Presence of phage-plasmids in multiple serovars of *Salmonella enterica*

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Abstract

Evidence is accumulating in the literature that the horizontal spread of antimicrobial resistance (AMR) genes mediated by bacteriophages and bacteriophage-like plasmid (phage-plasmid) elements is much more common than previously envisioned. For instance, we recently identified and characterised a circular P1-like phage-plasmid harbouring a $bla_{CTX\text{-}M\text{-}15}$ gene conferring extended-spectrum beta-lactamase (ESBL) resistance in *Salmonella enterica* serovar Typhi. As the prevalence and epidemiological relevance of such mechanisms has never been systematically assessed in Enterobacterales, in this study we carried out a follow-up retrospective analysis of UK *Salmonella* isolates previously sequenced as part of routine surveillance protocols between 2016 and 2021. Using a high-throughput bioinformatics pipeline we screened 47,784 isolates for the presence of the P1 lytic replication gene *repL*, identifying 226 positive isolates from 25 serovars and demonstrating that phage-plasmid elements are more frequent than previously thought. The affinity for phage-plasmids appears highly serovar-dependent, with several serovars being more likely hosts than others; most of the positive isolates (170/226) belonged to *S.* Typhimurium ST34 and ST19. The phage-plasmids ranged between 85.8–98.2kb in size, with an average length of 92.1kb; detailed analysis indicated a high amount of diversity in gene content and genomic architecture. 132 phage-plasmids had the p0111 plasmid replication type, and 94 the IncY type; phylogenetic analysis indicated that both horizontal and vertical gene transmission mechanisms are likely to be involved in phage-plasmid propagation. Finally, phage-plasmids were present in isolates that were resistant and non-resistant to antimicrobials. In addition to providing a first comprehensive view of the presence of phage-plasmids in *Salmonella*, our work highlights the need for a better surveillance and understanding of phage-plasmids as AMR carriers, especially through their characterisation with long-read sequencing.

Data Summary

All of the FASTQ files examined in this study have been uploaded to the Sequence Read Archive under BioProject PRJNA248792. Accessions of individual isolates which were found to contain phage plasmids are listed in Supplementary Table S1.

Impact Statement

Bacteriophage-like plasmids are increasingly being recognised as important mobile elements in many species of bacteria, particularly due to their involvement in the transmission of antimicrobial resistance (AMR); however, few studies of their overall prevalence in clinical datasets have been undertaken to date. In this study we have performed the first large-scale surveillance of human disease-associated *Salmonella* genomes for the presence of P1-like phage-plasmids, showing that they are more common than previously thought. Furthermore, we highlight how valuable information about the evolution and transmission of phage-plasmids in *Salmonella* and other Enterobacterales can be revealed by linking phage-plasmid prevalence and genetic diversity to epidemiologically relevant metadata such

as *S. enterica* serovar, outbreak clusters, time, and geography. Our work shows the ability to use sequencing data and scalable bioinformatics workflows for the detection and characterisation of these extrachromosomal elements, highlights the importance of screening for novel mechanisms of AMR transmission, and provides a foundation for further surveillance studies of phage-plasmid prevalence.

Introduction

The global threat of rising antimicrobial resistance (AMR) incidence is well known, particularly among genera of Enterobacterales such as *Escherichia* and *Salmonella* (1,2). AMR genes can be transferred between bacteria via horizontal gene transfer (HGT), which involves many diverse molecular mechanisms mediated by mobile and integrative elements including transposons and integrons, plasmids, bacteriophages and genomic islands, all of which are well known for their role in the transfer of AMR genes (3) as well as other genes such as virulence and fitness factors (4,5). Temperate bacteriophages, which typically integrate as prophages into the host chromosome, are another important mechanism of HGT and further contribute to the spread of AMR genes, toxins and virulence factors (4,6–8). Moreover, certain temperate bacteriophages have been found to exist in their prophage forms as low-copy number, extrachromosomal-plasmids and thus can replicate autonomously within bacterial cells while remaining latent (9,10).

Large-scale computational analysis of such bacteriophage-like plasmids, or "phage-plasmids", has shown that they are associated with multiple specific types of bacteriophages including P1, P7 and SSU5 (11). The P1-like phage-plasmid community belongs to the *Myoviridae* family and forms two distinct sub-groups based on relatedness of their gene content, with one containing P1 and its relatives, and the other containing phage-plasmid D6 (10,11). The plasmid replication types linked to the P1-like phage-plasmid group are consistently either IncY or p0111 (11,12), suggesting both types can theoretically be compatible and maintained in a single host.

There is increasing evidence from the literature that the horizontal spread of AMR genes by these phage-plasmids is more common than previously envisioned, especially among the P1, SSU5 and AB types (13). It is speculated that bacteriophages carrying AMR genes would confer an evolutionary advantage to their hosts, especially during latent infection as phage-plasmids. The mechanism involved in conferring evolutionary advantage is underpinned by several complex molecular pathways currently being elucidated (14); it would eventually result in phage-plasmids with AMR genes being positively selected. For instance, phage-plasmids carrying extended-spectrum beta-lactamase (ESBL) and colistin resistance genes have been identified in *Escherichia coli* (12,15–22), *Klebsiella pneumoniae* (23) and *Salmonella enterica* (13,24–26) to date. Among *S. enterica*, reports suggest that phage-plasmids are associated more specifically with the AMR genes $bla_{\text{CTX-M-15}}$ (25), $bla_{\text{CTX-M-27}}$ (24,26) and *mcr-1* (26).

We recently identified, characterised and described for the first time a circular P1-like phage-plasmid harbouring a $bla_{\text{CTX-M-15}}$ gene conferring high-level ESBL resistance within *S. enterica* serovar Typhi (25). Following this discovery, we wanted to determine the extent to which such elements may be distributed among all the subspecies and serovars of *Salmonella* in our collection, and whether these phage-plasmids play a significant role in the increasing spread of antibiotic resistance.

