Microevolution of antimicrobial resistance and biofilm formation of *Salmonella Typhimurium* during persistence on pig farms

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*Salmonella* Typhimurium and its monophasic variant *S. 4,[5],12:i:-* are the dominant serotypes associated with pigs in many countries. We investigated their population structure on nine farms using whole genome sequencing, and their genotypic and phenotypic variation. The population structure revealed the presence of phylogenetically distinct clades consisting of closely related clones of *S. Typhimurium* or *S. 4,[5],12:i:-* on each pig farm, that persisted between production cycles. All the *S. 4,[5],12:i:-* strains carried the *Salmonella* genomic island-4 (SGI-4), which confers resistance to heavy metals, and half of the strains contained the mTmV prophage, harbouring the *sopE* virulence gene. Most clonal groups were highly drug resistant due to the presence of multiple antimicrobial resistance (AMR) genes, and two clades exhibited evidence of recent on-farm plasmid-mediated acquisition of additional AMR genes, including an IncHI2 plasmid. Biofilm formation was highly variable but had a strong phylogenetic signature. Strains capable of forming biofilm with the greatest biomass were from the *S. 4,[5],12:i:-* and *S. Typhimurium DT104* clades, the two dominant pandemic clones found over the last 25 years. On-farm microevolution resulted in enhanced biofilm formation in subsequent production cycle.

*Salmonella* is the second most common cause of food borne disease in many countries world-wide and is associated with more deaths than any other foodborne disease in resource rich countries1,2. Pork and pork products are a leading source of human salmonellosis in the European Union3,4. The risk of pig meat contamination is exacerbated by the high prevalence of *Salmonella* in pigs arriving at slaughter, often in the absence of overt signs of disease5-8. Furthermore, antimicrobial resistance of *Salmonella* in pigs is a global concern, with resistance to at least one antimicrobial observed in 92% of *Salmonella* isolates in the UK9. Consequently, a reduction in the prevalence of *Salmonella* in pigs arriving at slaughter is a high priority for many countries, and subject to national control programmes7. Efforts to control *Salmonella* on farms is by a combination of endeavour to use *Salmonella*-free feed, good biosecurity measures, appropriate antibiotic usage, and implementation of an all-in all-out production system allowing for cleaning and disinfection between production cycles10,11. Although cleaning and disinfection of pig housing between production cycles is effective at reducing residual contamination, it is not completely effective as *Salmonella* may persist on water drinkers, feeders, and floor and wall surfaces, particularly if these are damaged12. The mechanisms used by *Salmonella* to persist in the farm environment are not known, but the ability to form biofilms is thought to be an important factor impacting survival in the environment21. The continued widespread use of antibiotics to treat or prevent disease has resulted in a high level of antibiotic resistance in *Salmonella* isolated from pigs, impacting the effectiveness of treatment on farm and dissemination into the food chain12,13.

Historically, *Salmonella enterica* serotype Typhimurium (*S. Typhimurium*), defined by the antigenic formula *4,[5],12:i:1,2,* is the serotype most frequently isolated on pig farms14-17. Since around 2005 the frequency of...
isolation of a monophasic variant of S. Typhimurium (S. 4,[5],12:i:-) has increased in many parts of the world.\textsuperscript{18–24} The epidemiological record of S. Typhimurium in livestock in England and Wales, and in Germany between 1970 and 2010 was characterized by a succession of dominant multidrug resistant (MDR) clones, namely DT204, DT104 and the current S. 4,[5],12:i:- DT193/DT120 strains.\textsuperscript{25–27} Initially, DT204 and DT104 emerged in cattle, but spread to pigs and poultry.\textsuperscript{26,27} In contrast, S. 4,[5],12:i:- DT193/DT120 strains emerged initially in pigs and have only recently spread to cattle and poultry in the UK where they remain minority types.\textsuperscript{28,29} In Ireland, a study of Salmonella sampled from manure on pig farms in 2009–2010 reported S. Typhimurium DT104 as the predominant type\textsuperscript{30} and S. 4,[5],12:i:- was first reported in pig herds in 2012, suggesting a rapid clonal replacement.\textsuperscript{29} A second MDR strain of S. Typhimurium phage type U288 emerged in UK pigs around 2001 accounting for up to 50% of all isolates but is rarely isolated from other livestock and poultry.\textsuperscript{27,30} A ubiquitous trait of historic and currently dominant strains of S. Typhimurium is MDR. S. Typhimurium DT104 exhibits a resistance profile of ACSSuT (ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracycline) encoded within a complex type I integron on Salmonella genomic island 1 (SGI-1), an integrative mobilisable element.\textsuperscript{31} S. Typhimurium U288 strains typically exhibit a resistance profile of ACSSuTIlm (including trimethoprim) due to two independent insertions into a pSLT plasmid.\textsuperscript{32} S. 4,[5],12:i:- DT193/DT120 strains exhibit an ASSuT resistance profile encoded on two resistance regions (RR1 and RR2) that are flanked by IS26 sequence and present adjacent to one another near the fljB locus that encodes the phase II flagelin protein.\textsuperscript{33} Insertion of RR1 and RR2 appears to have resulted in multiple deletions in the fljB locus and adjacent sequence that results in the monophasic phenotype of S. 4,[5],12:i:- but no apparent impact on pathogenicity.\textsuperscript{34,35} Although an MDR phenotype appears to be key to the success of epidemic strains, it does not explain the succession of dominant strains. A characteristic of the S. 4,[5],12:i:- DT193/DT120 epidemic strains is the presence of both cus and pco genes involved in copper and silver homeostasis present on an 80 kb genetic island, termed SGI-4.\textsuperscript{36} The cus and pco genes may impart an advantage for circulation in pig herds where pharmacological levels of copper are added to feed due to its non-specific antimicrobial properties.\textsuperscript{37} Further genotypic variation within the epidemic clade is generated by the acquisition, on multiple occasions, of a novel prophage (mTmV), encoding the SopE type III secretion system effector protein,\textsuperscript{38} that contributes to host cell invasion and induction of pro-inflammatory diarrhoea.\textsuperscript{39–40} To investigate persistence on pig farms and potential transmission from feed mills we determined the phylogenetic relationship of 138 S. 4,[5],12:i:- and S. Typhimurium strains isolated from pig farms in Ireland on two occasions crossing production cycles using whole genome sequencing.\textsuperscript{41} We then addressed the hypothesis that persistence on pig farms was associated with microevolution affecting drug resistance or biofilm formation, characteristics potentially selected during production or cleaning between production cycles. The distribution of AMR genes on mobile genetic elements was determined in order to study patterns of acquisition. The ability to form biofilms was investigated in the context of the phylogeny to investigate farm-specific phenotypes and to determine its association (if any) with persistence.

