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A unique megaplasmid contributes to stress tolerance and pathogenicity of an emergent *Salmonella enterica* serovar Infantis strain

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Summary

Of all known *Salmonella enterica* serovars, *S.* Infantis is one of the most commonly isolated and has been recently emerging worldwide. To understand the recent emergence of *S.* Infantis in Israel, we performed extensive comparative analyses between pre-emergent and the clonal emergent *S.* Infantis populations. We demonstrate the fixation of adaptive mutations in the DNA gyrase (*gyrA*) and nitroreductase (*nfsA*) genes, conferring resistance to quinolones and nitrofurans, respectively, and the carriage of an emergent-specific plasmid, designated pESI. This self-transferred episome is a mosaic megaplasmid (~280 kb), which increases bacterial tolerance to environmental mercury (*mer* operon) and oxidative stress, and provides further resistance to tetracycline, sulfamethoxazole and trimethoprim, most likely due to the presence of *tetRA*, *sulI* and *dfrA* genes respectively. Moreover, pESI carries the yersiniabactin siderophore system and two novel chaperone-usher fimbriae. *In vitro* studies established that pESI conjugation into a plasmidless *S.* Infantis strain results in superior biofilm formation, adhesion and invasion into avian and mammalian host cells. *In vivo* mouse infections demonstrated higher pathogenicity and increased intestinal inflammation caused by an *S.* Infantis strain harboring pESI compared with the plasmidless parental strain. Our results indicate that the presence of pESI that was found only in the emergent population of *S.* Infantis in Israel contributes significantly to antimicrobials tolerance and pathogenicity of its carrier. It is highly likely that pESI plays a key role in the successful spread of the emergent clone that replaced the local *S.* Infantis community in the short time of only 2–3 years.

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Introduction

Salmonella enterica is a facultative intracellular human and animal bacterial pathogen responsible for global pandemics of food-borne infections, posing a major threat to public health. This highly versatile pathogen can infect a broad range of hosts and cause different clinical outcomes (Rabsch *et al.*, 2001). The single species *S. enterica* includes more than 2600 serovars that share high sequence similarity and are taxonomically classified into six subspecies (Edwards *et al.*, 2002). Subspecies I serovars, adapted for mammals and avian hosts, are associated with more than 99% of all *Salmonella* infections in humans. The majority of non-typhoid *Salmonella* (NTS) infections in humans present as gastroenteritis; however, about 5% may be invasive and manifest as bacteremia or other extra-intestinal infections (Chen *et al.*, 2007). Many of the NTS serovars are capable of colonizing the intestines of livestock with potential risk of contaminating the food chain and therefore, salmonellosis is often associated with animal products and produce (Newell *et al.*, 2010).

Many of the *Salmonella* virulence factors are encoded on mobile elements with variable presence between different serovars and even across isolates within the same serovar (Hensel, 2004; Suez *et al.*, 2013). Genomic diversity between related bacteria is largely determined by gain of functions via horizontal gene transfer (HGT). Mobile and integrative genetic elements, including plasmids, bacteriophages, transposons, integrons, integrative and conjugative elements (ICEs), and pathogenicity islands are very important vehicles of HGT enabling transmission of

genetic information between bacteria. Horizontally acquired foreign DNA elements often encode genes that can profoundly affect the fitness, antimicrobial resistance (AMR) and the lifestyle of their bacterial host and therefore play a key role in bacterial evolution (Lawrence, 2005).

Salmonella enterica serovar Infantis (*S*. Infantis) is one of the most commonly isolated NTS serovars from clinical and veterinary sources. In the USA, *S*. Infantis was ranked ninth in the prevalence order in 2011, with 124 cases per 100,000 persons (CDC, 2012). In the EU, *S*. Infantis was ranked third immediately after serovars Enteritidis and Typhimurium (Anonymous, 2009). In recent years an emergence of *S*. Infantis has been reported in multiple countries around the globe including Belgium, France (Cloeckaert *et al*., 2007), Germany (Hauser *et al*., 2012), Hungary (Nogrady *et al*., 2008), Honduras (Liebana *et al*., 2004), Japan (Shahada *et al*., 2009), Australia (Ross and Heuzenroeder, 2008) and Israel (Gal-Mor *et al*., 2010).

The molecular mechanisms and the evolutionary processes involved in the emergence of new *Salmonella* strains are still largely enigmatic. Here, we applied whole genome sequencing and phenotypic comparisons between pre-emergent and emergent populations of *S*. Infantis. We demonstrate that the emergent strain is characterized by adaptive chromosomal mutations and a novel megaplasmid, which confers resistance to multiple drugs, heavy metals and disinfectants, but also enhances its virulence-associated phenotypes and its pathogenicity *in vivo*. These emergent-specific phenotypes seem to provide a significant advantage both in the environment and in the host, and therefore, most likely, contributed to the efficient dissemination and rapid spread of this particular *S*. Infantis clone.

Results

S. Infantis, which historically was not one of the five most prevalent serovars in Israel, has started to rapidly emerge between 2006-2007, and since 2009 (until, at least, the time of writing this report) is the most dominant serovar, comprising more than 33% of all Salmonellae isolates (Supporting Information Fig. S1). Recently, we showed that the emergent population is characterized by a distinct pulsed field gel electrophoresis (PFGE) pattern and a unique AMR profile (Gal-Mor *et al*., 2010). Here, to elucidate the genetic and evolutionary mechanisms involved in the dissemination of this particular *S*. Infantis clone, we have taken a comparative approach and studied genetic and phenotypic differences between pre-emergent strains (isolated during and prior to 2005) and recently emerged strains (isolated during and after 2007).

Phenotypic comparison between the pre-emergent and the emergent strains

To identify differences that may explain the rise and the spread of the emergent clone, we chose to compare a representative emergent isolate (no. 119944) that was isolated in 2008 and showed a typical emergent PFGE pulsotype and AMR profile to a pre-emergent strain (isolate no. 335-3) that was isolated in Israel in 1970. This isolate was sensitive to 16 examined antibiotics, as most of the pre-emergent isolates, and showed typical levels of biofilm formation and host cell adhesion that were similar to other pre-emergent isolates. Various phenotypic comparisons including growth in rich [Luria-Bertani, (LB)] and minimal (N-minimal pH 5.8) media, survival under nutrient-limiting conditions (phosphate-buffered saline at room temperature for 90 days), heat shock (55°C for 20 min), survival at 4°C (for 120 days), growth under dry conditions (37°C for 30 days), osmotic stress (6% NaCl), exposure to UV (312 nm), paraquat (100 mM), S-nitrosoglutathione (GNSO; 50 mM) and motility assays (0.3% agar plates for 5 h) did not show significant differences between the emergent and the pre-emergent strains (data not shown). To further screen for additional phenotypes, we have utilized the Biolog Phenotype MicroArray platform (Biolog, Hayward, CA) which allows quantitative testing of bacterial growth (cell respiration) under nearly 2000 culture conditions simultaneously (Bochner, 2009). Consistent with the results reported above, phenotypic arrays revealed no differences in carbon source utilization (190 compounds), and in the response to osmotic (96 conditions) and pH (96 conditions) stimuli (Supporting Information Fig. S2). Marginal growth advantage of the pre-emergent strain was found in the utilization of ammonia (Supporting Information Fig. S2 PM3, A2) and Leu-Met (Supporting Information Fig. S2 PM6, H11) as a nitrogen source and in the metabolism of several organic and inorganic phosphorus compounds (Supporting Information Fig. S2 PM4). These data suggest that both strains have similar growth requirements *in vitro* with only minor differences.

Nonetheless, the emergent strain presented a profound growth advantage in the presence of a wide array of antibiotic compounds from at least 12 different classes, including quinolones (e.g. nalidixic acid), dihydrofolate reductase inhibitor (trimethoprim), dihydropteroate synthase inhibitor (sulfisoxazole), nitro compounds (e.g. 2-nitroimidazole), aminoglycosides (e.g. streptomycin), tetracyclines (e.g. doxycycline) and others (Supporting Information Table S1), demonstrating a broad multidrug resistance (MDR) phenotype. These results are in close agreement and expand our previous antibiogram analysis showing that the emergent clone is resistant to nalidixic acid, nitrofurantoin, tetracycline and trimethoprim-sulfamethoxazole (SXT) (Gal-Mor *et al*., 2010).

The emergent strain harbors a self-conjugative megaplasmid conferring MDR phenotype

As many antibiotic resistance genes in *Enterobacteriaceae* are often encoded on self-transmissible or mobilizable plasmids (Carattoli, 2011), we tested possible plasmid-mediated resistance by conjugation experiments. Bacterial mating between the *Escherichia coli* J5-3 rifampin-resistant strain as a recipient and the emergent *S*. Infantis clone (isolate 119944) as the donor gave rise to transconjugants at a frequency of 4×10^{-6} that were resistant to tetracycline, sulfamethoxazole and trimethoprim, but not to streptomycin, nalidixic acid or nitrofurantoin (Gal-Mor *et al*., 2010 and Supporting Information Fig. S3A), indicating that the former three antibiotic resistance genes are encoded on a self-conjugative plasmid(s). To estimate the molecular size of the conjugative plasmid(s), we applied an S1 nuclease digest, which converts supercoiled plasmids into full-length linear molecules, followed by PFGE analysis (Barton *et al*., 1995). This approach demonstrated the presence of a single ~280 kb plasmid in the emergent strains (119944) and in the *E. coli* transconjugants, and the absence of plasmids from the pre-emergent (335-3) and recipient (J5-3) strains. *E. coli* R27 that was included as a control showed the expected presence of a single 168 kb plasmid (Supporting Information Fig. S3B). The exact same profile of a single ~280 kb plasmid was identified in other emergent isolates analyzed (e.g. isolate 122798; data not shown) and revised our previous estimation of the plasmid size (Gal-Mor *et al*., 2010). We concluded that tetracycline, sulfamethoxazole and trimethoprim resistance is conferred by a single self-transmissible plasmid, which was designated pESI (standing for plasmid of emerging *S*. Infantis).

Identification of the chromosomal loci responsible for nitrofurantoin and nalidixic acid resistance

Conjugation experiments suggested that the resistances to nalidixic acid and nitrofurantoin of the emergent clone are not encoded on the pESI plasmid and are therefore, chromosomally mediated. Currently, fluoroquinolones are one of the first-line drugs for treating severe *Salmonella* infections (Scallan *et al*., 2011). Resistance to quinolones and fluoroquinolones in Gram-negative bacteria is frequently associated with mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase (GyrA) (Griggs *et al*., 1996) or other topoisomerase subunits (Hopkins *et al*., 2005). Here we extended our previous analysis (Gal-Mor *et al*., 2010) and determined the DNA sequence of the QRDR in 13 emergent and 11 pre-emergent isolates from clinical, poultry and food sources. This analysis showed that 13/13 of the emergent isolates harbored a single nucleotide substitution from guanine to thymine at position 259 (G259T), resulted in the exchange of aspartic acid in position 87 to tyrosine (Asp87Tyr) and a nalidixic acid-resistant phenotype. Eight out of eleven pre-emergent strains had no change in the QRDR relative to the reference sequenced strain (*S*. Infantis strain SARB27) and were nalidixic acid-sensitive. Three pre-emergent isolates harbored as well at position 259, a guanine-to-adenine mutation (G259A) resulting in the exchange of the aspartic acid in position 87 to asparagine (Asp87Asn) and nalidixic acid resistance (Supporting Information Fig. S4). These results highlight the dominancy of point mutations that change amino acid 87 in GyrA in nalidixic acid-resistant strains of *S*. Infantis and show the fixation of a specific mutation in *gyrA* among the emergent isolates.