110 The Gastrointestinal Bacteria Reference Unit (GBRU) at the UK Health Security Agency
111 (UKHSA) is the UK national reference laboratory for enteric bacterial pathogens. Routine
112 whole genome sequencing (WGS) for all *Salmonella* spp. was implemented in 2014,
113 resulting in a collection of roughly 9000-10000 genomes per year from hundreds of different
114 serovars. In this study, we offer a retrospective examination of the genomic data associated
115 with this national surveillance. We developed a high-throughput process for detecting the
116 occurrence of P1-like phage-plasmids and applied it to examine the genomes of *Salmonella*
117 spp. isolates received by the reference laboratory. Our goal was to gain insight into the
118 distribution, prevalence, and connection of these phage-plasmids with antimicrobial
119 resistance genes.

120 # Materials and Methods

121 ## Data collection & bacterial strains

122 A total of 47,784 *Salmonella* spp. isolates were sequenced by GBRU between January 2016
123 and December 2021. All of the FASTQ files have been uploaded to the Sequence Read
124 Archive under BioProject PRJNA248792, where links to automatically generated genome
125 assemblies and annotated nucleotide sequences can also be found (Table S1). Ethical
126 approval for the detection of gastrointestinal bacterial pathogens from faecal specimens, or
127 the identification, characterisation and typing of cultures of gastrointestinal pathogens,
128 submitted to GBRU is not required as it is covered by the surveillance mandate of UKHSA.

129 ## Whole genome sequencing and bioinformatics

130 Genomic DNA was extracted using the Qiasymphony DSP DNA Midi Kit on the Qiasymphony
131 system (Qiagen, UK) to manufacturer's instructions. The Nextera XP kit (Illumina) was used
132 to prepare the sequencing library for sequencing on the HiSeq 2500 and NextSeq 1000
133 instruments (Illumina), run with the fast protocol. Trimmomatic v0.27 (27) was utilised to
134 remove bases with a PHRED score of <30 from the leading and trailing ends on the FASTQ
135 reads, with reads <50bp after trimming discarded. The eBurst Group (eBG) was determined
136 as previously described (28). Multilocus sequence type (MLST) assignment was performed
137 using MOST v2.18 (29), and the FASTQ reads underwent single nucleotide polymorphism
138 (SNP) typing and clustering using SnapperDB v0.2.9 (30), which additionally produced SNP
139 addresses for the isolates: these represent the relationship between isolates using
140 hierarchical single linkage clustering of pairwise SNP distances at several thresholds (250,
141 100, 50, 25, 10, 5 and 0 SNPs, respectively). Routine detection of AMR genes and plasmid
142 replication gene types was conducted using in-house GastroResistanceFinder v2.7, which
143 derives from GeneFinder (available at https://github.com/ukhsa-collaboration/gene_finder).

144 ## Detection of *repL* gene

145 The *repL* gene forms part of the L-replicon of the P1 family of temperate bacteriophages,
146 which is the active replicon for DNA replication during the lytic cycle (9); therefore, we used
147 the presence of *repL* as an indicator for carriage of a P1-like phage plasmid. For each of the
148 47,784 samples, reads were mapped to the *repL* gene sequence with the GEM mapper
149 v3.6.0 (31) in default mode. Alignments were filtered to keep those of sufficient quality
150 (having no more than 2 indels or clippings, clippings being defined as CIGAR operation "S" in
151 the SAM format (32) documentation – available at https://github.com/samtools/hts-specs –
152 such that the sum of the number of substitutions, indels and clippings would not exceed 5%
153 of the read length; and such that the sum of the number of mismatches and the lengths of
154 indels and clippings would not exceed 10% of the read length); all multimaps were kept.
155 Pileups were generated with Samtools (32,33) pileup v1.15.1 (using htslib (34) v1.16) and
156 parsed to determine coverage. The distribution of the fraction of gene bases having non-
157 zero coverage was observed to be bimodal, with peaks at either very small fractions (likely
158 corresponding to sequencing noise) or very high fractions (likely corresponding to true

159 positives). We considered a sample to be a potential positive for the detection of *repL*
160 whenever the number of gene bases supported by sequencing reads was >60% of the total,
161 which coincides with the position of the valley between the two peaks of the distribution.
162 While we did not observe correlation between the fraction of gene bases supported by
163 sequencing reads and read coverage (i.e., there were phage-plasmids with very low read
164 coverage but a complete *repL* gene) we further required the average coverage of bases
165 having non-zero coverage to be >1, to ensure that on average each gene base was
166 supported by more than one single sequencing read. This procedure produced 248
167 candidates. Further manual inspection based on metadata yielded 226 candidate samples
168 (Table S1).

169 Genome assembly and phylogenies

170 The 226 genomes in which the *repL* gene was present with high confidence according to the
171 alignment pipeline were assembled *de novo* from the paired-end reads using SPAdes v3.8.0
172 (35). The assembled genomes were visualised using Bandage v0.8.1 (36), with the built-in
173 BLAST (37) tool used to identify the phage-plasmid contig(s) associated with the *repL* gene
174 (NC_005856.1) and the IncY *repA* (K02380.1) and p0111 *repB* (AP010962.1) plasmid
175 replication genes, as well as to determine the location of any AMR genes (using the
176 Resfinder v4 database (38)). The putative phage-plasmid contigs were manually curated,
177 extracted and used to perform all downstream comparative genomic and phylogenetic
178 analyses. A total of 33/226 genome assemblies were of too poor quality to extract the
179 phage-plasmid contigs for further analysis, as determined by manual inspection in Bandage
180 and BLASTn against the P1 reference (NC_005856.1) revealing them to be spread across >5
181 contigs; we were unable to accurately determine their full sequence or to extract core gene
182 content. Panaroo v1.3.4 (39) was used to produce core gene alignments (present in >98% of
183 the remaining 193 phage-plasmids, as well as selected publicly available phage-plasmid
184 sequences (Table S2)), which were passed to FastTree v2.1.11 (40) to generate maximum-
185 likelihood trees using the GTR CAT method of nucleotide substitution and 100 bootstrap
186 replicates. Trees were visualised alongside the metadata using Microreact (41).