Materials and Methods

Bacterial strains and culture conditions. Isolation of Salmonella on ten pig farms located across three provinces of the Republic of Ireland on two sampling occasions in successive production cycles was described previously.\textsuperscript{42} Briefly, ten farrow-to-finish pig farms (farms A–J) with a history of high Salmonella prevalence (> 50%) were sampled twice between March 2012 and June 2013. Samples were collected across all production stages and from faeces, feed, water and the farm environment including floors, walls, water drinkers and troughs. S. Typhimurium or S. 4,[5],12:i:- was isolated from all farms except farm E. Therefore, isolates from nine farms were included in this study. Isolation from feed from three commercial feed mills (mills B, C and D) and one home compounder (mill E) were described previously.\textsuperscript{29} We retain farm and mill designations for ease of reference. For routine culture, strains were grown in Luria Bertani Broth with shaking at 37 °C. For determination of red, dry and rough (RDAR) phenotype, strains were cultured on Congo Red agar plates for 48 hours at 28 °C. For conjugation experiments, donor and recipient strains were cultured for 18 hours in 5 ml of LB broth at 37 °C without shaking for 24 hours at 26 °C. Ex-conjugants were enumerated on LB agar supplemented with nalidixic acid (50 µg/ml) or nalidixic acid (50 µg/ml)/chloramphenicol (25 µg/ml) and incubated at 30 °C for 18 hours, and CFU enumerated. Transfer of pSTM6-275 to the recipient strain was determined by PCR using primers 5′-TTTCTCTGAGTCACTGTATTACAC-3′ and 5′-GGCTCACTACGTTGTGCTA-3′, that annealed specifically to IncHI2 plasmids replicon. MIC for antibiotics was performed using a microdilution assay, as previously described.\textsuperscript{43}

Biofilm assay. The formation of biofilm on polystyrene was assessed using a microtitre plate assay as previously described.\textsuperscript{44} Bacterial strains were cultured overnight in 5 ml LB broth without salt at 37 °C under static conditions. The OD₆₀₀nm of the culture was subsequently adjusted to 0.02 using LB broth without salt, and 200 µl aliquots were dispensed in eight wells (8 technical replicates per biological replicate) of a microtiter plate. The 96-well plates were then incubated at 22 °C to simulate the environmental temperature of pig farms, without shaking, for 24 or 48 hours, after which the supernatant was removed, and the cells washed with 200 µl of tryptone salt (Oxoid Limited), before fixing with 300 µl of pure ethanol for 20 minutes. Biomass was stained with crystal violet and the OD was read at 595 nm. At least two biological replicates were performed.

Genomic DNA preparation and sequencing. The genomic DNA of 104 S. 4,[5],12:i:- and 34 S. Typhimurium strains was isolated using a Wizard® Genomic DNA purification kit (Promega). Sequencing libraries were prepared using the Low Input Transposase-Enabled (LITE) library developed at the Earlham institute.
DNA sequence analysis and phylogenetic reconstruction. For phylogenetic reconstruction, single nucleotide polymorphisms (SNPs) were identified in the whole genome sequences of 138 S. 4,[5],12:i:- and S. Typhimurium strains with reference to S. Typhimurium strain SL1344 by aligning sequence using BWA MEM\(^{43}\), variant calling with Freebayes\(^{44}\) and SNP filtering using vcflib/vcftools\(^{45}\), using Snippy v1.0\(^{46}\). Maximum-likelihood trees were constructed using a general time reversible (GTR) substitution model with gamma correction for among-site rate variation with RAxML v8.0.20\(^{47}\). Rapid bootstrapping was performed with 450 replicates. Suspected recombined sequence was removed to improve the accuracy of the phylogeny using Gubbins v2.3.4\(^{48}\). The size of the reference genome in which variant sites were used for phylogenetic reconstruction changed depending on the dataset used. For 138 isolates mapped to strain SL1344, variant and invariant sites alignment was 3,824,106 bases long, corresponding to the 78.4% of the reference genome, for the subset of these used for analysis of variation in biofilm formation this was 2,328 informative SNPs in 4,213,370 bases (86%) of the SL1344 reference genome. The phylogeny of the IncHI2 plasmid was based on 162 variant sites within the 193,845 base-long core-genome (70% of reference plasmid).