As opposed to nalidixic acid, *Salmonella* resistance mechanisms to nitrofurantoin are much less characterized. Some studies have suggested that in *E. coli*, nitrofuran resistance may be associated with mutations in the type I (oxygen insensitive) nitroreductase genes *nfsA* and *nfsB* (McCalla *et al*., 1978; Bryant *et al*., 1981; Sandegren *et al*., 2008). These nitroreductases are presumed to reduce the inactive compound of nitrofurans into a toxic intermediate with an antimicrobial activity (Peterson *et al*., 1979).

In agreement with this notion, sequence comparison of the *nfsA* gene in 13 emergent and 11 pre-emergent strains showed that 13/13 of the emergent strains contained an adenine-to-guanine substitution resulting in a nonsense mutation at position 159 of NfsA and in a nitrofurantoin resistance phenotype, while none of the nitrofurantoin-sensitive pre-emergent strains (11/11) carried this mutation (Fig. 1A). To examine whether a premature stop codon is indeed responsible for the resistance to nitrofurantoin, we complemented the resistant isolate (119944), containing a truncated version of NfsA, with an intact copy of *nfsA* cloned into an ampicillin-resistant, low-copy-number vector (pWSK29). As a control, we introduced an empty vector into this strain. While both strains were able to grow on LB or LB + nitrofurantoin (due to plasmid loss, in the absence of ampicillin selection), when the strains were plated on a medium supplemented with both nitrofurantoin and ampicillin, only the strain that carried the empty vector, but not the intact *nfsA* gene, was able to grow (Fig. 1B). These results established that nitrofurantoin is harmful to *S*. Infantis in the presence of a functional NfsA, and resistance can be mediated by a single-step nonsense mutation in this gene. To the best of our knowledge, this is the first report demonstrating the genetic basis of nitrofurantoin resistance in *Salmonella*.

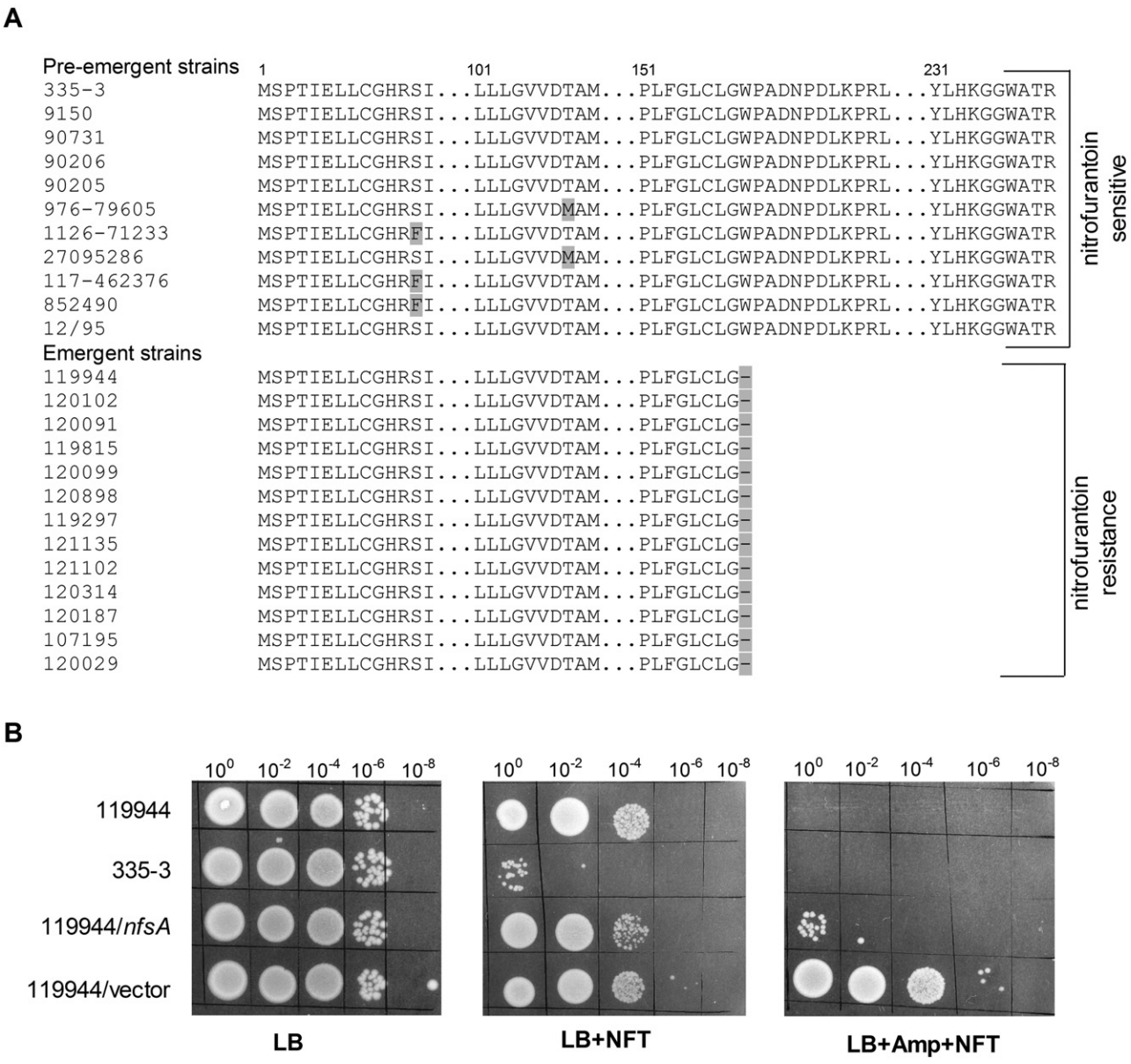


Fig. 1. A premature stop codon in the nitroreductase gene *nfsA* is responsible for nitrofurantoin resistance in emergent *S.* Infantis.
A. Amino acid sequence of NfsA from 11 pre-emergent and 13 emergent isolates. A stop codon at position 159 is indicated by the dash (–) sign. Polymorphism is highlighted in grey.
B. Serial dilutions of emergent (119944), pre-emergent (335-3) and emergent strain harboring a functional copy of *nfsA* (119944/*nfsA*), or the vector only (119944/vector) were plated on LB, LB supplemented with nitrofurantoin (LB + NFT) and LB supplemented with nitrofurantoin and ampicillin plates (LB + Amp + NFT). Bacterial growth after 16 h incubation at 37°C is shown.

pESI is a unique chimeric megaplasmid

To gain further information regarding genetic differences between pre-emergent and emergent strains, we applied a comparative genomics approach and sequenced the entire genome of 119944 and 335-3 strains to a draft level (see Materials and Methods). Pairwise comparison confirmed the presence of 119944-specific contigs that corresponded to the identified conjugative plasmid (pESI). Sequence analysis of pESI revealed that it is a previously uncharacterized plasmid, containing several modular genetic regions involved in plasmid transfer, maintenance, antimicrobial resistance and virulence. pESI harbors a leading and a transfer region that is highly similar to the corresponding region in the R64 plasmid (Sampei *et al.*, 2010) (AP005147; 97% identity over 72.7 kb), belonging to the incompatibility IncI1 group. The leading and the transfer region in pESI (Fig. 2) consists of

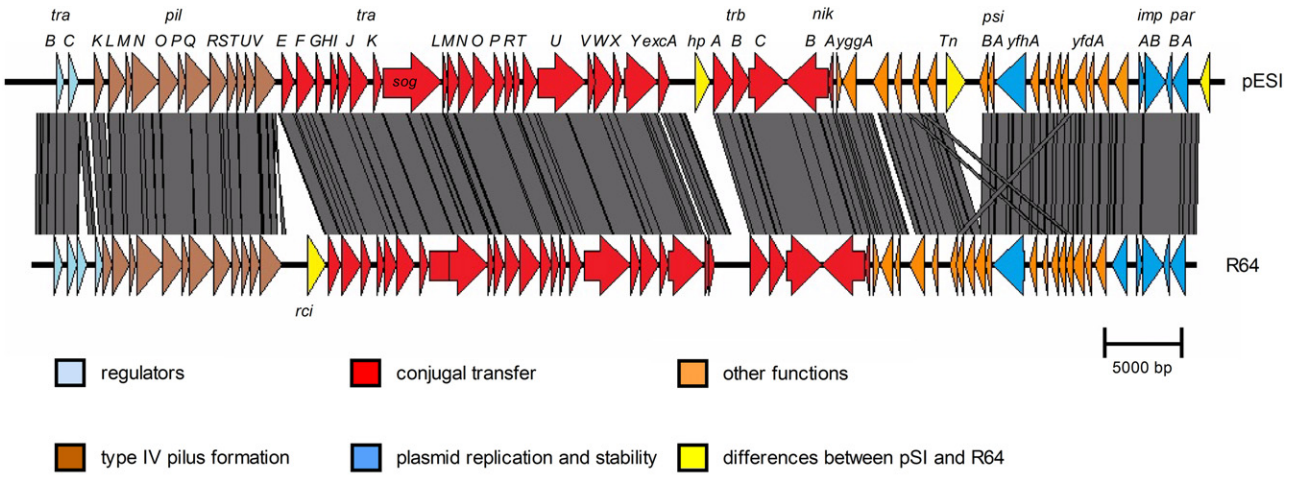


Fig. 2. The transfer region of pESI is homologous to IncI plasmids. Pairwise comparison of the transfer region of pESI and comparison to the incI1 R64 plasmid (AP005147 position 1-74713) was performed using the Easyfig tool. Homologous regions are colored in different shades of grey. Differences between pESI and R64 are shown in yellow.

six functional gene clusters including: (i) the regulatory operon *traBC*; (ii) the *pil* cluster (12 genes) encoding a type IV pilus; (iii) the *tra*/*trb* (25 genes) involved in the conjugal transfer; (iv) the *oriT* and the *nikAB* genes required for relaxome formation involved in plasmid transfer; (v) the leading region (20 genes from *yggA* to *impB*), containing the first DNA segment to enter the recipient cell during conjugation; and (vi) the *parAB* genes responsible for the active partitioning of the replicated plasmid during cell division. A noteworthy difference between the pESI and the R64 plasmids is the absence of Rci in pESI. Rci is a shufflon-specific recombinase that functions as a biological switch to select one of seven C-terminal segments of the PilV proteins in the R64 thin pilus (Sampei *et al*., 2010).