187 Of the 193 phage-plasmid sequences, 39 were determined using Bandage to have
188 assembled into single, circular contigs, while a further 69 were single, linear contigs. For
189 individual analyses, phage-plasmid contigs were annotated using Bakta v1.8.2 (42), and
190 visualised and compared using Easyfig (43) and Clinker (44). To examine the taxonomic
191 context of the phage-plasmids we compared a representative subset of 25 sequences from a
192 range of serovars to the full collection of prokaryotic dsDNA viruses on the ViPTree server
193 (45), producing a proteomic dendrogram based on tBLASTx genome-wide sequence
194 similarity.

195 Phylogenetic analysis of plasmid replication type

196 To examine how the plasmid replication type varied across the *repL*-positive phage-plasmid
197 tree, we performed ancestral state reconstruction (46) and a phylogenetic randomisation

test. First, to determine if the two replication types were distributed randomly across the tips of the tree without regard to tree structure, we performed a simple label-switching test. The observed states, IncY (n=79/193) and p0111 (n=114/193), were randomly reassigned to new terminal nodes. This process was repeated 100 times. In each case, a Welch t-test was performed using the t.test function in R package *stats* v 4.3.1 (47) to assess whether the distribution of Euclidean genetic distances between IncY and p0111 individuals differed between the observed and the tip-randomised dataset. The mean Bonferroni-corrected p-value is 1.07e-15, indicating that plasmid replication type is not distributed randomly across the tips of the tree if one disregards tree structure.

However, as the presence of large clades with near-zero branch lengths might inflate the appearance of clustering and vertical inheritance in the replication type, we needed to account for phylogenetic structure. We performed ancestral state reconstruction using the discrete maximum-likelihood method via the functions pml and ancestral.pml in R package *phangorn* v 2.11.1 (48) to infer the likely replication type state on the internal branches of the phage-plasmid tree, identifying 17 changes in replication type along the tree (Fig. S1). To determine whether these 17 changes occurred randomly across the phylogenetic history of the sample, we performed a phylogenetically-informed randomisation test. The 17 changes were randomly reassigned to new branches, with probability of reassignment proportional to branch length, and IncY/p0111 states were simulated from root to tips according to these change-points with the phen.sim function in R package *treeWAS* v 1.1 (49). We performed 100 simulations and repeated the Welch t-test as described above to determine whether the observed replication type states showed any greater propensity for vertical inheritance than one might expect despite the number of changes inferred.

221 ## Results

Serovar	No.	Prev. phm.	Prev. surv.	ST(s)	Years	Rep type(s)	AMR
Typhimurium	170	84%	19%	34, 19	2015-2021	IncY (63) p0111 (107)	None
Oslo	9	4.0%	0.31%	1370	2016	IncY (5) p0111 (4)	None
Livingstone	6	2.7%	0.21%	457, 638	2018-2019, 2021	IncY (4) p0111 (2)	None
Indiana	5	2.2%	0.90%	17	2018	p0111	None
Mbandaka	4	1.8%	0.81%	413, 1602	2016, 2018, 2020	IncY (3) p0111 (1)	None
Mikawasima	4	1.8%	0.76%	1815	2016-2018	IncY	None
Virchow	4	1.8%	1.4%	16	2016-2018, 2020	IncY (2) p0111 (2)	None
Infantis	3	1.3%	4.3%	32	2018	p0111 (3)	None
Stanley	3	1.3%	1.6%	29	2018-2019	IncY (3)	None
Agona	2	0.90%	2.3%	13	2019	p0111 (2)	None
Typhi	2	0.90%	3.2%	1	2019	IncY (2)	$bla_{CTX-M-15}$
Adjame	1	0.44%	0.11%	3929	2021	IncY	None
Bareilley	1	0.44%	1.2%	203	2018	p0111	None
Corvallis	1	0.44%	0.42%	1541	2016	p0111	None
S. enterica 45:b:-	1	0.44%	0.66%	2524	2016	IncY	None
Enteritidis	1	0.44%	27%	11	2016	IncY	None
Florian	1	0.44%	0.002%	7166	2020	p0111	None
Florida	1	0.44%	0.021%	351	2017	IncY	None
Minnesota	1	0.44%	0.31%	548	2019	IncY	None
Mississippi	1	0.44%	0.25%	4711	2020	IncY	None
Monschaui	1	0.44%	0.08%	443	2017	IncY	None
Ohio	1	0.44%	0.12%	329	2017	IncY	None
Pomona	1	0.44%	0.084%	451	2016	p0111	None
Rissen	1	0.44%	0.19%	469	2017	IncY	None
Telaviv	1	0.44%	0.0063%	1068	2016	p0111	None

222 **Table 1** – Summary of metadata for the 226 *Salmonella* isolates possessing the *repL* gene;
223 see Table S1 for individual isolate information. Values within brackets indicate frequency of
224 the Rep type(s). In the column "Prev. surv." ("prevalence in surveillance") we report the
225 percentage of occurrences in the 47,784 *Salmonella* spp. isolates that were sequenced by
226 GBRU between January 2016 and December 2021; in the column "Prev. phm." (short for
227 "prevalence in phage-plasmids") we report the percentage of occurrences in the set of
228 sequences that were found to contain phage-plasmids as a result of the current study.

