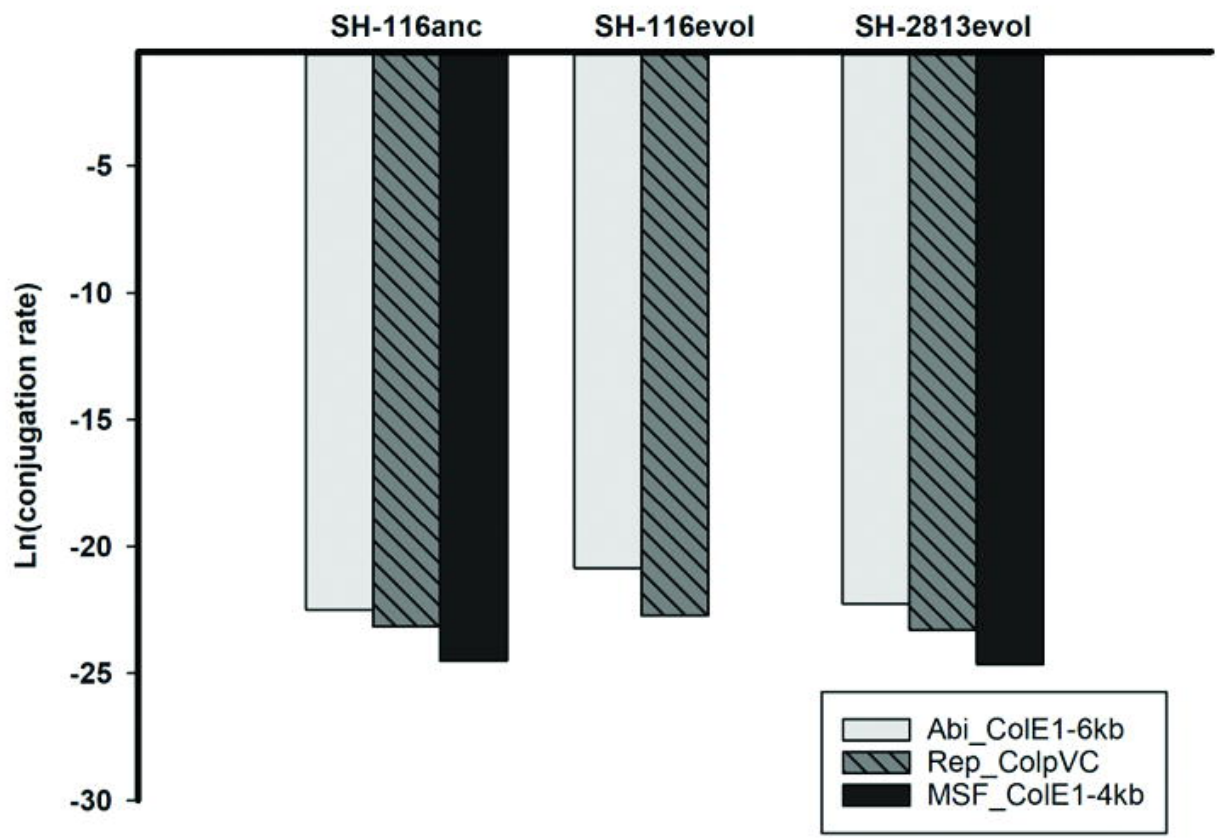
2
4
6
8
10
12
14
0
0.5
1
1.5
2
2.5
3

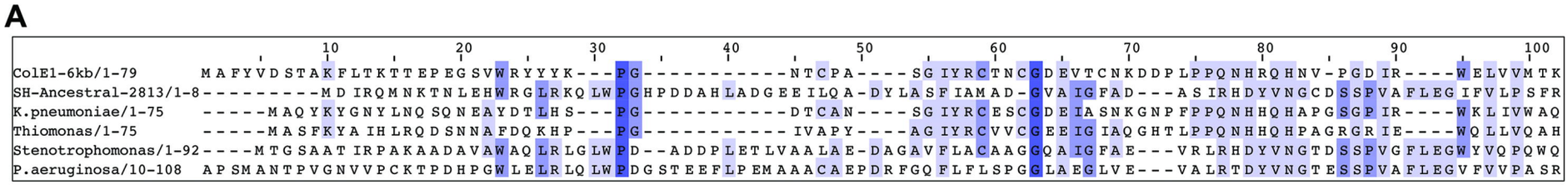

A
B
C
D
E
Conjugation/mobilization
Accessory gene
Hypothetical protein
Replication, stability, and recombination protein
ColE1 mbeA1-282
ColE1 ECOLI1-115