Surprisingly, sequence alignments (blastn) of distinct origin of replication (*ori*) probes from 18 different incompatibility plasmid groups (Carattoli *et al*., 2005) did not reveal a typical IncI1 origin of replication in pESI. Further examinations using PCR with conserved primers from the IncI1 origin of replication and a Southern blot analysis, using an IncI probe also failed to identify an IncI1 *ori* sequence in pESI (Supporting Information Fig. S5A). Instead, the replication origin and incompatibility region, *oriV* of IncP-1α plasmids (found in pBS228, AM261760; RK2, J01780; and pTB11, AJ744860; Supporting Information Fig. S5B) was found between the *merA* and *tetR* genes (see below). To experimentally determine the incompatibility nature of pESI, we further tested the ability of an IncP-1α (pRK2) and IncI1 (pCVM29188_101) plasmids to cohabitate with pESI. Conjugation experiments showed that while the IncP-1α plasmid was able to self-transfer to a *S*. Infantis host cell containing pESI, the IncI1 plasmid was not transferred at all to this strain, although it was readily conjugated into a plasmidless *S*. Infantis (Table 1). A successful coexistence of pESI and pRK2 plasmids in the same bacterial host cell as well as the lack of modules known to be involved in IncP plasmid replication and maintenance such as the TrfA (IncP replication initiator) and KorA/B (which control the copy number of the plasmid) (Adamczyk and Jagura-Burdzy, 2003) suggested that pESI does not belong to the IncP-1α class, while conjugation experiments demonstrated a clear IncI1 incompatibility. Nevertheless, the absence of a

Table 1. Conjugation frequencies of IncP-1α and IncI1 plasmids into *S*. Infantis strains.

	S. Infantis 335-3	*S*. Infantis 335-3/ pESI
IncP-1α (pRK2)	$3.5 \times 10^{-1} - 7.5 \times 10^{-1}$	$2.8 \times 10^{-2} - 7.9 \times 10^{-1}$
IncI1 (pCVM29188_101)	$3.2 \times 10^{-3} - 7.7 \times 10^{-3}$	0

The frequency of a successful trans-conjugation is shown. An azide-resistant derivative of *S*. Infantis 335-3 harboring or lacking pESI was used as the recipient strain in conjugation experiments with *E. coli* strains carrying pRK2 (Amp^R) or pCVM29188_101 (Amp^R) as the donors strains. Trans-conjugants were isolated on LB plates supplemented with 100 $\mu g\ ml^{-1}$ azide (to select for the recipient strain) and 100 $\mu g\ ml^{-1}$ ampicillin (to select for pRK2 and pCVM29188_101) after 6 h conjugation at 37°C. Trans-conjugation frequency was calculated as the ratio between the obtained $azide^R$+Amp^R CFU (transconjugants) and the donor strain CFU. Transconjugants resulted from conjugation into *S*. Infantis 335-3/ pESI were also tested for Tc^R phenotype to confirm the pESI stability. The results of two independent experiments are reported.

canonical IncI1 *ori* and the presence of a conserved IncP-1α *oriV* sequence indicate that pESI is a chimeric plasmid that evolved by recombination between at least two different (IncI1 and IncP-1α) ancestral plasmids.

The stability of pESI is presumed to benefit from the presence of three type II addiction (toxin–antitoxin) systems that were found in the plasmid. CcdAB and VagCD are organized in one cluster and a third addiction system termed MazEF/ PemKI is encoded in a different operon 33.5 kb away. Such systems are believed to contribute to plasmid maintenance and prevalence by postsegregational killing mechanisms during vertical transmission in the daughter cells (Van Melderen, 2010).

Antibiotics resistance, mercury tolerance and yersiniabactin siderophore genes are integrated into pESI

Plasmid sequence analysis showed that pESI presents a modular structure, in which several mobile genetic elements (MGEs) are integrated in intergenic regions between backbone modules. Upstream to the *traBC* genes, a region of 20.4 kb was found to harbor clusters of drugs and heavy metals resistance genes (Fig. 3A) comprised of two MGEs adjacently inserted. The left arm contains a transposon Tn21-like element with resistance genes to streptomycin and spectinomycin (*aad*A1), ethidium bromide and quaternary ammonium compounds (*qacEΔ1*), and a dihydropteroate synthetase (*sulI*) conferring sulphonamide resistance. This MGE also encodes a mercury resistance, *mer* operon, which is often linked to antibiotic resistance genes on the same mobile element (Davis *et al*., 2005). Like in the Tn21 transposon, the pESI *mer* operon consists of the regulatory genes *merR* and *merD* and the structural genes *merT*, *merP, merC* and *merA* (*urf2* and *merE* are unknown reading frames) (Liebert *et al*., 1999). On the right arm of this region, another mobile element similar to transposon Tn1721 is also integrated. This mobile element carries the Tn1721 tranposase *tnpA* gene and the *tet*AR operon, conferring resistance to tetracycline (Fig. 3A). Additional MGE harbored on pESI is a class I integron, encoding the gene *dfrA1* (conferring resistance to trimethoprim) and *orfC*, which can be found on the chromosome and in some plasmids of many *Salmonella* strains and other Gram-negative species.

pESI was also found to carry a region of ~29 kb containing the entire yersiniabactin (Ybt) biosynthetic, regulatory and transport operons (11 ORFs; Fig. 3B). Yersiniabactin is a siderophore-dependent iron uptake system essential for *Yersinia* virulence in mice and is often encoded on a MGE known as the High Pathogenicity Island (HPI). The HPI is found among *Enterobacteriaceae* virulent strains including *Citrobacter, Enterobacter, Klebsiella, Photorhabdus, Salmonella, Serratia,* and *E. coli* (Perry and Fetherston, 2011). In pESI, the yersiniabactin locus is associated with transposase genes on the 3′ end and possesses a putative IS element similar to members from the IS21 or IS256 families (found in *E. coli* and *Y. pestis*). These elements are presumed to mediate the *ybt* mobility and dissemination as an autonomous unit separately from the rest of the HPI. A semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis demonstrated the induction of *ybt* under iron-deficiency conditions (Fig. 3C), indicating that this operon is expressed from pESI and adequately regulated in the emergent *S*. Infantis strain.

To gain a broader perspective about the distribution of pESI and its MGEs among the *S*. Infantis population, we examined their prevalence in the pre-emergent and emergent strains. The results presented in Table 2 showed that all of the emergent strains (13/13), but none (0/11) of the pre-emergent strains harbored pESI backbone. Similarly, MGEs encoding *sulI*, *qacEΔ1* and the yersiniabactin genes were absent from all of the pre-emergent strains, but were present in all of the emergent isolates. The *tetAR* operon was found only in one pre-emergent isolate (1174-62376); and the *mer* operon was found in only two pre-emergent isolates (976-79605 and 1174-62376). In addition, 3/11 pre-emergent strains harbored class I integrons, but these were different from the pESI integron: two isolates (1174-62376 and 852-490) harbored an identical 1.2 kb integron encoding the *aadA1* gene, and isolate 976-79605 carried a 2 kb integron encoding aminoglycoside 6′-N-acetyltransferase (*aac6-Il*) and OXA-21 beta-lactamase (*bla*OXA-21). Collectively this analysis demonstrated that none of the pre-emergent strains carried the pESI backbone and that an independent distribution of pESI-integrated MGEs was sporadic and uncommon prior to the dissemination of pESI in the emergent *S*. Infantis population.

The contribution of pESI to mercury and oxidative stress tolerance

Several genes encoded on the pESI plasmid including the *mer* operon and the *qacEΔ1* gene suggested that in addition to antibiotic resistance, this plasmid may confer bacterial resistance to other microbicides and toxic compounds including mercury. Growth studies in the presence of 25 mM mercury clearly showed that while the growth of the pre-emergent 335-3 strain was completely inhibited, the growth of the emergent strain was not affected (Fig. 4A). To examine the contribution of the pESI to *S*. Infantis fitness in the presence of heavy metals, the pESI plasmid was transferred by conjugation into an azide-resistant derivative of the pre-emergent strain (335-3 Az^R).