Targeted assembly of IncHI2 plasmid sequence was performed using a bespoke pipeline; sequence reads were mapped against S. Typhimurium L01157-10 assembled sequence using Snippy\(^{46}\) and the unmapped reads were then isolated and assembled using VelvetOptimiser\(^{46}\). The contigs generated were then merged and their ability to circularise tested with Circlator\(^{50}\). The homology of the IncHI2 plasmids with the pSTM6-275 plasmid (accession number CP019647.1) was visualised using the BRIG\(^{51}\).

The presence of sequence reads mapping to the sopE, SGi-4 and fljAB loci was investigated using SRST2 v0.1.7\(^{52}\) with custom databases. Matches with >90% coverage and <10% sequence divergence were reported as present. In silico detection of AMR genes, integrons and plasmids from ResFinder, INTEGRALL, and PlasmidFinder databases was performed using ARIBA\(^{53,54}\) and visualised using ggtee\(^{58}\).

Results

Farm-specific genotypes of S. Typhimurium and S. 4,[5],12:i:- in Ireland can persist across production cycles. To establish the phylogenetic relationship of S. Typhimurium on nine pig farms and four feed mills in Ireland, we determined the whole genome sequence of 34 S. Typhimurium and 104 S. 4,[5],12:i:- (Table 1) from a previous study\(^{61}\). A maximum likelihood phylogenetic tree constructed using 2,427 informative SNPs from the core genome of the farm isolates and three reference strains SO4698-09 (S. 4,[5],12:i:- DT193/120, NCTC13348 (DT104) and SO1960-05 (U288) indicated the farm and feed isolates were present in five major clades (clades A to E, Fig. 1). In each case, clades consisted of strains distinguishable from a hypothetical common ancestor of the clade by 5–30 SNPs in the core genome. The majority of strains (104) were in clade A and were closely related to strain SO4698-09, a reference strain for the current MDR S. 4,[5],12:i:- which is part of a current pandemic\(^{34}\). Clade A contained at least one strain from seven of the nine farms. Clade E contained six strains from two farms and these were closely related to S. Typhimurium DT104 (NCTC13348), a reference strain for a previously dominant MDR pandemic clone\(^{39,60}\). Three strains clustered with a reference strain for phage type U288 (strain SO1960-05), a representative from an ongoing epidemic of S. Typhimurium in pigs in the UK\(^{81}\). The remaining strains formed two distinct but closely related subclades (B and C). We observed a strong phylogenetic signature for strains from each farm, with the majority of strains isolated from each farm closely related to one another. S. Typhimurium and S. 4,[5],12:i:- were isolated from a wide range of sources on most farms, including the environment within the pens and troughs, water drinkers, feed and in the freshly voided faeces (Fig. 1). In many cases, identical or very closely related strains (<5 SNPs in core genome) were isolated from multiple pens, troughs, feeders and pigs, on the same farm suggesting their spread on the farm.

In four cases, strains isolated from two different farms or a farm and feed mill had identical or fewer than 5 SNPs in the core genome and therefore likely represented transmission events (Fig. 1). The core genome sequence of one S. 4,[5],12:i:- strain from farm B was identical to that of a number of strains from farm A. Indistinguishable strains from three different farms were identical to one another, and to an isolate recovered from soybean meal sampled from the home compounder mill E. Two strains from feed mill D were identical in core genome sequence.

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**Table 1.** Farm, production stage, source of isolation and antimicrobial resistance profile of the S. 4,[5],12:i:- and S. Typhimurium strains used in the study, from Burns et al. 2015 and Burns et al. 2018. *FW: farrowing; G: gilts; W1: 1st stage weaner; W2: 2nd stage weaner; D: dry sow; F: finisher.* 1Environmental samples from swabs from the pen, water drinkers, feed troughs and feed bins.
to a strain isolated from farm J, and different by a single SNP from an isolate from mill B. Also, a single isolate from mill C was identical to two strains from farm A.

On six of the nine farms, Salmonella Typhimurium or S. 4,[5],12:i:- strains were isolated on two separate occasions, 8 to 9 months apart, from successive production cycles (Fig. 1). In each case most strains isolated on the second sampling were closely related to the previously isolated strains, and the tree topology was consistent with direct descent from earlier strains, suggesting that these strains either persisted on the farm between production cycles or were reintroduced from a common source.

Diverse antimicrobial resistance genes are associated with farm-specific strains. Multidrug resistance is common in S. Typhimurium, particularly strains isolated from farm animals in which antibiotic use is widespread62. We therefore analysed the whole genome sequence of strains to determine variation in AMR
genes. Furthermore, since these genes are commonly encoded on plasmids we also identified replicon sequences (Supplementary Fig. 1).

Generally, the presence of AMR genes correlated with phenotypic resistance in laboratory tests with few exceptions (Supplementary Fig. 1). Only 13 strains lacked resistance genes entirely. The distribution of AMR genes differentiated phylogenetic clades, but considerable variation occurred within clades containing strains that differed by 5–10 SNPs, indicating rapid and ongoing gene flux on farms (Fig. 2). Strains within the epidemic clade of S. 4,[5],12:i:- and S. Typhimurium DT104 encode MDR on the chromosome, located on mobile genetic elements and S. Typhimurium U288 in integrons and transposons on a pSLT-like plasmid. Six S. Typhimurium strains that were closely related to the previous epidemic DT104 type strain (NCTC13348),...
encoded genes consistent with the common ACSSuT penta-resistance profile of this clone, as expected. They also encoded the qacE (Delta 1) gene, conferring resistance to quaternary ammonium compounds, previously described. Most S. 4,[5],12:i:- contained the genes associated with the typical ASuT tetra-resistance profile associated with the current epidemic clone. The presence of the blaTEM, strA, strB, sul2 and tetA genes in S. 4,[5],12:i:- strain correlated with an IncQ replicon sequence (repA) present within the RR1 and RR2 region of the chromosome. However, 14 S. 4,[5],12:i:- had lost various combinations of the blaTEM, strA, strB, sul2 and tetA genes, likely due to mobilisation through various IS26 elements within RR1 and RR2 regions. In addition, a large proportion of the strains carried dfrA14 gene (trimethoprim) which may have inserted into strA, as suggested by its concomitant absence as described in several plasmids. Four strains closely related to a representative S. Typhimurium U288 strain (clade D), were resistant to ampicillin (blaTEM-1b), streptomycin (strA, strB), sulphonamides (sul2, sul3) and tetracycline (tetA or tetB) consistent with previous descriptions of a U288 strain. However, additional resistance genes to aminoglycosides (aadA1/aadA2 and/or aph), chloramphenicol (cmi), trimethoprim (dfrA12), macrolides (mefB) and quaternary ammonium compounds (qacH) previously unreported in this clonal group were present in some strains.