229 ## Phage-plasmid prevalence

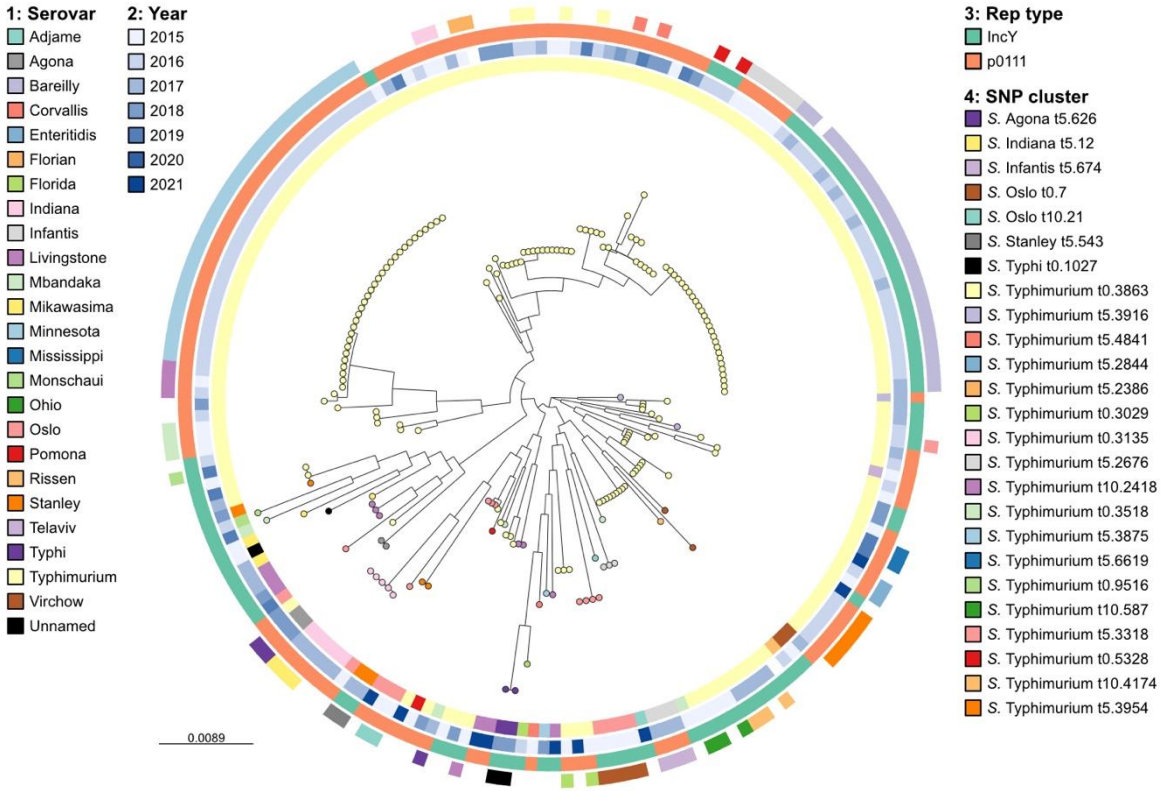
230 Using our mapping pipeline, the *repL* gene was detected in the reads of 226 isolates (0.47%),
231 comprising 0.2% to 1.1% of the dataset depending on the year (Table 1, Table S1). The *repL*
232 gene was present in 25 different *Salmonella* serovars and was most prevalent among
233 isolates of *S.* Typhimurium (n=170), followed by *S.* Oslo (n=9), *S.* Livingstone (n=6), and *S.*
234 Indiana (n=5). Table 1 also shows serovar prevalence for the whole set of 47,784 isolates.
235 Comparison of serovars for which phage-plasmids were detected suggests that they tend to
236 occur much less than expected in *S.* Enteritidis (0.44% with phage-plasmids vs. 27%
237 prevalence of *S.* Enteritidis within our whole *Salmonella* collection); less than expected in *S.*
238 Agona (0.9% vs. 2.3%), *S.* Infantis (1.3% vs. 4.3%), *S.* Typhi (0.9% vs. 3.2%); much more than
239 expected (84% vs. 19%) in *S.* Typhimurium, *S.* Oslo (4.0% vs. 0.31%), and *S.* Livingstone (2.7%
240 vs. 0.21%); and more than expected in *S.* Indiana (2.2% vs 0.9%), *S.* Mbandaka (1.8% vs.
241 0.81%), and *S.* Mikawasima (1.8% vs. 0.76%). Phage-plasmids were also not detected in a
242 large number of serovars often occurring in the full surveillance datasets (the most frequent
243 being *S.* Newport, corresponding to 3.3% of the 47,784 isolates; *S.* Paratyphi A, 1.6%; *S.*
244 Kentucky, 1.5%; and *S.* Braenderup, 1.5%).

245 ## Phage-plasmid features

246 To explore the structure and content of the putative phage-plasmids, we performed *de novo*
247 assembly of the 226 *repL*-positive *Salmonella* genomes and extracted the contigs upon
248 which the *repL* gene was located, where possible (n=193). Analysis of contigs assessed to be
249 circular (n=39) showed that the phage-plasmids ranged between 89.8–97.6kb in size with an
250 average length of 93.1kb. Expanding this to include all phage-plasmids that had assembled
251 into a single circular or linear contig (n=108) gave a range of 85.8-98.2kb (average 92.1kb),
252 except for four isolates of *S.* Typhimurium which had notably shorter or longer phage-
253 plasmid sequences of 76-79kb and 104-109kb (Table 1, Table S1). Additionally, two other *S.*
254 Typhimurium genomes had phage-plasmids located on single contigs >115kb in length;
255 these were putatively determined to instead be integrated into chromosomal sequences of
256 these isolates (Fig. S2); long-read sequencing would be necessary to corroborate this
257 finding.