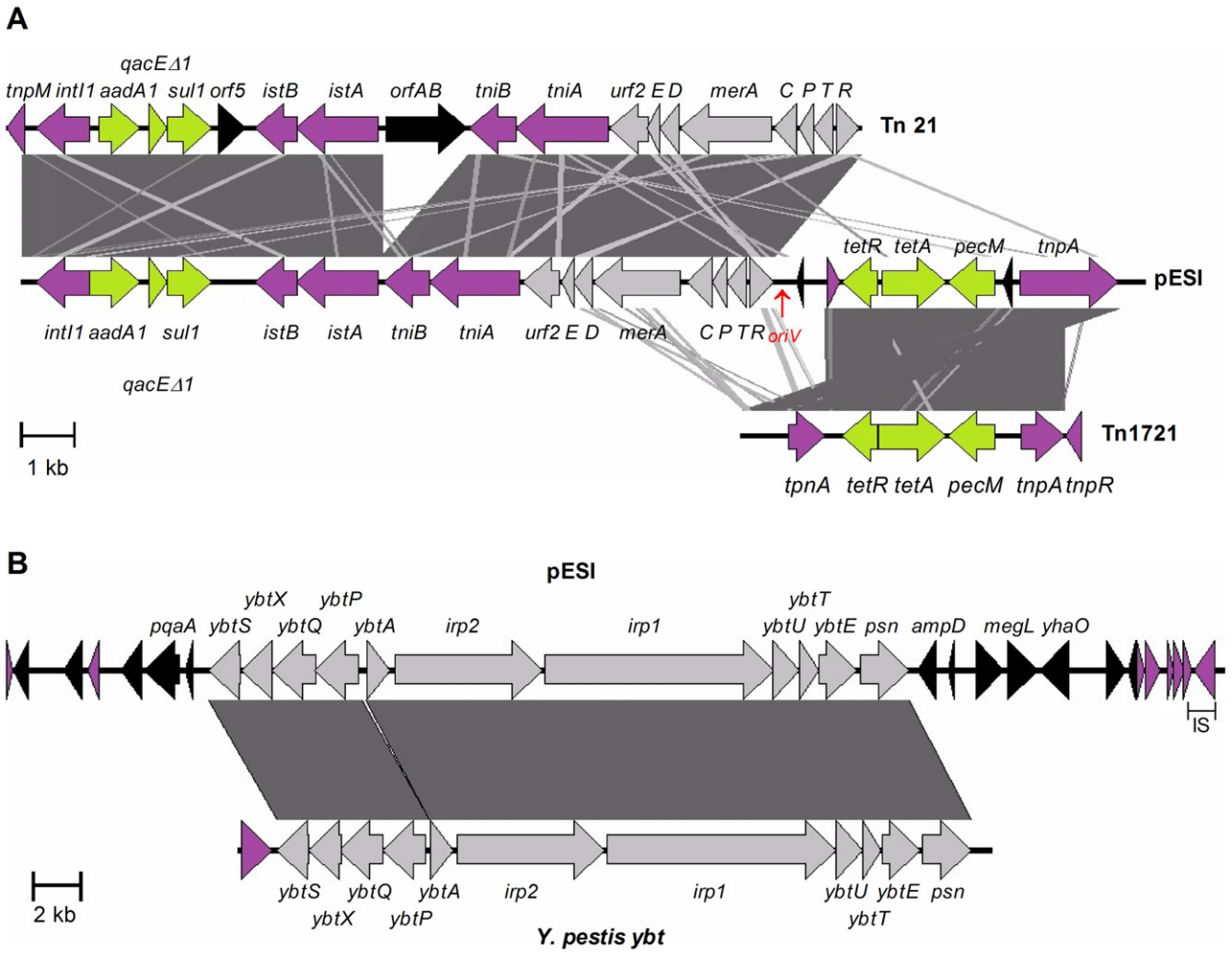


Fig. 3. Integration of antimicrobial resistance, mercury tolerance (*mer*) and yersiniabactin (*ybt*) modules into pESI.
A. The presence of resistance genes and comparison with Tn21 (AF071413 positions 3689–19672) and Tn1721 (JX424423 positions 91911–98279) transposons is shown. Mobile element genes are represented by purple arrows, antimicrobial resistance genes are shown in green and the *mer* genes in grey. The location of an IncP *oriV* is indicated by a red arrowhead.
B. Pairwise comparison of the yersiniabactin (*ybt*) biosynthetic, regulatory and transport operons of *Yersinia pestis* (AF091251 position 1501-32801) and the corresponding region in pESI is shown. Mobile element genes are shown in purple, the *ybt* genes in grey, and IS element is shown by a horizontal bar.
C. Semi-quantitative RT-PCR analysis of *irp2* and *rpoD* transcripts. *S.* Infantis 119944 RNA was extracted from cultures grown in N-minimal medium under iron-deficient conditions (–iron) or under iron-enriched conditions in the presence of 20 μM $FeCl_3$ (+iron). RT-PCR reactions with genomic DNA (gDNA) or with purified RNA only (-RT) were included as positive and negative controls respectively.

Table 2. Distribution of pESI and its mobile genetic elements among emergent and pre-emergent isolates.

Isolate	pESI backbone	*tetAR*	*sulI*	*qacEΔ1*	*mer*	class I integron	yersiniabactin
Pre-emergent strains							
9150	–	–	–	–	–	–	–
90731	–	–	–	–	–	–	–
90206	–	–	–	–	–	–	–
335–3	–	–	–	–	–	–	–
90205	–	–	–	–	–	–	–
976–79605	–	–	–	–	+	2 kb (*aac6-II*, *bla*OXA-21)	–
1126–71233	–	–	–	–	–	–	–
270–95286	–	–	–	–	–	–	–
1174–62376	–	+	–	–	+	1.2 kb (*aadA1*)	–
852–490	–	–	–	–	–	1.2 kb (*aadA1*)	–
12/95	–	–	–	–	–	–	–
Emergent strains							
120102	+	+	+	+	+	1 kb (*dfrA1*)	+
120091	+	+	+	+	+	1 kb (*dfrA1*)	+
119815	+	+	+	+	+	1 kb (*dfrA1*)	+
120099	+	+	+	+	+	1 kb (*dfrA1*)	+
119944	+	+	+	+	+	1 kb (*dfrA1*)	+
121135	+	+	+	+	+	1 kb (*dfrA1*)	+
121102	+	+	+	+	+	1 kb (*dfrA1*)	+
120314	+	+	+	+	+	1 kb (*dfrA1*)	+
120187	+	+	+	+	+	1 kb (*dfrA1*)	+
119297	+	+	+	+	+	1 kb (*dfrA1*)	+
107195	+	+	+	+	+	1 kb (*dfrA1*)	+
120898	+	+	+	+	+	1 kb (*dfrA1*)	+
120029	+	+	+	+	+	1 kb (*dfrA1*)	+

The presence of the pESI backbone, *tetAR*, *sulI*, *qacEΔ1,* class I integron, the *mer* operon (*merA*), and yersiniabactin (*irp2*) were examined by PCR using the primers listed in Table S3. Obtained PCR products of class 1 integrons were subsequently sequenced to determine their AMR gene content. To maximize specificity, pESI backbone was screened by primers corresponding to the ORF (hp) upstream to *trbA* (similar to hypothetical protein from *E. coli* KO11FL, YP_005280318), which was not found in any other plasmid in the database. All isolates were also plated on to LB-agar plates supplemented with tetracycline (20 μg ml^{-1}), sulfamethoxazole (50 μg ml^{-1}) and trimethoprim (50 μg ml^{-1}) for phenotypic conformation.

Mercury growth inhibition of the pre-emergent strain was relieved by introducing pESI into this strain (Fig. 4A). As a control, we also studied bacterial growth in the presence of a different heavy metal, cadmium. In contrast to mercury, propagation of all *S*. Infantis strains was equally repressed by 700 μM of cadmium (Fig. 4B), demonstrating the specific beneficial effect of pESI in the presence of toxic mercury.

Similarly, survival experiments of *S*. Infantis strains in the presence of bactericidal concentrations of hydrogen peroxide (40 mM) showed that the emergent strain is significantly more resistant than the pre-emergent strain (Fig. 4C). The enhanced resistance to hydrogen peroxide could be genetically acquired by introducing pESI into the pre-emergent strain (Fig. 4D), indicating that the plasmid can also confer improved resistance to hydrogen peroxide, perhaps by the efflux system that mediates antiseptic resistance encoded by the *qacEΔ1* gene.

pESI harbors two unique chaperon-usher fimbriae

In pathogenic bacteria fimbriae often function as virulence factors, which play an important role in attachment to surfaces, biofilm formation, adhesion to and infection of host cells (Thanassi *et al*., 2012). Sequence analysis identified the presence of two chaperone-usher fimbria clusters, located about 6 kb from each other on pESI. The chaperone-usher secretion pathway, commonly used by Gram-negative bacteria, consists of a dedicated periplasmic chaperone and an integral outer membrane assembly platform known as the usher. The larger chaperone-usher fimbria cluster on pESI spans ~9.3 kb and encodes a putative K88-like fimbria. This cluster harbors putative usher (*feaD*), chaperon (*feaE*), the major subunit (*feaG*), and minor rod subunits (*feaCFHI*), an unknown reading frame (*orf*) and regulatory genes (*feaAB*, Fig. 5A). Interestingly, this cluster also contains additional truncated copies of the regulatory gene *feaA* and the structural *feaJ* gene (*feaA´* and *feaJ´* respectively). Nucleotide blast analysis showed partial homology to fimbriae operons encoded on various plasmids of *S.* Dublin (CP001143, DQ115388), *S.* Pullorum (JN885081), *S*. Gallinarum (AF005899), *Shigella sonnei* (HE616529.1) and *E. coli* (AP009243.1, CP002730.1, CP002968.1) strains.

The second, smaller fimbria cluster of 5.1 kb encodes a previously uncharacterized chaperone-usher fimbria, which we designated Infantis plasmid-encoded fimbria