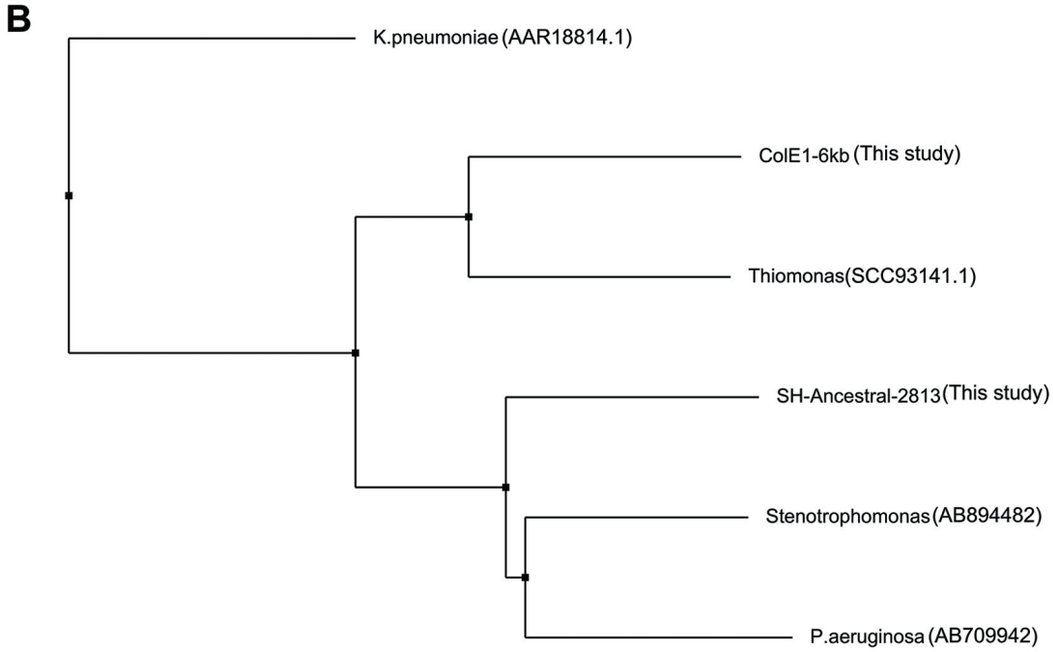
A

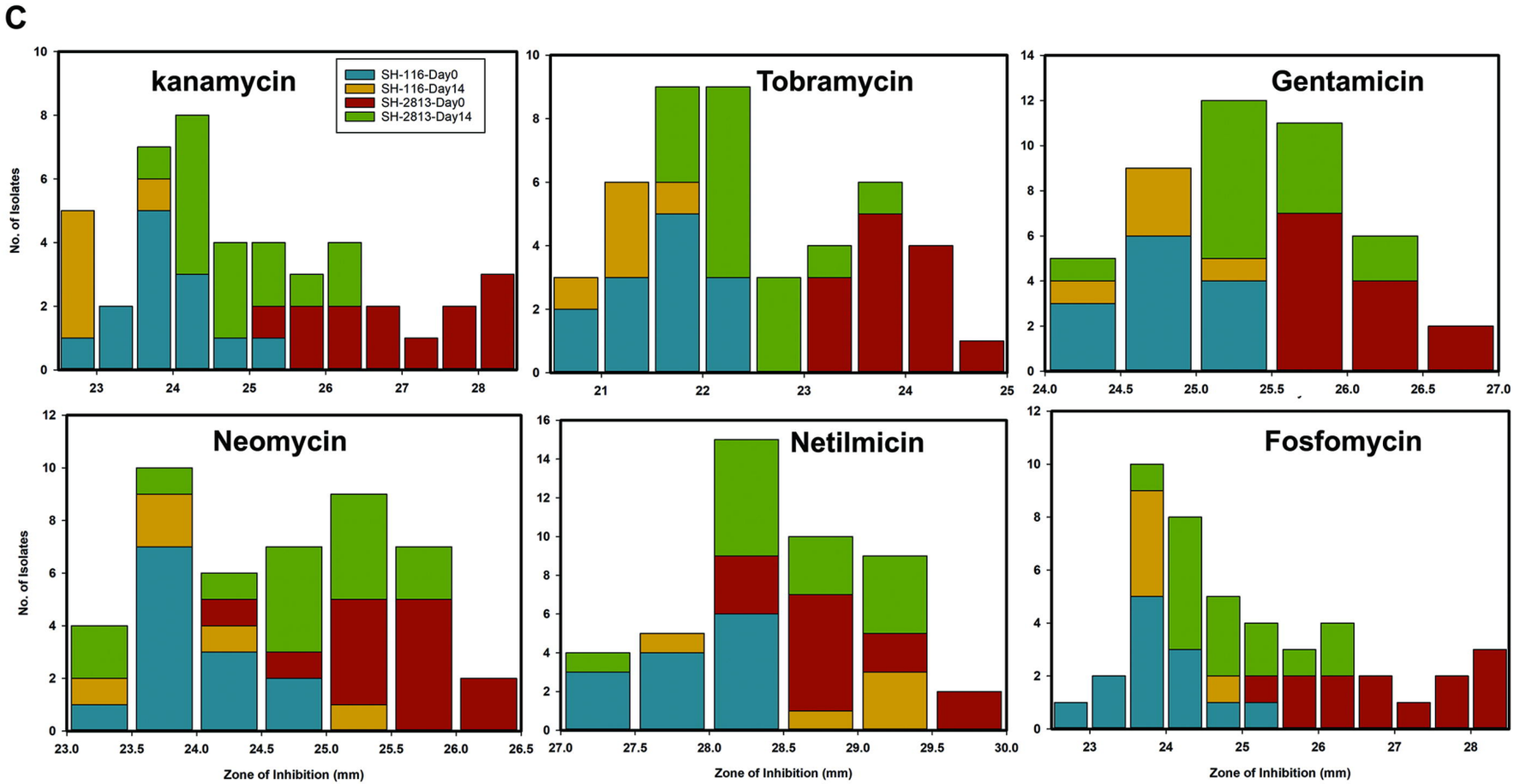

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fitness Relative to SH-2813Nal
SH-116anc
SH-116evol
SH-2813evol