258 A search against a database of plasmid replication genes revealed that 94 of the phage-
259 plasmids had the IncY plasmid replication type and 132 had the p0111 replication type. To
260 assess the level of vertical or horizontal transmission from the correlation between phage-
261 plasmid phylogeny and plasmid replication type, a maximum-likelihood tree based on a
262 51,260bp alignment of the 54 core genes from the 193 phage-plasmid contigs was
263 constructed (Fig. 1). Statistical analysis showed that the two types are not distributed
264 completely randomly across the tips of the tree, if one disregards the tree structure (p-value
265 = 1.07e-15). That excludes a scenario where transmission occurs in a fully horizontal
266 manner. We then conducted a different analysis that considers the structure of the
267 phylogenetic tree. Ancestral state reconstruction (46) revealed that 17 changes in
268 replication type had occurred along the evolutionary history of the phage-plasmid sample

269 (Fig. S1). Comparison to simulated data suggested that these changes occur randomly,
270 falling with greater probability on longer branches, and result in a distribution of replication
271 types that does not differ statistically from a random distribution once branch lengths are
272 considered (p-value = 0.15). Vertical inheritance of replication type thus occurs among
273 clones and some close relatives, but it is not maintained over greater genetic distances. This
274 supports a mixed model of transmission, whereby, as expected, both vertical and horizontal
275 gene transfer mechanisms play a role.



276
277 **Fig. 1** – Midpoint-rooted maximum-likelihood tree of 51,260bp alignment of 54 core genes
278 from the 193 *repL*-positive phage-plasmid contigs, with tips coloured by serovar. Rings from
279 inner to outer: serovar; year; replication gene type; host isolate SNP cluster based on 0-10
280 SNP threshold of SNP address (Table S1).

281 ## Genetic diversity

282 In almost all cases, phage-plasmid sequences are grouped according to the host isolate SNP
283 clustering (as determined by 0-10 SNP thresholds, described by the SNP addresses in Table
284 S1) but are not necessarily closely related to others from the same serovar (Fig. 1, Fig. 2).
285 However, there are examples of cross-serovar similarity, with a phage-plasmid from an *S.*
286 Stanley that is highly similar to several from *S.* Typhimurium (Fig. S3). Additionally, there are
287 also phage-plasmids from closely related, epidemiologically-linked *S.* Typhimurium strains
288 (forming a 10-SNP cluster based on SNP address) that display considerable genetic diversity
289 and even possess different plasmid replicon types (Fig. 3). In relation to the P1 phage-
290 plasmid itself, several regions are variable (Fig. S4) including: 47.5kbp - 53.6kbp (encoding a
291 type 3 restriction-modification enzyme); 65.7kbp - 68.1kbp (encoding autolytic and phage

proteins); 76.3kbp - 78.9kbp (encoding phage tail fibre proteins). However, there are large sections of conserved sequence surrounding the previously mentioned variable regions which contain a variety of phage and plasmid associated genes.

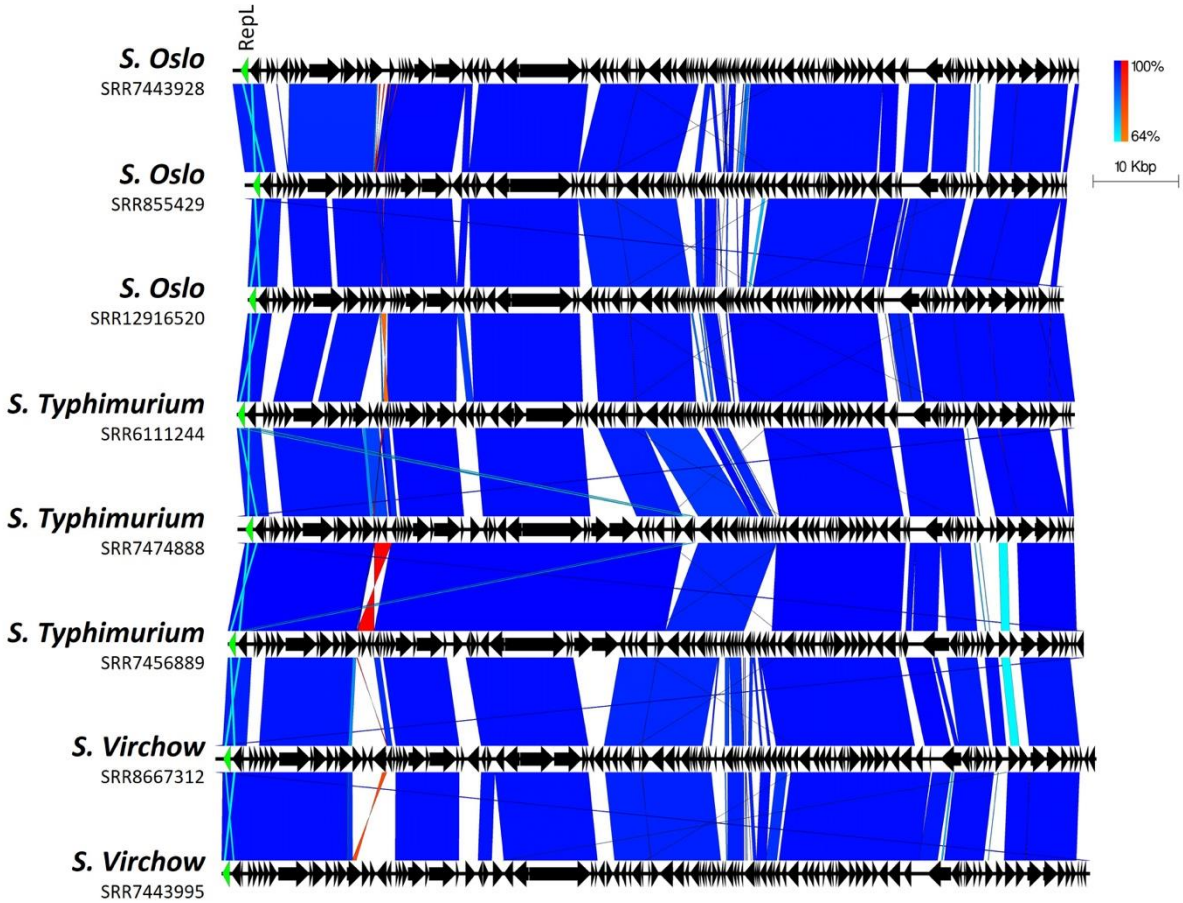


Fig. 2 – Plot showing how diversity occurs in sections in-between highly conserved regions, even among isolates belonging to the same serovar. Arrows indicate gene direction; scale bars indicate level of nucleotide similarity for forward (blue) and reverse (red) sequences. The *repL* gene is highlighted in green.