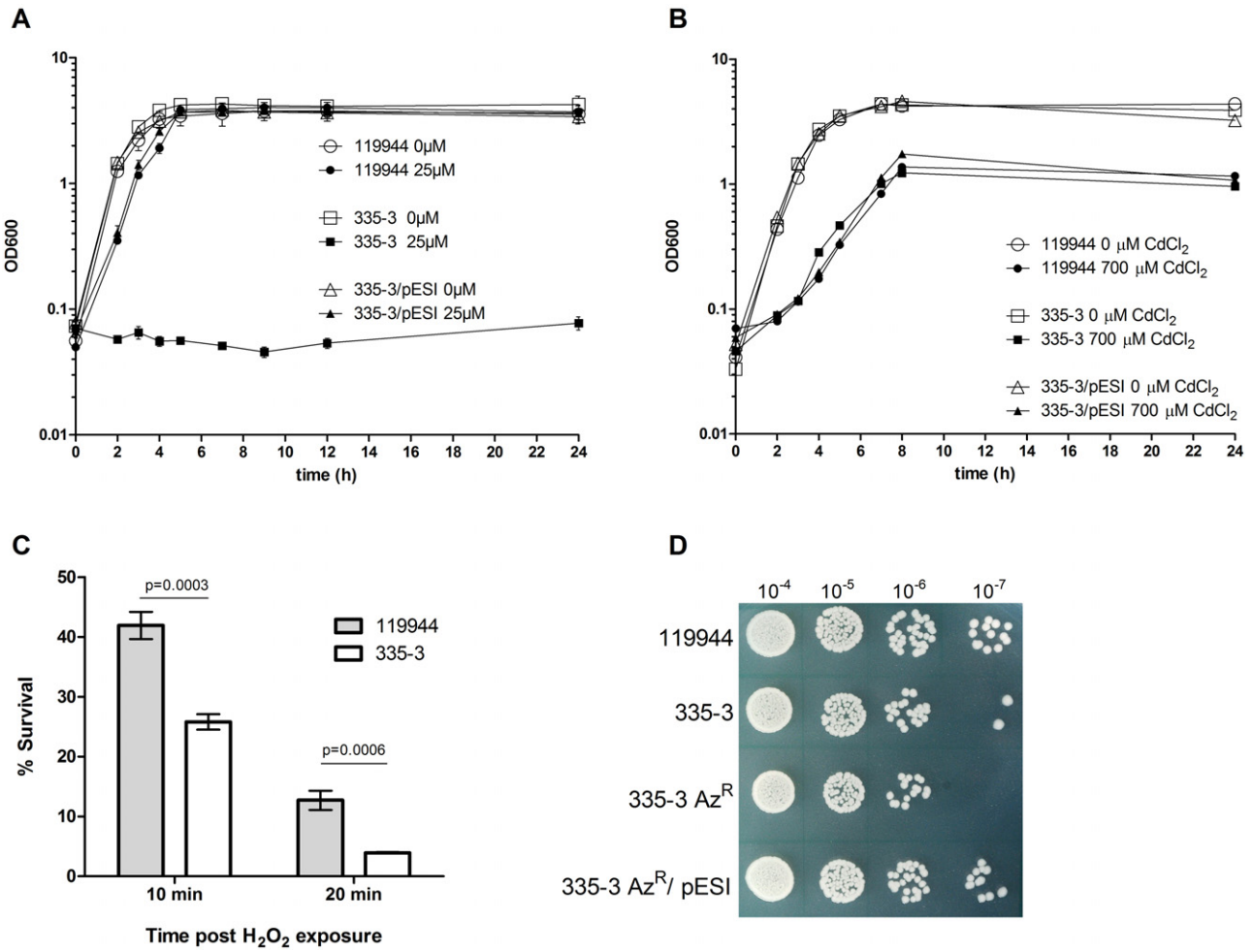


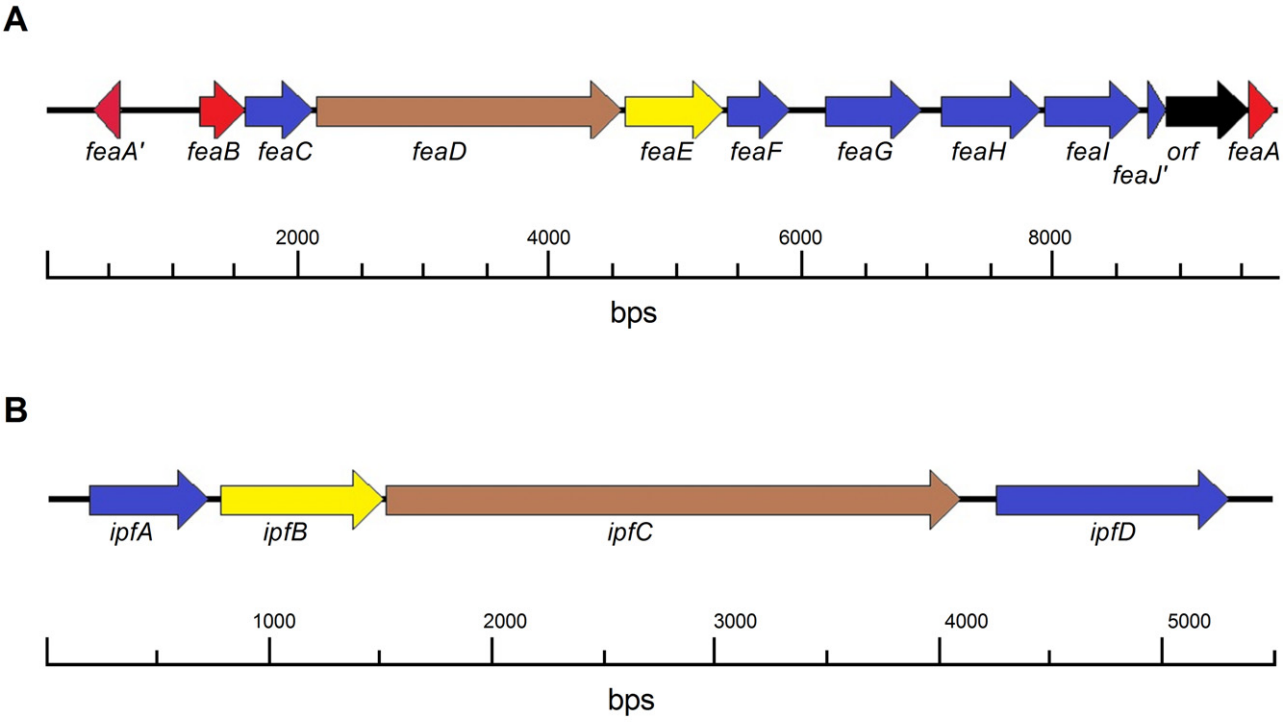
Fig. 4. pESI confers resistance to mercury and hydrogen peroxide.
A. Pre-emergent (335-3), emergent (119944) and azide-resistant derivative of the pre-emergent strain harboring the pESI plasmid (335-3/pESI) were grown in minimal medium in the absence and presence of 25 μM $HgCl_2$. Optical density (OD600) values at each time point are shown as the mean ± standard error of the mean (SEM) of three biological repeats (cultures).
B. The same strains were grown in minimal medium in the absence and presence 700 μM CdCl2.
C. Survival of the emerging and pre-emergent strains in the presence of 40 mM H_2O_2. Results show the average of five repeats in a representative experiment.
D. The emergent strain(119944), pre-emergent(335-3), azide-resistant derivative (335-3 Az^R) and 335-3 Az^R harboring the plasmid were exposed to 40 mM H_2O_2 for 20 min. Serial dilutions were plated on LB plates and incubated for 16 h at 37°C for bacterial enumeration.

(*ipf*). This cluster contains four ORFs encoding a putative usher (*ipfC*), a chaperon (*ipfB*), a rod subunit (*ipfA*) and a fimbrial adhesin subunit (*ipfD*, Fig. 5B). Blastn analysis identified partial homologous sequences in the integrative and conjugative element, ICESe3 in *S. enterica* subsp. VII strain SARC16 (FN298495) and in *S. enterica* subsp. arizonae (subsp. IIIa; CP000880.1). Therefore, this is the first report describing a genetically related fimbria in an *S. enterica* subsp. I isolate. Expression studies using semi-quantitative RT-PCR showed that both pESI-encoded fimbriae are readily expressed under *in vitro* conditions (growth in LB to the late logarithmic phase), known to induce *Salmonella* invasion (Ibarra *et al.*, 2010), as shown in Fig. 5C.

pESI increases biofilm formation, adhesion and invasion into host cells

Phenotypic comparison between five pre-emergent isolates (335-3, 1126-71233, 90205, 90206 and 90731) and five emergent isolates (119815, 119944, 121135, 121102 and 120314) showed superior biofilm formation by the emergent strains (Fig. 6A). Similarly, the emergent strains inclined to adhere better to human epithelial cells (HeLa) than most of the pre-emergent strains examined (Fig. 6B).

Based on the above results, we speculated that pESI may play a role in these virulence-associated phenotypes. As *S.* Infantis is often associated with poultry



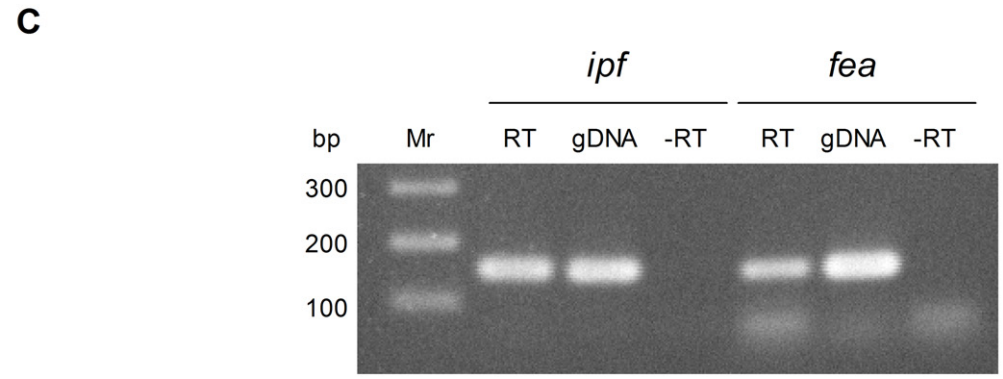


Fig. 5. Gene organization and expression of the K88-like and Ipf fimbriae.
A. K88-like pESI-encoded fimbria operon.
B. Ipf pESI-encoded fimbria operon.
Usher genes are shown in brown, chaperons in yellow, fimbria subunits in blue and regulatory genes in red.
C. Semi-quantitative RT-PCR analysis of *feaD* and *ipfC*. RT-PCR reactions with genomic DNA (gDNA) or with purified RNA only (-RT) were included as positive and negative controls respectively.

(Cloeckaert *et al*., 2007; Nogrady *et al*., 2008; Shahada *et al*., 2009; Gal-Mor *et al*., 2010), we also included in the assays below avian fibroblasts (CEF-DF1 cells) and studied biofilm formation, adhesion and invasion of the azide-resistant pre-emergent strain carrying the plasmid (335-3 Az^R/pESI) in comparison to a plasmidless strain (335-3 Az^R). Clearly, introducing pESI into the 335-3 Az^R strain resulted in a significant increase in biofilm formation (Fig. 7A), adhesion (Fig. 7B and C) and invasion into host cells (Fig. 7D and E). These results indicated that certain genes encoded on the plasmid are playing an important role in these virulence-associated phenotypes and therefore are expected to provide a significant advantage during host infection as well as in the environment because of an improved biofilm formation.

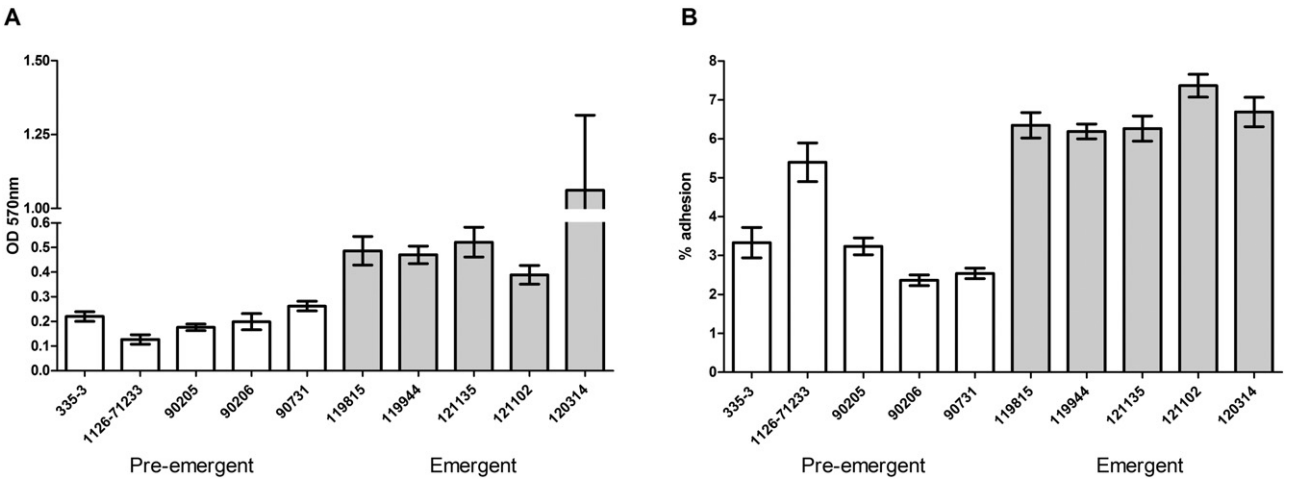


Fig. 6. Biofilm formation and host cell adhesion by emergent and pre-emergent strains.
A. Biofilm formation of five pre-emergent isolates (335-3, 1126-71233, 90205, 90206, and 90731) and five emergent isolates (119815, 119944, 121135, 121102, and 120314) was compared after 48 h growth in cholesterol-coated tubes. The mean ± SEM of six biological repeats is shown for a representative experiment.
B. Adhesion of the above strains to human epithelial HeLa cells was studied in the presence of cytochalasin D as explained in the Experimental Procedures. The proportion of adhered bacteria from the infected inoculum (% of adhesion) is shown as the mean ± SEM of four biological repeats of a representative experiment.

pESI contributes to S. *Infantis pathogenicity* in vivo

To examine the contribution of pESI to the virulence of the emergent strain *in vivo*, we used the streptomycin-pretreated mouse model of salmonellosis (Barthel *et al*., 2003). C57BL/6 mice were infected intragastrically with the azide-resistant derivative of the pre-emergent strain (335-3 Az^R) and with this strain harboring the pESI (335-3 Az^R/pESI). At one day post infection, *S*. Infantis colonization of the pESI harboring strain in the cecum, colon and ileum was higher than the colonization of the parental strain (Fig. 8A). Also, the cecum weight of the mice infected with the 335-3 Az^R/pESI strain was significantly lower than the one in mice infected with the 335-3 Az^R strain, indicating more severe inflammation in the former (Fig. 8B). To evaluate histopathological changes, tissues were fixed in formalin and embedded in paraffin. Sections were stained with H&E and assessed for pathological changes. As expected, cecal tissues from 335-3 Az^R/pESI-infected mice showed significantly higher inflammation and more severe tissue damage (Fig. 8C), (evidenced by a moderate submucosal edema, increased inflammatory cell infiltrate in the submucosa and lamina propria, mucosal hyperplasia, crypt destruction and focal epithelial desquamation (Fig 8 D–E). Similar results were also obtained when we compared the emergent 119944 isolate to the wild type (i.e. not azide-resistant) pre-emergent (335-3) strain (Supporting Information Fig. S6). These results demonstrated clear differences in the virulence of the *S*. Infantis strains and established that the presence of pESI enhances the virulence and the ability to induce inflammation.