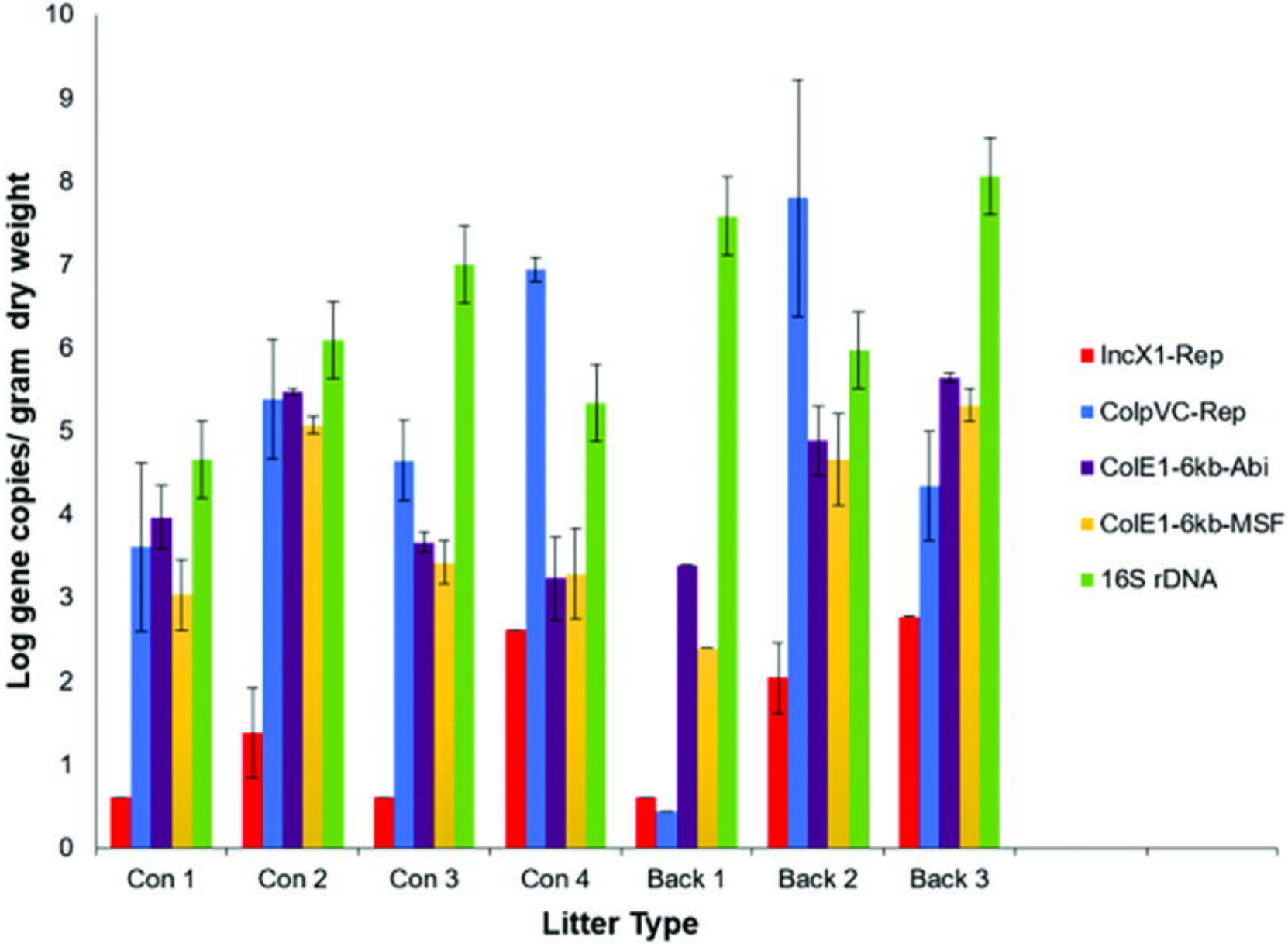
B


SH-116anc
SH-116evol
SH-2813evol
-5
-10
-15
-20
-25
-30
Ln(conjugation rate)
Abi_ColE1-6kb
Rep_ColpVC
MSF_ColE1-4kb


A
10 20 30 40 50 60 70 80 90 100
ColE1-6kb/1-79 MAFYVDSTAKFLTKTTEPEGSVWRYYYK---PG----------NTCPA----SGIYRCTNCGDEVTCNKDDPLPPQNHRQHNV-PGDIR----WELVVMTK
SH-Ancestral-2813/1-8---------MDIRQMNKTNLEHWRGLRKQLWPGHPDDAHLADGEEILQA-DYLASFIAMAD-GVAIGFAD---ASIRHDYVNGCDSSPVAFLEGIFVLPSFR
K.pneumoniae/1-75 -----MAQYKYGNYLNQSQNEAYDTLHS---PG----------DTCAN----SGIYRCESCGDEIASNKGNPFPPQNHHQHAPGSGPIR----WKLIVWAQ
Thiomonas/1-75 -----MASFKYAIHLRQDSNNAFDQKHP---PG----------IVAPY----AGIYRCVVCGEEIGIAQGHTLPPQNHHQHPAGRGRIE----WQLLVQAH