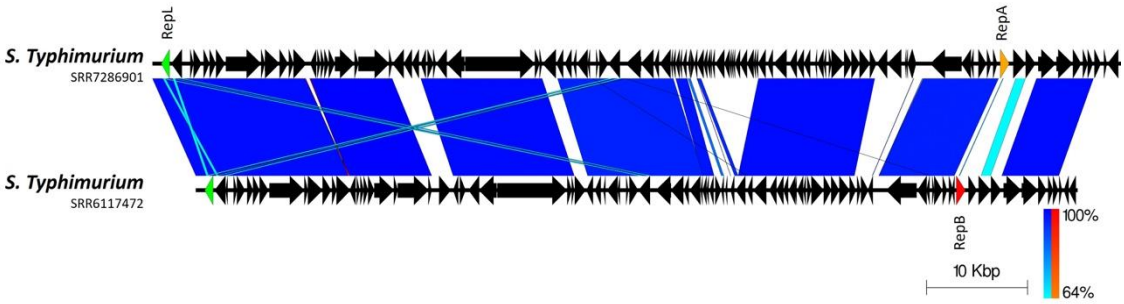
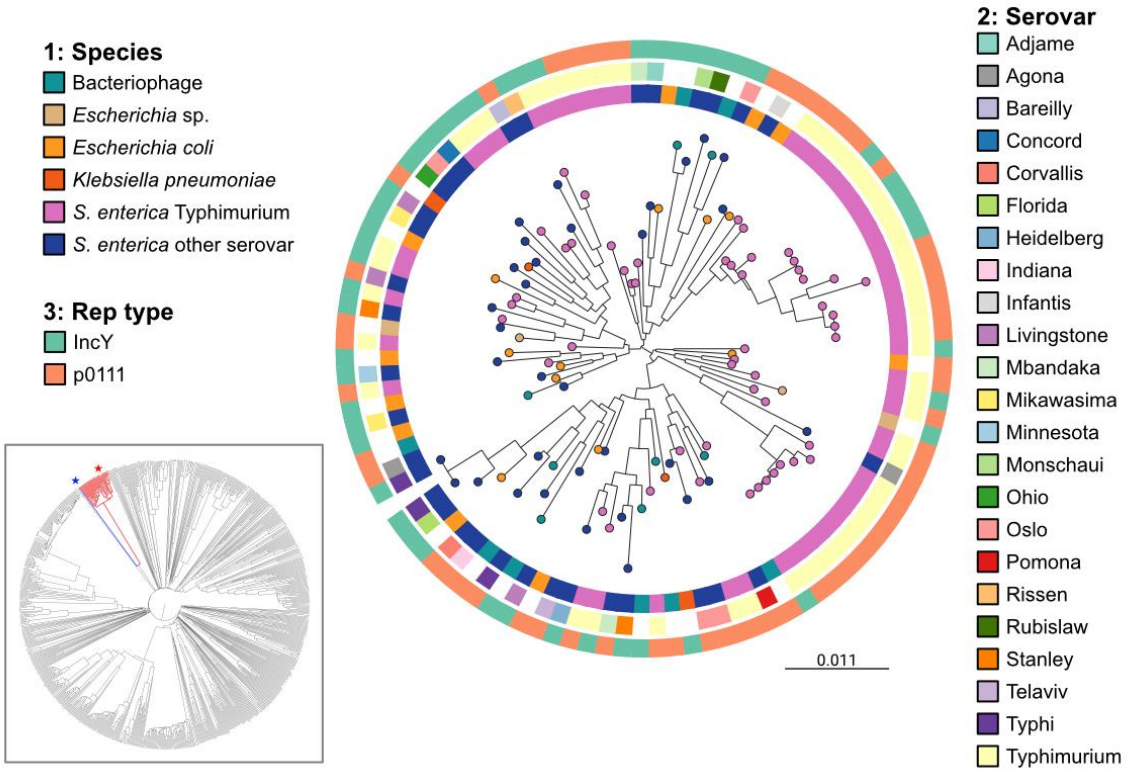


Fig. 3 – Plot showing variability in the sequences of two phage-plasmids from *S.* Typhimurium isolates that belong to the same 10-SNP cluster. Arrows indicate gene direction; scale bars indicate level of nucleotide similarity for forward (blue) and reverse (red) sequences. The *repL* gene is highlighted in green; the IncY *repA* in orange and the p0111 *repB* in red.

306 We next performed a BLASTN search of the GenBank database to identify other phage-
307 plasmids that were related to those in our collection, including from other species of
308 Enterobacterales. We produced a tree containing selected representative phage-plasmid
309 sequences from our collection, alongside 31 publicly available sequences labelled as either
310 phage-plasmids, plasmids or bacteriophages (Table S2). This confirmed the high amount of
311 genetic diversity and additionally showed that there is no apparent correlation between
312 phage-plasmid sequence and species (Fig. 4). We did however identify several sequences
313 (typically labelled as either plasmid or bacteriophage) that are highly similar to phage-
314 plasmids from our collection (Fig. S5). To further examine the taxonomic context of our
315 phage-plasmid dataset, we compared 25 representative sequences taken from a range of
316 serovars to a large public collection of prokaryotic dsDNA viruses, confirming that the P1-
317 like phage-plasmids are monophyletic, and that the closest relative to this clade is
318 *Escherichia* phage D6 (inset Fig. 4, Fig. S6).

319



320

321 **Fig. 4** – Midpoint-rooted maximum-likelihood tree of 42,606bp alignment of 46 core genes
322 from 107 phage-plasmids, including 31 from public databases, with tips coloured by species.
323 Rings from inner to outer: species; *S. enterica* serovar; replication gene type. Inset figure:
324 unrooted proteomic tree of 825 prokaryotic dsDNA viruses from the ViPTree server (45)
325 showing the single clade containing P1-like phage-plasmids (including P1, P7, SJ46 and 25
326 from this study) coloured in red. Phage P1 is highlighted with a red star, and phage D6 by a
327 blue star.