Discussion

An emerging pathogen is defined as the causative agent of an infectious disease, which appears in a new host population or whose incidence is increasing in an existing population as a result of long-term changes in its epidemiology (Cleaveland *et al*., 2007). Approximately 160 newly emerging infectious diseases caused by bacterial pathogens have been discovered in the past 7 decades (Jones *et al*., 2008), including the emergence of multiple-antibiotic resistant strains of *S.* Typhimurium, such as the definitive phage type (DT) 104 (Rabsch *et al*., 2001; Velge *et al*., 2005). A better understanding of the evolutionary processes leading to the emergence of such pathogens is essential to improve prevention and intervention strategies against future emerging pathogens.

Our previous work reported a sudden emergence of *S*. Infantis in Israel resulting from the efficient spread of just one clone of *S*. Infantis rather than the dissemination of several biotypes simultaneously (Gal-Mor *et al*., 2010). This local emergence of a new *S*. Infantis clone could be a part of a wider phenomenon and a global increase in *S*. Infantis incidence observed recently in other countries as well (Liebana *et al*., 2004; Cloeckaert *et al*., 2007; Nogrady *et al*., 2008; Ross and Heuzenroeder, 2008; Shahada *et al*., 2009; Hauser *et al*., 2012).

In the present study, we have applied whole genome sequencing and other comparative approaches to identify adaptive changes in the emergent clone. The ability to compare the emerged *S*. Infantis population to multiple local pre-emergent strains (isolated from 1970 to 2005) provided a unique opportunity to identify the genetic

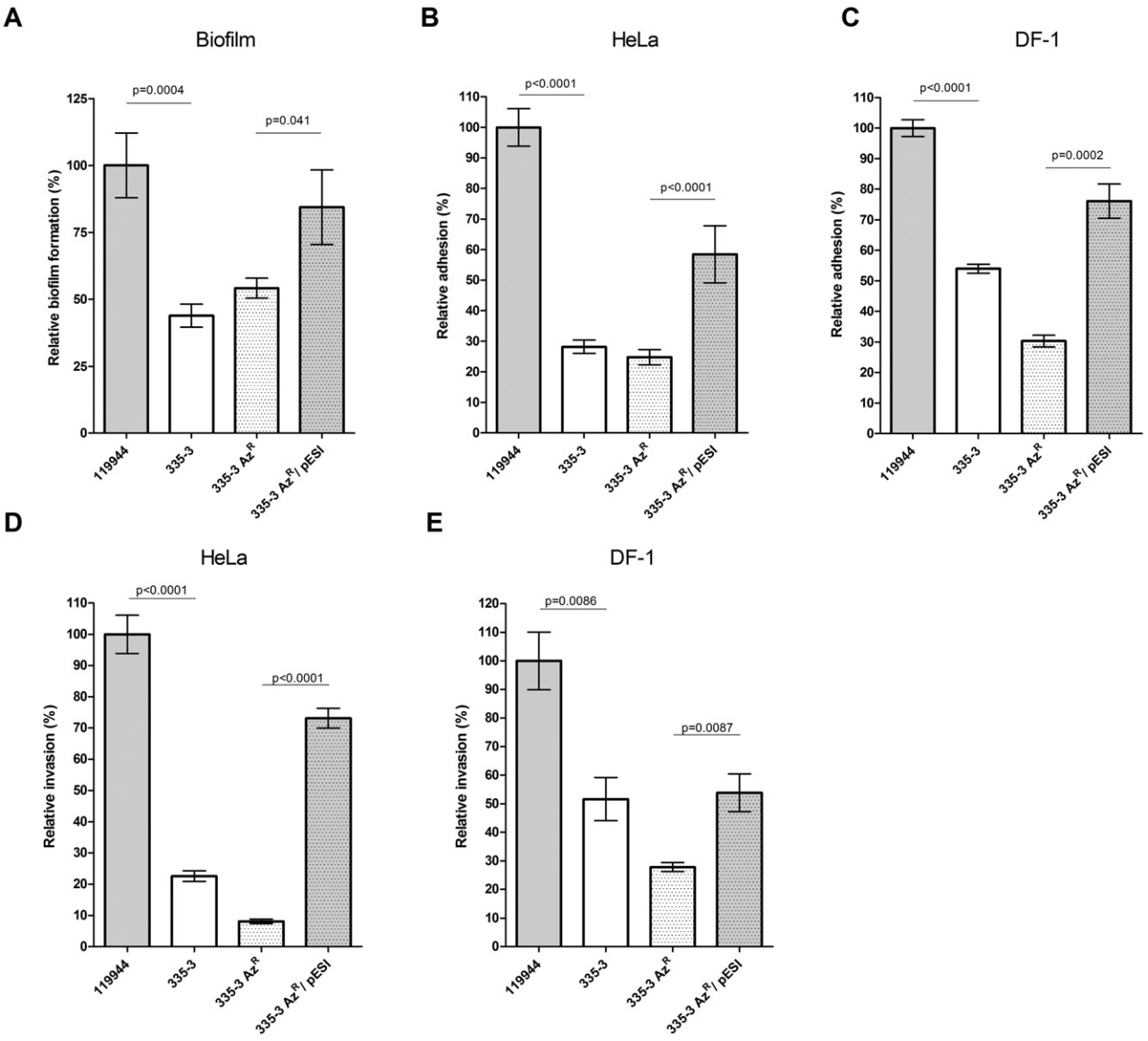


Fig. 7. Enhanced virulence-associated phenotypes of the emergent strain are mediated by pESI.
A. Biofilm formation of the emergent (119944), pre-emergent (335-3), azide-resistant derivative of 335-3 (335-3 Az^R) and the 335-3 Az^R strain containing pESI (335-3 Az^R/pESI) were compared after 48 h growth in cholesterol coated tubes.
B. Adhesion of the above strains to human epithelial HeLa cells in the presence of cytochalasin D.
C. Adhesion of the above strains to CEF-DF-1 chicken fibroblasts.
D. Invasion of *S.* Infantis strains to HeLa host cells.
E. Invasion of *S.* Infantis strains to CEF-DF-1 cells.
The relative phenotypes in panels A-E are shown in relation to *S.* Infantis 119944 and represent the mean of at least four biological repeats of a representative experiment.

changes that were selected during the emergence and to study the molecular mechanisms underlying this process.

HGT of foreign DNA coding for novel phenotypes is considered a key player in the rapid evolution of bacterial pathogens (Ochman *et al.*, 2000; Ochman and Moran, 2001). Our study provides an excellent up-to-date experimental proof for this concept, revealing the emergent-specific, previously uncharacterized mega-plasmid, termed pESI.

Interestingly, pESI exhibits a mosaic structure that evolved by recombination between at least two ancestral plasmids and multiple insertions of mobile genetic units into its backbone. As a result, pESI harbors an *oriV* of IncP-1 α plasmids (Supporting Information Fig. S5), but the transfer region of an IncI1 plasmids (Fig. 2). In