Stenotrophomonas/1-92----MTGSAATIRPAKAADAVAWAQLRLGLWPD--ADDPLETLVAALAE-DAGAVFLACAAGGQAIGFAE---VRLRHDYVNGTDSSPVGFLEGWYVQPQWQ
P.aeruginosa/10-108 APSMANTPVGNVVPCKTPDHPGWLELRLQLWPDGSTEEFLPEMAAACAEPDRFGQFLFLSPGGLAEGLVE---VALRTDYVNGTESSPVAFLEGVFVVPASR


B
K.pneumoniae (AAR18814.1)
ColE1-6kb (This study)
Thiomonas(SCC93141.1)
SH-Ancestral-2813 (This study)
Stenotrophomonas (AB894482)
P.aeruginosa (AB709942)


C
kanamycin
SH-116-Day0
SH-116-Day14
SH-2813-Day0
SH-2813-Day14
Tobramycin
Gentamicin
Neomycin
Netilmicin
Fosfomycin
No. of Isolates
Zone of Inhibition (mm)


Log gene copies/ gram dry weight
10
9
8
7
6
5
4
3
2
1
0
Con 1
Con 2
Con 3
Con 4
Back 1
Back 2
Back 3
Litter Type
IncX1-Rep
ColpVC-Rep
ColE1-6kb-Abi
ColE1-6kb-MSF
16S rDNA