Antimicrobial resistance

AMR genes were detected in all 170 of the *S.* Typhimurium isolates and in 25/57 of the isolates belonging to other serovars (Table S1). Preliminary visual assessment of the whole genome sequences indicated that the majority of AMR genes were present on the chromosomes of these isolates rather than being associated with the phage-plasmid contigs. However, we did confirm the presence of $bla_{\text{CTX-M-15}}$ genes encoding ESBL resistance on phage-plasmids from two *S.* Typhi isolates (Fig. 5), both of which were associated with travel to Iraq. We have previously reported and described the phage-plasmid sequence in detail for one of these isolates (25). Further comparison with publicly-available plasmid and phage-plasmid sequences showed that the flanking region of $bla_{\text{CTX-M-15}}$ is variable, and that the gene is located next to an IS*Ec9*/IS*Ecp1* element in the *S.* Typhi isolates that is similar to that alongside the $bla_{\text{CTX-M-55}}$ gene of *E. coli* phage-plasmid JL22 (ON018986), but different to that next to the $bla_{\text{CTX-M-27}}$ in *S.* Indiana phage SJ46 (KU760857).

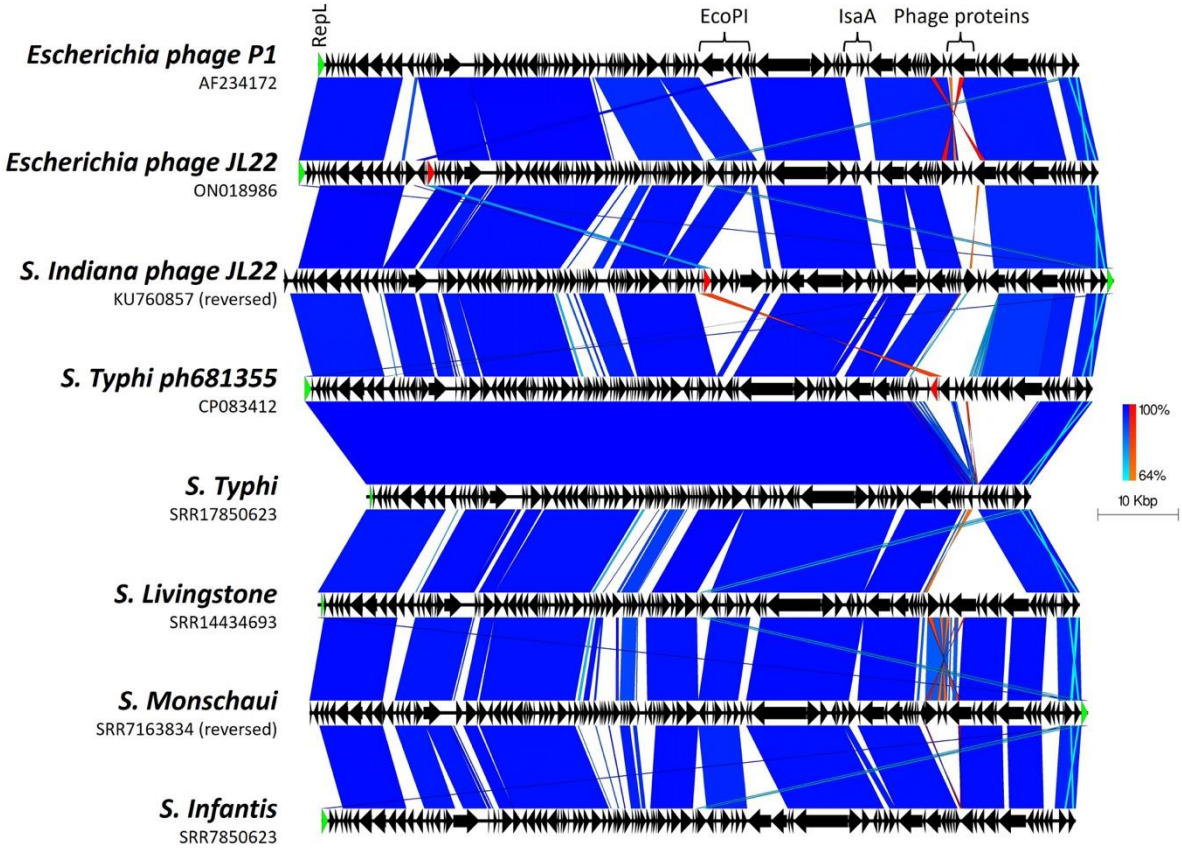


Fig. 5 – Comparison of *S.* Typhi ESBL-carrying phage-plasmid (CP083412: $bla_{\text{CTX-M-15}}$) to other ESBL-carrying phage-plasmids (ON018986: $bla_{\text{CTX-M-55}}$ & KU760857: $bla_{\text{CTX-M-27}}$). Arrows indicate gene direction, scale bars indicate level of nucleotide similarity for forward (blue) and reverse (red) sequences. The *repL* gene is highlighted in green. In the figure we show that those encoding ESBL genes (CP083412, ON018986 & KU760857) shown by the red arrows, are encoded within variable loci, and not within the same region.

Discussion

New and emerging research based on WGS highlights the prevalence of many different phage-plasmids in the bacterial world (11,12,19,26). We performed retrospective genomic surveillance for the presence of P1-like phage-plasmids within a collection of almost 48,000 *Salmonella* isolates received over a five year period. Our work shows that phage-plasmids are distributed among multiple serovars of *Salmonella* and have been present within the UK population since at least 2016. While there is growing evidence of a significant phage-plasmid presence among genera of Enterobacterales (11,13,19), targeted large-scale genomic surveillance of *Salmonella* had not previously been performed.