Acquisition of plasmids enhanced antimicrobial resistance on two farms. Two clusters of strains in clade A (S. 4,[5],12:i:-) and clade B encoded additional AMR genes that correlated with the presence of plasmid replicons IncHI2 and IncI1, respectively (Supplementary Fig. 1). In each case strains containing the additional replicon were direct descendants of a common ancestor that was sensitive to these antibiotics and lacked the replicon, and in the case of clade B there was evidence from the tree structure for this having occurred on the farm. In the first example, 13 S. 4,[5],12:i:- strains encoded additional resistance genes, aadA1/aadA2, aph, cmi, dfrA12, mefB, sul2, sul3, and qacH, conferring resistance to aminoglycosides, chloramphenicol, trimethoprim, macrolides, sulphonamides, and quaternary ammonium compounds. Ten of these strains were from farm J and three strains from two feed mills. All of the strains with the IncHI2 replicon were closely related, with the exception a single S. Typhimurium strain from farm J. IncHI2 plasmids have previously been reported in epidemic S. 4,[5],12:i:- strains in China and Australia, including pSTM6-275. Comparison of assembled whole genome sequences with that of pSTM6-275 indicated a high level of sequence identity to 90% of the plasmid. All of the strains with the IncHI2 replicon were closely related, including the S. Typhimurium strain Fig. 3B), and relatively distantly related to pSTM6-275. The IncHI2 plasmid from S. Typhimurium strain 3593A was close to the root of the plasmid clade associated with farm J and two of the mills, suggesting a recent common source. Moreover, the plasmid from this isolate carried the qacEDelta1 gene, which was absent from the plasmids from S. 4,[5],12:i:-. S. 4,[5],12:i:- strain 3508A was able to transfer resistance to aminoglycosides, chloramphenicol, trimethoprim and sulphonamides by conjugation in vitro with a frequency of 4 × 10−5 per recipient. Although the plasmid could not be visualised by horizontal gel electrophoresis, as described for large plasmids previously, sequence was detected by PCR amplification using specific oligonucleotide primers. Also, the presence of mefB in U288-like isolates (Clade D) from farm J and in IncHI2-positive isolates may suggest its plasmid-independent mobilisation between distinct genotypes on this farm.

An example of probable on-farm acquisition of a plasmid encoding AMR genes was observed on farm G. Sixteen S. Typhimurium strains from this farm formed a phylogenetically distinct clonal group (clade B, Fig. 1), of which were pan-susceptible and four acquired blaTEM-1b, strA, strB, sul2 and tetA AMR genes that correlated with the presence of an IncI1 replicon (Supplementary Fig. 1). All three MDR strains were present on distal branches of the phylogenetic tree and were isolated during a second sample collection on the farm, suggesting that a strain that persisted over production cycles on the farm had acquired the IncI1 plasmid.

SGI-4, fljB locus and sopE gene flux in S. 4,[5],12:i:- farm strains. We previously reported the microevolution and genotypic variation of S. 4,[5],12:i:- strains in the UK and Italy. S. 4,[5],12:i:- strains were distinct from other Salmonella reported previously in encoding an 80 kb genetic island termed SGI-4, adjacent to the yidC and Phe-tRNA loci, that encodes genes conferring enhanced resistance to copper and potentially other metals, such as silver and arsenic. Three of 78 strains investigated previously lacked SGI-4, due to deletion. All of the S. 4,[5],12:i:- strains in the current study encoded SGI-4, further supporting the potential significance of this island in the success of the clone. A second reported source of genotypic variation was the acquisition of a novel phage termed MfN, that carried the sopE gene. Approximately half of the S. 4,[5],12:i:- strains contained the sopE gene, with strains from three farms and two feed mills containing the gene. Further genotypic variation resulted from multiple deletions in and adjacent to the fljB locus (Supplementary Fig. 2). Deletion of the fljB locus was associated with the lack of the second phase flagella antigen, but deletions varied in size as previously reported, affecting from 3 to 25 genes. Finally, the absence of IncFI plasmids within the epidemic monophasic variant is noteworthy.