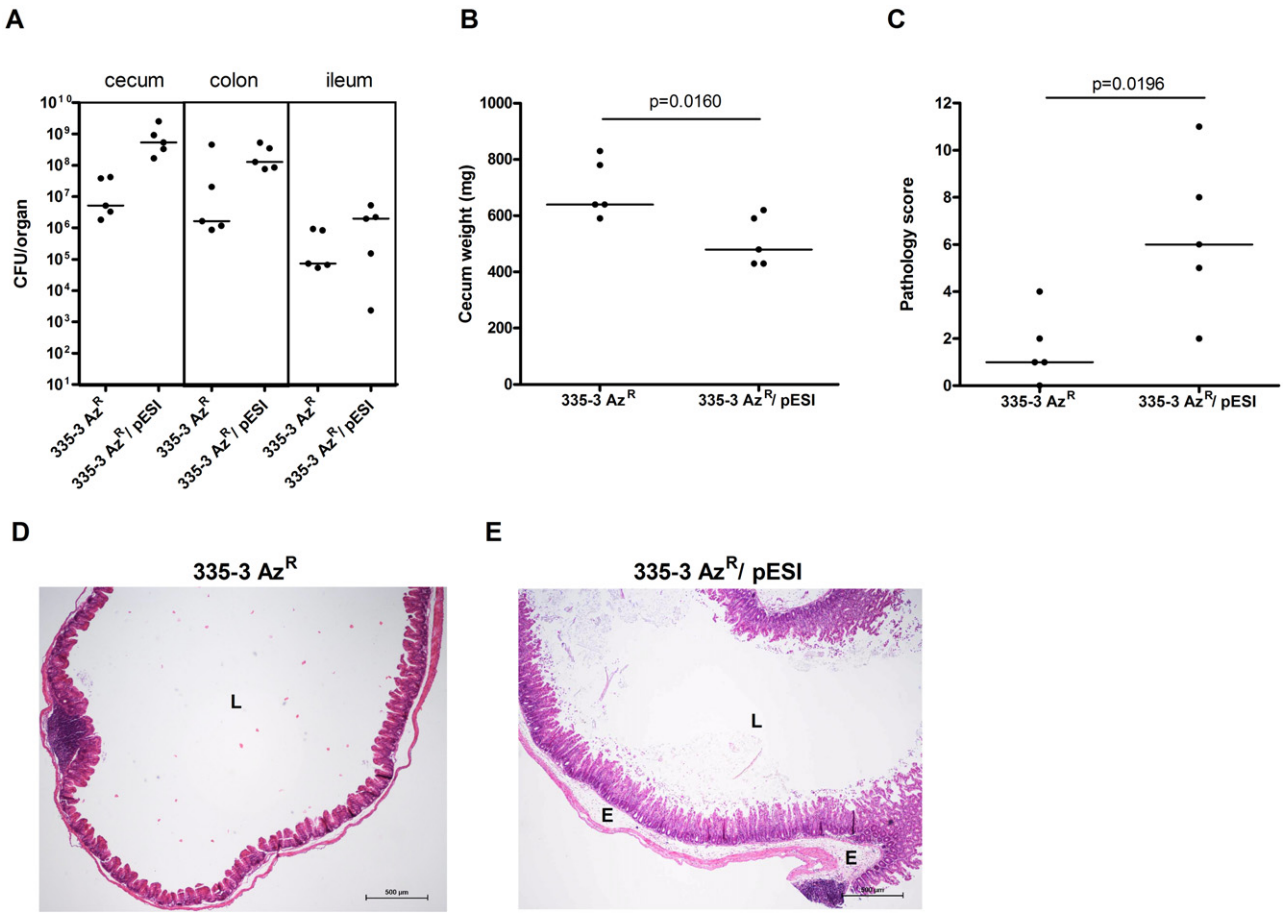


Fig. 8. The carriage of pESI increases *S.* Infantis pathogenicity *in vivo*. Two groups of five female C57BL/6 mice were treated with streptomycin and 24 h later infected orally with the azide-resistant derivative of the pre-emergent strain (335-3 Az^R) and with this strain harboring the pESI (335-3 Az^R/pESI).
A. At 24 h post infection, colonization and bacterial burden were examined at gastrointestinal sites (cecum, colon and ileum).
B. Cecum weight was determined.
C. Pathology scoring (0–25) was performed for H&E stained sections of the cecal mucosa.
D–E. Representative micrographs at an original magnification of 40× are shown. Bar = 500 μm. L indicates the lumen and E indicates submucosal edema.

addition, it contains various mobile elements encoding AMR genes, a mercury resistance operon and the yersiniabactin siderophore (Fig. 3). We specifically showed that the presence of the plasmid provides resistance to several antibiotic compounds including tetracycline, sulfamethoxazole and trimethoprim (Supporting Information Fig. S2), likely due to the *tetRA*, *sull* and *dfrA* genes respectively. Although the *aad*A1 gene (encoding streptomycin adenyltransferase) was also found in pESI, conjugation experiment showed that streptomycin-resistant phenotype is not linked to the pESI transfer. This might be explained by the presence of a lysine to arginine substitution identified at position 41 of this protein in comparison to homologous proteins (e.g. NP_052897) and the presence of a paralogous gene (E-value 2e-64; 59% similarity) in the chromosome. Additional phenotypes mediated by this plasmid are resistant to toxic concentrations of inorganic mercury, conferred by the *mer* operon, and increased hydrogen peroxide tolerance (Fig. 4).

Besides the yersiniabactin siderophore and resistance to heavy metals, antibiotics and antiseptics, we revealed that pESI encodes at least two previously uncharacterized chaperon-usher fimbria operons (a K88-like and the Ipf fimbria; Fig. 5), which may play a role in host tropism and pathogenicity. Interestingly, porcine enterotoxigenic *E. coli* (ETEC) strains harboring the K88 fimbria were shown to be associated with enteritis and edema disease in swine, and were found to possess an enhanced ability to adhere to porcine intestinal epithelia (Nagy and Fekete, 2005). Indeed, *In vitro* assays showed that the emergent clone presents eminent biofilm formation and adhesion to mammalian host cells (Fig. 6). Furthermore, we demonstrated that these fimbria clusters are readily expressed under conditions known to induce *Salmonella* invasion

(Ibarra *et al.*, 2010) and that a functional transfer of the plasmid to a pre-emergent strain significantly enhanced biofilm formation, adhesion and invasion into host cells (Fig. 7). Notably, *in vivo* infection experiments using the streptomycin-pretreated mouse model demonstrated higher pathogenicity and increased intestinal inflammation caused by a *S.* Infantis strain carrying pESI in comparison with its parental plasmidless strain (Fig. 8), possibly due to the activity of the siderophore yersiniabactin and/or the identified chaperone-usher fimbriae. Collectively, our results showed an eclectic combination of genes encoded on the plasmid that increase *S.* Infantis fitness under various environmental conditions, but also affect host–pathogen interactions and enhance *S.* Infantis pathogenicity in a plasmid-dependent manner.

Aside from plasmid-encoded AMR genes, we identified two point mutations on chromosomal loci that also contribute to the MDR phenotype of the emergent strain. We showed that a single nucleotide substitution from guanine to thymine at position 259 (G259T) in *gyrA* resulted in the exchange of aspartic acid in position 87 to tyrosine (Asp87Tyr) and is associated with nalidixic acid resistance phenotype (Supporting Information Fig. S4). Additionally, we demonstrated, for the first time in *Salmonella*, that a point mutation in the nitroreductase gene, *nfsA*, resulted in a premature stop codon at position 159 of NfsA, conferring resistance to nitrofurantoin (Fig. 1). Therefore, we concluded that the overall MDR phenotype (for tetracycline, sulfamethoxazole, trimethoprim, nalidixic acid and nitrofurantoin) characterizing the emergent strain is conferred by both adaptive point mutations in the chromosome and HGT event of genes from exogenous origin.

Antimicrobial compounds are widely used in medicine and agriculture, not only to treat and prevent infections in animal and produce, but also as growth promoters in poultry and other livestock (McEwen and Fedorka-Cray, 2002). The fact that the multiple resistance phenotypes were positively selected during *S.* Infantis emergence suggests that the acquisition of antibiotic resistance is likely necessary to survive in the ecological niches and habitats of *S.* Infantis. This finding emphasizes both the role and the risk of the selective pressure imposed by overuse and misuse of antimicrobials and the potential of concomitant selection of virulence factors encoded in the same elements with the AMR genes.

The fitness cost of antimicrobial resistance is debatable. Some studies have suggested that certain mechanisms, in particular those conferring resistance to fluoroquinolones in *S.* Typhimurium, claim a significant fitness cost and could therefore be counter-selected under an antibiotic-free environment (Giraud *et al.*, 2003). Growth studies under various conditions showed very similar growth of both pre-emergent and emergent strains (Supporting Information Fig. S2 and Fig. 4) and therefore do not support the existence of a significant fitness cost in the case of pESI. Considering the MDR phenotype conferred by pESI, its contribution to virulence and the likely lack of a fitness cost, the circulation of this plasmid or its derivatives in the environment may pose a considerable health risk due to the potential of its transfer to other Salmonellae or other bacterial species. Two factors that may enhance this risk are the plasmid stability within the bacterial host and the chance of plasmid transfer via conjugation. Sequencing analysis showed the presence of at least three independent addiction systems that are involved in plasmid integrity and stability (MazEF/PemKI, CcdAB and VagCD) in pESI. At least under laboratory conditions, repeated passages of the 119944 strain under antibiotic-free selection at 45°C did not lead to plasmid loss (data not shown), indicating its stability even in the absence of selective pressure. Additionally, we showed that the conjugation frequency of pESI was 4×10^{-6} between *Salmonella* and *E. coli* species and 4×10^{-5} between different strains of *S.* Infantis, demonstrating the potential risk of inter and intra-species transfer of this plasmid.

The role of plasmid acquisition in the evolution of pathogens is fundamental. Plasmid gain by different bacteria has allowed the occupation of new ecological niches, the exploration of new hosts, and the rise of new adapted pathogens. One of the most known examples is the speciation of *Shigella* spp. from nonpathogenic *E. coli* through the acquisition of a large mosaic virulence plasmid (pINV). This plasmid is an essential virulence determinant and encodes the molecular machinery necessary for tissue invasion and the intracellular lifestyle of *Shigella* (Schroeder and Hilbi, 2008). *Pantoea agglomerans* is another example of a bacterium associated with many plants as a common epiphyte and endophyte that has evolved into a host-specific (gypsophila and beet) tumorigenic pathogen by acquiring a plasmid (pPATH) containing the genes required for gall formation and host specificity (Barash and Manulis-Sasson, 2009). The pathogenic gram-positive bacteria *Bacillus anthracis* is also believed to have evolved from the common soil bacterium *Bacillus cereus* by acquiring two virulence plasmids (pXO1 and pXO2), both of which are required by the bacterium to cause anthrax (Pilo and Frey, 2011).

Here, we demonstrated how the presence of a self-transmissible, autonomous and mosaic genetic element provides new phenotypic traits and largely contributes to the fitness of its bacterial host including resistance to multiple antibiotics and biocides, heavy metal tolerance and enhancement of virulence. Our results suggest that pESI carriage, accompanied by adaptive chromosomal mutations, facilitated the emergence of a fitter, resistant and more virulent strain. This specific clone rapidly

replaced the local *S*. Infantis population in a short time of 2–3 years (Supporting Information Fig. S1). Taken all together, these data reveal a very recent evolutionary change in the pathogenicity and stress tolerance of a local *S.* Infantis population, demonstrate the role of a novel megaplasmid in its dissemination and present a possible paradigm to explain rapid emergence of new bacterial pathogens in environmental reservoirs.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains utilized in this study are listed in Supporting Information Table S2. Pre-emergent (isolated in Israel before 2006) and emergent (isolated in Israel during and after 2007) *S*. Infantis isolates were obtained from the Israeli National *Salmonella* Reference Center after serotyping according to the White–Kauffmann–LeMinor scheme (Guibourdenche *et al*., 2010) by agglutination with O- and H-antigen specific sera. A spontaneous azide-resistant *S*. Infantis 335-3 strain was induced on LB plates containing 100 μg ml^{-1} azide and was used in mating with strain 119944 for pESI conjugation. Bacterial cultures were routinely maintained in LB (BD Difco, Sparks, MD) liquid medium at 37°C and plated onto LB or xylose lysine deoxycholate (XLD; BD Difco) agar plates. When appropriate, antibiotics were added to the medium as follows: tetracycline (20 μg ml^{-1}), nitrofurantoin (64 μg ml^{-1}), trimethoprim (50 μg ml^{-1}), nalidixic acid (20 μg ml^{-1}), sulfamethoxazole (50 μg ml^{-1}) and rifampin (100 μg ml^{-1}).