S. Typhimurium and S. 4,[5],12:i:- farm isolates exhibit diverse expression of cellulose, curli fimbriae and the ability to form biofilm. The ability to form a biofilm is thought to increase survival in the environment by increasing resistance to desiccation, shear forces, and antimicrobial compounds such as biocides and antibiotics. Cellulose and curli fimbriae constitute two of the key components forming the extra-cellular matrix (ECM) which covers Salmonella biofilms and production correlates with the thickness of the biofilms. The majority of both S. 4,[5],12:i:- (98.1% %) and S. Typhimurium (76.7%) strains exhibited a red, dry and rough (RDAR) phenotype on Congo red agar after 72 hours at 28 degrees, indicating production of cellulose and curli fimbriae. The remaining strains produced a pale and smooth phenotype. The ability of farm strains to form biofilm on a polystyrene surface in 96-well plates was similar for S. Typhimurium and S. 4,[5],12:i:- strains.
**Figure 3.** Sequence similarity of IncH12 plasmids from Irish farms with the pSTM6-275 plasmid. (A) The extent of the homology between the Irish IncH12 plasmids with the pSTM6-275 was visualised with BRIG. Sequence assembly contigs from each sample were blasted against the pSTM6-275, which was used as reference. Each circle represents the plasmid from each of the 14 Irish isolates. The colour gradient indicates the degree of nucleotide identity. Genes in pSTM6-275 are represented by arrows (outer circle). Genes encoding antimicrobial or heavy metals resistance genes are annotated. A detailed list of gene presence/absence is present in Supplementary Table 4. (B) Phylogenetic relatedness of IncH12 plasmids. Maximum-likelihood phylogeny of the thirteen IncH12 plasmid sequences based on 162 core-genome SNPs identified using pSTM6-275 as reference. Due to the high proportion of missing data, plasmid from isolate 1495 C was excluded from the phylogenetic analysis.
at 22 °C for 24 and 48 hours incubation (Fig. 4A). Both S. Typhimurium and S. 4,[5]12:i:- strains formed a significantly greater biofilm after 48 hours of incubation at 22 °C, compared to 24 hours (p < 0.005, Fig. 4A) and biofilm formation at 37 °C was low for both S. Typhimurium and S. 4,[5]12:i:- strains (data not shown).

Despite the mean biofilm formation being similar for both S. Typhimurium and S. 4,[5]12:i:- strains, considerable variability in biofilm formation was observed after 48 hours incubation at 22 °C (Fig. 4A). In general, there was a high degree of congruence of the amount of biomass in biofilm and the phylogenetic relatedness of strains (Fig. 4B,C). S. Typhimurium exhibited a greater variation in biofilm formation than S. 4,[5],12:i:- strains. Most of the S. Typhimurium strains that formed strong biofilms were closely related to the DT104 reference strain (Clade E). The remaining S. Typhimurium strains in three separate clades (Clades B, C and D) generally formed moderate or weak biofilms, with the exception of two strains that formed strong biofilms. Despite the fact that all the S.

Figure 4. Biofilm formation by S. Typhimurium and S. 4,[5]12:i:- strains and correlation with the phylogeny. The biofilm-forming ability of the isolates was investigated with the microtiter plate assay. (A) Scatterplot of the OD<sub>595nm</sub> values measured for S. 4,[5],12:i:- and S. Typhimurium after 24 and 48 hours of incubation at 22 °C. (B) Comparison of strains from each clade (A1, A2, B–E) to form biofilm. The OD<sub>595nm</sub> values measured after 48 hours of incubation were plotted based on the topology of the phylogenetic tree. Pairwise Mann-Whitney test (95% of confidence interval) was performed and p < 0.05 were indicated as; p < 0.05 for all except clade E isolates (DT104-like) (#); p < 0.05 for all clades (**) p < 0.05 for all except clade A1 (+). (C) Maximum-likelihood phylogenetic tree as Fig. 1 with a subset of isolates for which biofilm formation was determined. The heatmap shows OD<sub>595nm</sub> measured after incubation at 22 °C for 48 hours. The clade designation is indicated.
4,[5],12:i:- strains were very closely related, biofilm formation appeared bimodal, with a large number of strains forming moderate biofilm levels (clade A2) and eight strains accumulating significantly more biomass (clade A1). All nine S. 4,[5],12:i:- strains that exhibited the greatest level of biofilm formation were isolated from farm B, and were distinct from all other S. 4,[5],12:i:- strains by virtue of the presence of a non-synonymous substitution present in the add gene, encoding a phosphogluconate dehydratase that participates in the Entner-Doudoroff glycolytic pathway.

Discussion

S. Typhimurium and the monophasic variant S. 4,[5],12:i:- are the most commonly isolated serotype from pigs, especially in finisher herds where intestinal carriage at slaughter is a primary risk factor for contamination of the food chain. An understanding of the colonisation, persistence and transmission of S. Typhimurium on farms, and variation in genotype and phenotype is therefore key to the rational design of interventions aimed at amelioration of the risk to food safety. Whole genome sequencing provided the resolution to distinguish virtually all strains, and where no differences in sequence were detected, the confidence that these were the same strain isolated on two separate occasions. Furthermore, in this study phylogenetic reconstruction based on sequence variation in the core genome was used to infer ancestry, and to interpret gene flux associated with AMR on the farm.

The phylogenetic relationship of strains from farms in Ireland suggested that each had been colonised in the recent past by a clone of S. Typhimurium or S. 4,[5],12:i:- and undergone limited sequence divergence. Most strains isolated from each farm differed by 0–12 SNPs in the core genome and were more closely related to one another, than to strains isolated from other farms. However, in some cases identical or near identical strains were isolated from more than one farm or feed mill, particularly within the S. 4,[5],12:i:- clade. For example, a subclade of S. 4,[5],12:i:- formed from 41 strains from farm A, also contained a strain from each of farms B and D, and three were distinct from farm G. This pattern suggested either contamination of multiple farms from a common source, or direct transmission between the farms. The latter cannot be excluded as information regarding movement of animals between farms was not collected, but introduction through a common source is more likely, based on the implication of feed as an important source of Salmonella on farms. That S. 4,[5],12:i:- strains from three commercial feed mills, one home feed compounder and at least some of the strains from seven of the nine farms were essentially clonal indicated a close relationship and recent transmission. In particular, the clonality of a group of strains from farm A with that from mill C, which supplied the farm, is consistent with at least initial transmission from contaminated feed to the farm at some point in the recent past.

The strong farm-specific phylogenetic signature was striking and the maintenance of this structure across two sampling occasions spanning 6–9 months suggested that Salmonella was most likely persistent on these farms. Furthermore, there was a moderate level of sequence divergence of strains isolated on each farm since they shared a common ancestor, suggesting that they may have been present on the farm for several years. S. 4,[5],12:i:- on farm J exhibited the greatest sequence divergence with many strains having accumulated 10–15 SNPs since sharing a common ancestor. Based on the reported short-term substitution rate of 1–2 SNPs per genome per year for Salmonella Typhimurium, the common ancestor for farm-specific clusters existed in the last 2–7 years. This is also consistent with previous studies that used subgenomic typing methods. The population structure of S. Typhimurium and S. 4,[5],12:i:- strains was consistent with considerable transmission on each farm both within pens (between pigs of similar age) and between pens housing multiple production stages. Salmonella differing by 0–5 SNPs were isolated from multiple pens housing pigs at various stages of weaning, fattening, or breeding from freshly voided faeces, the environment within a pen, that included the floor, walls and feed trough, feed, and drinking water and drinking nipple. While we cannot exclude multiple and continuous acquisitions of Salmonella from the same feed source, the relatively low incidence of Salmonella contamination in feed reported previously would suggest that this is more likely to be transmission within the farm due to ineffective biosecurity.

Consistent with previous reports, antimicrobial resistance genes were common in S. Typhimurium and S. 4,[5],12:i:-. Most S. Typhimurium DT104 and S. 4,[5],12:i:- DT193/DT120 strains contained genes consistent with the widely reported resistance profile, with occasional loss of one or more of the genes as previously reported. However, there were also two clear cases of acquisition of additional AMR genes on plasmids within a clade of S. 4,[5],12:i:- strains on farm J, and within a S. Typhimurium clade on farm G (clade B, Fig. 1). The first case involved acquisition of a large plasmid with extensive sequence similarity to pSTM6-275, an IncH1 plasmid encoding heavy metal resistance and AMR genes in S. 4,[5],12:i:-, reported previously in Australia. The pSTM6-275 plasmid was present in 11 strains that had been isolated on two separate occasions from farm J, and three S. 4,[5],12:i:- strains isolated from two different feed mills. All of the strains containing pSTM6-275 were S. 4,[5],12:i:-, except for a single farm isolate of S. Typhimurium. Strains of S. 4,[5],12:i:- with or without the plasmid were present in samples from both 2012 and 2013, suggesting plasmid gain or loss was occurring on the farm. To be a possible source of the pSTM6-275-like plasmid, as it was present in strains from two feed mills which were almost identical to plasmid-containing strains on farm J. In a second example, we observed evidence of AMR gene flux mediated by an Inc11 plasmid on farm G. While all the strains from the first sampling date lacked the Inc11 plasmid, a large proportion almost identical strains from the same clade carried the plasmid nine months later. These observations are consistent with the acquisition of the plasmid on the farm, from an unknown source.

The mechanisms by which Salmonella persist on farms is not known, but the ability to form biofilms is thought to be key to survival in the environment. Our analysis suggested that distinct clones of S. Typhimurium and S. 4,[5],12:i:- strains were isolated from farms spanning at least two production cycles which strongly suggests that they persisted in the environment despite the cleaning and disinfection that is normal practice for pig facilities between batches of pigs via a combination of mechanical means and chemical disinfection. The benefit of enhanced cleaning and disinfection compared to standard procedures was recently demonstrated.
Furthermore, the clonal structure of *Salmonella* on each farm and observation of farm-specific clones suggest that persistence on the farm is the most likely reason for *Salmonella* presence on farms and not reintroduction onto the farm from an external source. We observed considerable variability in the ability of strains to form biofilms on polystyrene, with particularly large degree of variation in biofilm formation in *S*. Typhimurium strains. Strains from clades B, C and D (U288) generally exhibiting limited biofilm formation while clade E (DT104) exhibited the greatest biofilm formation (Fig. 4). S. 4,[5],12:i:- strains generally formed moderate to strong biofilms, and one sub-clade composed of strains from a single farm exhibited a significantly greater biomass in their biofilm compared to other S. 4,[5],12:i:- strains, similar to previous observations. Most of the S. 4,[5],12:i:- strains circulating in Portugal could produce a strong biofilm and the ability of the strains to form biofilms increased between 2006 and 2011, suggesting that this provided an advantage for persistence. Similarly, closely related S. 4,[5],12:i:- strains on farm B differed in biofilm formation with the second sampling time point exhibited significantly greater biofilm formation than those isolated earlier. The former group of strains differed from the latter by a single nucleotide polymorphism that resulted in an amino acid substitution in the *edd* gene, encoding an enzyme in the Entner-Doudoroff (ED) metabolic pathway, that was previously implicated in biofilm formation of *Campylobacter jejuni*. However, the ability to form a biofilm was not essential for persistence on the farm, since strains of clade B generally formed weak biofilms but were isolated from successive production stages on farm G.

Together these data highlight the remarkable clonality of *S*. Typhimurium or S. 4,[5],12:i:- on individual pig farms. However, gene flux frequency was high enough that even within highly clonal groups changes in coding capacity has the potential to affect virulence, flagella antigen expression, and antimicrobial and heavy metal resistance genes. Furthermore, variation in ability to form biofilms was dependent on the genotype of *S*. Typhimurium or S. 4,[5],12:i:-, supported by a strong phylogenetic signature for this phenotype, suggesting that intervention strategies aimed at decreasing the incidence of *Salmonella* on pig farms may vary in effectiveness depending on the prevalent genotype.