Next generation sequencing and bioinformatics

Paired-ends sequencing using the Illumina Genome Analyzer IIx platform (Illumina, Inc., San Diego, CA) and pyrosequencing on a Roche/454 GS FLX sequencer (454 Life Sciences, a Roche company, Branford, CT) were both used to determine the whole genome sequence of the emergent (119944) and the pre-emergent (335-3) strains to a draft level. The obtained reads (combined from both platforms) provided more than 230-fold coverage and were subjected to de-novo assembly using the CLC Genomics Workbench 5.5.1 package (CLC-bio, Aarhus, Denmark). Genome assembly of the emergent strain generated 110 contigs (contig N50 size = 165303 bp) with a total length of 4.97 Mb, while the pre-emergent strain was assembled into 112 contigs (contig N50 size = 152157 bp) of 4.65 Mb. Genome sequences of both strains were annotated using the RAST tool (Aziz *et al*., 2008), and the IS element analysis was done using the ISFinder tool (Siguier *et al*., 2006). This whole genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accessions ASRF00000000 and ATHK00000000 for strains 119944 and 335-3 respectively. The versions described in this paper are versions ASRF01000000 and ATHK01000000.

Conjugation experiments

Plasmid transfer by conjugation was performed using the emergent strain (119944) as the donor and a rifampin-resistant *E. coli* J5-3 as a recipient. Both strains were grown in LB for 16 h with aeration, and 1 ml from each strain was harvested by centrifugation and resuspended in 100 μl of LB medium. Equal amounts (10 μl; ~ 2×10^9 CFU) from each culture were mixed and spotted onto LB-agar plates followed by incubation at 37°C for 6 h. Resulting transconjugants were resuspended in LB and serial dilutions were plated on LB-agar plates supplemented with tetracycline (20 μg ml^{-1}) and rifampin (100 μg ml^{-1}). Colony PCR was performed to confirm the plasmid presence using the primers Fim Fw and Fim Rv, and K88 Fw and K88 Rv. A similar experiment was done with an *S*. Infantis azide-resistant 335-3 strain as a recipient, and transconjugants were isolated under tetracycline (20 μg ml^{-1}) and azide (100 μg ml^{-1}) selection.

Biofilm formation

200 μl from overnight cultures grown in LB (OD 600 = 4.5) were added to 15 ml cholesterol-coated polypropylene tubes. Negative control included broth only. The tubes were incubated aerobically at 37°C under continuous shaking (250 RPM) for 48 h. Planktonic cells were discarded and attached cells were fixed for 1 h at 60°C. Fixed bacteria were stained with 400 μl of 0.1% crystal violet for 10 min at room temperature. The tubes were washed with phosphate buffered saline (PBS) and the dye bound to the adherent bacteria was resolubilized with 1 ml of 33% acetic acid. The optical density of each tube was measured at 570 nm.

Molecular biology and nfsA cloning

Primers used in this study are listed in Supporting Information Table S3. Oligonucleotides were purchased from IDT (Leuven, Belgium) and PCR was carried out using ReddyMix PCR (Thermo Scientific, Waltham, MA) for determination of genetic elements distribution or with PfuUltra II Fusion HS DNA Polymerase (Stratagene, Santa Clara, CA) for cloning. The intact sequence of *nfsA* was amplified with the primers nfsAFclone and nfsARclone using *S*. Infantis 335-3 as a template. Amplified fragment was digested with *Eco*RI and *Xba*I and ligated using T4 DNA ligase (NEB, Ipswich, MA) into pWSK29 after gel purification. pWSK29::*nfsA* was transformed into electrocompetent *S*. Infantis strain 119944.

RT-PCR

RNA was extracted from *Salmonella* cultures that were subcultured into fresh medium (LB, N-minimal or N-minimal supplemented with 20 μM $FeCl_3$) and grown aerobically to late exponential phase using the Qiagen RNAprotect Bacteria Reagent and the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including an on-column DNase digest. Purified RNA was secondarily treated with an RNase-free DNase I followed by ethanol-precipitation and 150 ng of DNase I-treated RNA were subjected to a first strand cDNA synthesis, using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the kit protocol. 2 μl from the reverse transcription reaction were used as a template for a PCR amplification using the primers listed in Supporting Information Table S3.

Tissue culture conditions and bacterial infection

The human epithelial HeLa and avian fibroblasts CEF-DF1 cell lines were purchased from ATCC (Manassas, VA). All cell lines were cultured in a high-glucose (4.5 g l^{-1}) Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM pyruvate and 2 mM L-glutamine. HeLa cells were cultured at 37°C in a humidified atmosphere with 5% CO_2, and CEF-DF1 cells were maintained at 39°C under 10% CO_2. Cells were seeded at 5×10^4 cells ml^{-1} in a 24-well tissue culture dish 18 to 24 h prior to bacterial infection, and experiments were carried out using the gentamicin protection assay as was previously described (Gal-Mor *et al*., 2006). Cells were infected at a multiplicity of infection (MOI) of ~1:100 with *Salmonella* strains that had been subcultured from an overnight culture and grown for 3 h to late logarithmic phase under aerobic conditions. At the desired time points post infection (p.i.), cells were washed three times with PBS and harvested by addition of lysis buffer (0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100 in PBS). Appropriate dilutions were plated on LB-agar plates for bacterial enumeration. *Salmonella* invasion was determined by the number of intracellular *Salmonella* 2 h p.i. divided by the number of infecting bacteria. For the cytochalasin D (CD)-dependent adhesion assay, cells were prepared in 24-well plates as described earlier. One hour prior to infection, 10 μg ml^{-1} CD (Sigma-Aldrich, St. Louis, MO) was added to the cells and was kept throughout the infection. At 2 h p.i., cells were washed three times in PBS and processed for CFU determination by serial dilution.

Mouse infection model

All animal experiments were conducted consistent with the ethical requirements of the Animal Care Committee of Schleswig-Holstein. Female C57BL/6 mice (Charles River, Sulzfeld, Germany) were infected at the age of 8–10 weeks. Food and water were provided *ad libitum*. Streptomycin (20 mg per mouse) was given by oral gavage 24 h prior to infection with 3×10^6 bacteria (*S.* Infantis 119944, *S.* Infantis 335-3 or *S.* Infantis 335-3/pESI) in 100 μl HEPES buffer (100 mM, pH 8.0). Control mice were given 100 μl HEPES buffer. At 24 h post infection, mice were euthanized and tissues were harvested aseptically for bacterial enumeration and evaluation of histopathology. Tissues were collected on ice and homogenized in 1 ml PBS with a Tissuelyzer homogenizer (Qiagen). Serial dilutions of the homogenates were plated on MacConkey agar plates, incubated overnight and counted to calculate bacterial tissue burdens.

Histology

Tissues were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. Sections (5 μm) were stained with Hematoxylin and Eosin (H&E). Tissue pathology was scored as previously described (Grassl *et al*., 2008).

Statistics

An unpaired *t*-test with two tails was used to determine the significance of the differences between the compared data, and $P < 0.05$ was considered to be statistically significant.

Ethics statement

Animal experiments were performed in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment, Kiel, Germany (permit# V312–7224.123-3). Clinical isolates were obtained from the National *Salmonella* Reference Center (Israel) and were processed anonymously. All experiments and data analysis were approved by the institutional review board of the Sheba Medical Center (approval# 7072-09-SMC) and conducted accordingly.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Experimental procedures.
Fig. S1. The emergence of *S*. Infantis in Israel. *Salmonella* is a mandatory reportable pathogen in Israel and each *Salmonella* isolate received at the National *Salmonella* Reference Center is being serotyped and documented. The prevalence of laboratory-confirmed *S*. Infantis isolates is shown as the percentage from all *Salmonella* isolates that were obtained and serotyped at the National *Salmonella* Reference Center 1995 to 2013. For the year 2013, *S*. Infantis proportion was calculated for the months of Jan–Sep. Data about *S*. Infantis prevalence between 1995–2009 were also included in our previous report (Gal-Mor *et al*., 2010) and are included here to provide a complete and updated description of S. Infantis epidemiology over 19 years.
Fig. S2. A consensus pairwise comparison of the Phenotypic Microarray (PM) results that were generated from the analysis of 20 plates (1920 conditions) in duplicate runs. Each position represents the growth of isolate 119944 (red) and 335-3 (green) under a certain condition (nutrient source, antimicrobial, pH, salt concentration etc.) over 24 h (carbon and nutrient) or 48 h (sensitivity) time period. Yellow color indicates equal (and therefore overlapping) growth of both isolates.
Fig. S3. The emergent strain harbors a single ~280 kb plasmid conferring tetracycline, sulfamethoxazole and trimethoprim resistance. (A) Mating experiments between a plasmidless *E. coli* J5-3 strain and *S*. Infantis 119944 strain. Bacterial growth of the donor (*S*. Infantis 119944), recipient (*E. coli* J5-3) and two randomly selected transconjugant isolates grown on LB plates under different selections is shown. (B) *S*. Infantis emergent clone (119944), pre-emergent strain (335-3), *E. coli* transconjugant (TC-1), the recipient strain (J5-3) and *E. coli* R27 (carrying a 168 kb plasmid as a positive control) were digested with S1 nuclease followed by PFGE. The identified linearized plasmids are indicated by arrow heads.
Fig. S4. Nalidixic acid resistance in *S*. Infantis is associated with point mutations at position 87 in GyrA. The amino acid sequence of the QRDR (positions 61 to 122) of the DNA gyrase subunit A (GyrA) in *S*. Infantis SARB27 (ZP_09726429) was compared against the sequence that was determined in pre-emergent ($n = 11$) and emergent ($n = 13$) isolates. Nalidixic acid resistance (+) or sensitive (–) phenotype is indicated. Amino acid substitutions all found at position 87 are highlighted in grey.
Fig. S5. pESI harbors an IncP-1α oriV, but not an IncI1 origin of replication. (A) Southern blot analysis of the *S*. Infantis 119944, *E. coli* DH10BT1/ pCVM29188_101 (as a positive control), *E. coli* C600/pRK2 (as a negative control) and S. Infantis 335-3 genomes using a Dig-labeled IncI-1α *oriV* probe. (B) Multiple alignment of the *oriV* sequence present in pESI against the origin of replication of IncP-1α plasmids pBS228 (AM261760), RK2 (J01780) and pTB11 (AJ744860) is shown. Alignment was generated and formatted using Multalin version 5.4.1. High consensus is shown in red and low consensus sequences are in blue.
Fig. S6. The virulence of the emergent S. Infantis clone *in vivo*. C57BL/6 mice were treated with streptomycin and 24 h later infected orally with the *S*. Infantis emergent strain (119944) and the pre-emergent strain (335-3), or given HEPES buffer only (uninfected). At 24 h post infection, colonization and bacterial burden were examined at gastrointestinal sites (cecum, colon and ileum) (A). Pathology scoring (0–25) was performed for H&E stained sections of the cecal mucosa (B). Representative micrographs at an original magnification of 40 $\times$ are shown (C). Bar = 100 μm; L indicates the lumen and E indicates submucosal edema. Two independent mice infection experiments were performed, the results of one representative experiment are shown.
Table S1. Differences in antimicrobial resistance between the emergent and the pre-emergent strains.
Table S2. Bacterial strains and plasmids used in this study.
Table S3. Primers used in this study.