References

1. EFSA. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA Journal* 12, https://doi.org/10.2903/j.efsa.2014.3547 (2014).
2. Kirk, M. D. *et al.* World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. *PLoS Med* 12, e1001921, https://doi.org/10.1371/journal.pmed.1001921 (2015).
3. EFSA, E. U. *Summary report on zoonoses, zoonotic agents and food-borne outbreaks 2015*. *EFSA Journal* 14, 4634, https://doi.org/10.2903/j.efsa.2016.4634 (2016).
4. Hazards, E. P. O. B. Scientific Opinion on an estimation of the public health impact of setting a new target for the reduction of Salmonella in turkeys. *EFSA Journal* 10, 2616 (2012).
5. Boyen, F. *Non-typhoidal Salmonella infections in pigs: a closer look at epidemiology, pathogenesis and control*. * Vet Microbiol* 130, 1–19, https://doi.org/10.1016/j.vetmic.2007.12.017 (2008).
6. Wood, R. L., Pospischil, A. & Rose, R. Distribution of persistent Salmonella typhimurium infection in internal organs of swine. *Am J Vet Res* 50, 1015–1021 (1989).
7. Andres, V. M. & Davies, R. H. Biosecurity Measures to Control Salmonella and Other Infectious Agents in Pig Farms: A Review. *Comprehensive Reviews in Food Science and Food Safety* 14, 317–335 (2015).
8. Baptista, F. M., Dahl, J. & Nielsen, L. R. Factors influencing Salmonella carcass prevalence in Danish pig abattoirs. *Prev Vet Med* 95, 231–238, https://doi.org/10.1016/j.prevmed.2010.04.007 (2010).
9. Miller, A. J. *et al.* Salmonella serovars and antimicrobial resistance patterns on a sample of high seroprevalence pig farms in England and Wales (2003-2008). *Zoonoses and public health* 58, 549–559, https://doi.org/10.1111/j.1600-0777.2011.01402.x (2011).
10. Martelli, F. *et al.* Evaluation of an enhanced cleaning and disinfection protocol in Salmonella contaminated pig holdings in the United Kingdom. *PLoS One* 12, e0178897, https://doi.org/10.1371/journal.pone.0178897 (2017).
11. Steenackers, H., Hermans, K., Vanderleyden, J. & De Keersmaecker, S. C. J. *Salmonella biofilms: An overview on occurrence, structure, regulation and eradication*. *Food Research International* 45, 502–531 (2012).
12. Bolton, D. J., Ivory, C. & McDowell, D. A study of Salmonella in pigs from birth to carcass: serotypes, genotypes, antibiotic resistance and virulence profiles. *Int J Food Microbiol* 160, 298–303, https://doi.org/10.1016/j.ijfoodmicro.2012.11.001 (2013).
13. Prendergast, D. M. *et al.* Application of multiple locus variable number of tandem repeat analysis (MLVA), phage typing and antimicrobial susceptibility testing to subtype Salmonella enterica serovar Typhimurium isolated from pig farms, pork slaughterhouses and meat producing plants in Ireland. *Food Microbiol* 28, 1087–1094, https://doi.org/10.1016/j.fm.2011.02.013 (2011).
14. Visscher, C. F. *et al.* Serodiversity and serological as well as cultural distribution of Salmonella on farms and in abattoirs in Lower Saxony, Germany. *Int J Food Microbiol* 146, 44–51, https://doi.org/10.1016/j.ijfoodmicro.2011.01.038 (2011).
15. Gebreyes, W. A., Altier, C. & Thakur, S. Molecular epidemiology and diversity of Salmonella serovar Typhimurium in pigs using phenotypic and genotypic approaches. *Epidemiol Infect* 134, 187–198, https://doi.org/10.1017/S0950268805004723 (2006).
16. Gebreyes, W. A. *et al.* Characterization of antimicrobial-resistant phenotypes and genotypes among Salmonella enterica recovered from phenotypic strains from farms, transport trucks, and from pigs after slaughter. *J Food Prot* 67, 698–705 (2004).
17. Rowe, T. A. *et al.* Salmonella serotypes present on a sample of Irish pig farms. *Vet Res* 153, 453–456 (2003).
18. Andres-Barranco, S., Vico, J. P., Marin, C. M., Herrera-Leon, S. & Mainar-Jaime, R. C. *Characterization of Salmonella enterica Serovar Typhimurium Isolates from Pigs and Pig Environment-Related Sources and Evidence of New Circulating Monophasic Strains in Spain*. *J Food Prot* 79, 407–412, https://doi.org/10.4315/0362-028X.JFP-15-430 (2016).
19. Arguello, H. *et al.* Prevalence, serotypes and resistance patterns of Salmonella in Danish pig production. *Res Vet Sci* 95, 334–342, https://doi.org/10.1016/j.rvsc.2013.04.001 (2013).
20. Hauser, E. *et al.* Pork contaminated with Salmonella enterica serovar 4,[5],12:i:-, an emerging health risk for humans. *Appl Environ Microbiol* 76, 4601–4610, https://doi.org/10.1128/AEM.02991-09 (2010).
21. Bonardi, S. *Salmonella in the pork production chain and its impact on human health in the European Union*. *Epidemiol Infect* 145, 1513–1526, https://doi.org/10.1017/S095026881700363X (2017).
22. Hopkins, K. L. *et al.* Multiresistant Salmonella enterica serovar 4,[5],12:i:- in Europe: a new pandemic strain? *Euro Surveill.* 15, https://doi.org/10.2807/eue.15.22.19580-en (2010).
23. Antunes, P., Mourau, J., Pestana, N. & Peixe, L. Leakage of emerging clinically relevant multidrug-resistant Salmonella clones from pig farms. *J Antimicrob Chemother* 66, 2028–2032, https://doi.org/10.1093/jac/dkr228 (2011).
65. Kim, S. et al. An Additional Novel Antimicrobial Resistance Gene Cluster in Salmonella Genomic Island 1 of a Salmonella enterica Serovar Typhimurium DT104 Human Isolate. *Foodborne Pathogens and Disease* 6, 471–479, https://doi.org/10.1089/fpd.2008.0199 (2009).

66. Bosse, J. T. et al. Identification of dfrA14 in two distinct plasmids conferring trimethoprim resistance in Actinobacillus pleuropneumoniae. *J Antimicrob Chemother* 70, 2217–2222, https://doi.org/10.1093/jac/dkv121 (2015).

67. Dyall-Smith, M. L., Liu, Y. & Billman-Jacobe, H. Genome Sequence of an Australian Monophasic Salmonella enterica subsp. enterica Typhimurium Isolate (TW-S66) Carrying a Large Plasmid with Multiple Antimicrobial Resistance Genes. *Genome Announc* 5, https://doi.org/10.1128/genomeA.00793-17 (2017).

68. Billman-Jacobe, H. et al. pSTM6-275, a Conjugative IncHI2 Plasmid of Salmonella enterica That Confers Antibiotic and Heavy-Metal Resistance under Changing Physiological Conditions. *Antimicrob Agents Chemother* 62, e02357–e02317, https://doi.org/10.1128/AAC.02357-17 (2018).

69. Feasey, N. A. et al. Drug resistance in Salmonella enterica ser. Typhimurium bloodstream infection, Malawi. *Emerg Infect Dis* 20, 1957–1959, https://doi.org/10.3201/eid2011.141175 (2014).

70. Romling, U. Genetic and phenotypic analysis of multicellular behavior in Salmonella typhimurium. *Methods Enzymol* 336, 48–59 (2001).

71. Aarestrup, F. M., Hasman, H., Olsen, I. & Sørensen, G. International spread of bla(CMY-2)-mediated cephalosporin resistance in a multiresistant Salmonella enterica serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. *Antimicrob Agents Chemother* 48, 1916–1917 (2004).

72. Ökoro, C. K. et al. High-resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal Salmonella typhimurium disease. *Clin Infect Dis* 54, 953–963, https://doi.org/10.1093/cid/cir1302 (2012).

73. Hawkey, J. et al. Evidence of microevolution of Salmonella Typhimurium during a series of egg-associated outbreaks linked to a single chicken farm. *BMC Genomics* 14, 800, https://doi.org/10.1186/1471-2164-14-800 (2013).

74. Weaver, T. et al. Longitudinal study of Salmonella 1,4,[5],12:i:- shedding in five Australian pig herds. *Prev Vet Med* 136, 19–28, https://doi.org/10.1016/j.prevetmed.2016.11.010 (2017).

75. Lim, S. K., Byun, J. R., Nam, H. M., Lee, H. S. & Jung, S. C. Phenotypic and genotypic characterization of Salmonella spp. isolated from pigs and their farm environment in Korea. *J Microbiol Biotechnol* 21, 50–54 (2011).

76. Michael, G. B. & Schwarz, S. Antimicrobial resistance in zoonotic nontyphoidal Salmonella: an alarming trend? *Clin Microbiol Infect* 22, 968–974, https://doi.org/10.1016/j.cmi.2016.07.033 (2016).

77. Boyd, D. et al. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of Salmonella enterica serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J Bacteriol* 183, 5723–5732, https://doi.org/10.1128/JB.183.19.5723-5732.2001 (2001).

78. Vestby, L. K., Moretto, T., Langrud, S., Heir, E. & Nesse, L. L. Biofilm forming abilities of Salmonella are correlated with persistence in fish meal- and feed factories. *BMC Vet Res* 5, 20, https://doi.org/10.1186/1746-6148-5-20 (2009).

79. Seixas, R., Machado, J., Bernardo, E., Vilela, C. & Oliveira, M. Biofilm formation by Salmonella enterica serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic, and socio-geographic analysis. *Curr Microbiol* 66, 670–677, https://doi.org/10.1007/s00284-014-0523-x (2014).

80. Peyru, G. & Fraenkel, D. G. Genetic mapping of loci for glucose-6-phosphate dehydrogenase, gluconate-6-phosphate dehydrogenase, and gluconate-6-phosphate dehydrogenase in Escherichia coli. *J Bacteriol* 95, 1272–1278 (1968).

81. Vegge, C. S. et al. Glucose Metabolism via the Entner–Doudoroff Pathway in Campylobacter: A Rare Trait that Enhances Survival and Promotes Biofilm Formation in Some Isolates. *Front Microbiol* 7, 1877, https://doi.org/10.3389/fmicb.2016.01877 (2016).

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**Author Contributions**

This project was conceived by E.T., G.D. and R.A.K., the experiments were performed by E.T., data analysis and expertise was provided by E.T., G.D., M.B., C.M.B., E.M.M. and R.A.K., material and expertise of samples was provided by P.G.L. and G.G., the manuscript was drafted by E.T. and R.A.K., and all authors (E.T., G.D., M.B., C.M.B., E.M.M., P.G.L., G.G. and R.A.K.) provided critical input and approved the final manuscript.

**Additional Information**

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