P1-like phage-plasmids have been reported in a small number of individual strains of *Salmonella* (24–26), however previous large-scale genomic surveillance studies of the Enterobacterales have primarily focused on *E. coli*. Our work shows that the overall prevalence of P1-like phage-plasmids among the clinical serovars of *S. enterica* appears to be considerably lower than in *E. coli*, being just ~0.5% compared to 7-12.6% (15,19). However, this may vary depending on country or isolate source, as a higher prevalence has been found in *Salmonella* from pork in China (24). Interestingly, this study also suggested an association between the phage-plasmid sequences and their host strains, with closely related *S. enterica* isolates (<5 SNPs) possessing identical or extremely similar phage-plasmids; though only among specific strains of *S.* Derby and *S.* Indiana that were sampled from the same facility. The difference in prevalence between our collection and studies such as theirs may be a result of the clinical bias that is inherent in our dataset. We saw hints of this when selecting public phage-plasmid sequences for comparison: many of the P1-like sequences from *S. enterica* and other Enterobacterales that are available online were sampled from animals, food, or the environment. It would therefore be beneficial to extend such screening to other large genomic (and metagenomic) datasets that contain comparatively more food and environmental samples to gain an accurate view of overall prevalence in *S. enterica*.

We confirmed the presence of P1 phage-plasmids in multiple *Salmonella* serovars, with the majority (84%) occurring in *S.* Typhimurium. This represents a significantly higher fraction than the fraction of *S.* Typhimurium recorded in our surveillance database during the same time period. Interestingly, it has been reported that isolates belonging to *S.* Typhimurium have a higher prophage burden than many other serovars of *Salmonella* (50), possibly because phage-mediated recombination plays a role in the periodic emergence of epidemic clones of *S.* Typhimurium (8). We also found in this study that other serovars seem to be especially amenable to acquiring other such elements – examples are *S.* Oslo, *S.* Livingstone and *S.* Indiana. Perhaps even more importantly, we notice that some serovars very rarely acquire phage-plasmids, with the fraction of isolates containing phage-plasmids being much smaller than what would be expected from the fraction of surveillance isolates. That is especially true for *S.* Enteritidis (0.44% vs. 27%, or ~50 times less); other examples are *S.* Agona, *S.* Infantis and *S.* Typhi. While the exact mechanism of affinity between serovars and

390 phage-plasmids is likely to depend on several factors, we speculate that a broad link with
391 the level of host specificity of an organism might exist, with generalists like *S.* Typhimurium
392 and *E. coli* requiring a higher level of genome adaptability via acquisition of malleable
393 extrachromosomal mobile elements; though this does not explain the low prevalence
394 observed in *S.* Enteriditis.

395 Similar to previous studies of P1-like phage-plasmids, we report that the majority of those
396 detected in our dataset fall into a narrow size range of 85.8-98.2kb, with the 92.1 kb
397 average matching well to those from other collections. Others have suggested that this
398 small size range may be due to a limit on the amount of genetic material that can be
399 packaged within the virion head, although there have been reports of larger phage-plasmids
400 ranging up to 155kb (51). The structure of the P1 phage-plasmids when comparing between
401 *Salmonella* strains are diverse, but nonetheless share a common genomic architecture. Our
402 results, alongside those of other studies (12,24), highlight large regions of conserved
403 sequence interspaced with more variable regions, which mainly encode hypothetical genes
404 and phage related genes rather than plasmid coding sequences. However, the conserved
405 regions also contain phage genes along with some plasmid associated genes.

406 Based on SNP typing of the corresponding *Salmonella* chromosomes there is an association
407 between the phage-plasmid sequences and chromosomal diversity, with closely related (<10
408 SNPs) isolates possessing identical or highly similar phage-plasmids. We also detected
409 several P1-like phage-plasmids in online databases that share considerable sequence
410 similarity with specific sequences from our collection. These public phage-plasmid
411 sequences derive from various sources and span multiple years, continents and even host
412 species; implying widespread horizontal transmission. Another study also detected an
413 identical phage-plasmid that was present in two serovars of *S. enterica* sampled from the
414 same facility (24). In combination with the link to chromosomal relatedness this suggests
415 that horizontal transfer may be followed by maintenance of the phage-plasmids during
416 clonal expansion of strains, with subsequent loss of the phage-plasmid occurring
417 sporadically in some isolates, rather than there having been long-term vertical transmission
418 and evolution at a serovar or species level. This is reinforced by our discovery that there is
419 only limited phylogenetic clustering of the two different plasmid replicon types: one type is
420 maintained across each shallow clade but frequent switching in replication type breaks this
421 association when considering deeper lineages. Additionally, our results suggest that overall
422 genetic diversity of the phage-plasmids is not consistent with the corresponding population
423 structure of their hosts at either a serovar or species level, which has also been observed in
424 other studies (52) and is indicative of their extensive recombination and horizontal method
425 of acquisition.

426 Phage-plasmids are known to be involved in the horizontal transmission of AMR genes,
427 antiseptic resistance genes (13) and virulence factors such as toxins (4), as well as being
428 involved in bacterial survival (51). The P1-like family in particular is the most common type
429 of phage-plasmid to be associated with AMR gene presence (13), with a prevalence that lies

430 between that of phages and plasmids. In our collection, P1-like phage-plasmids were
431 present both in isolates that were genotypically resistant and susceptible to antibiotics, with
432 patterns depending on the serovar. Most of these AMR genes were observed to be located
433 on the chromosome, and so while it is difficult to be certain without utilising long-read
434 sequencing, we did not find an association with AMR in almost all of the phage-plasmids in
435 this study. However, we previously described an IncY P1-like phage-plasmid carrying bla_{CTX-}
436 $_{M-15}$ (25) and in our current study we identified an identical phage-plasmid sequence, also
437 possessing the $bla_{CTX-M-15}$ gene, in an isolate that is genetically closely related (0 SNPs
438 distance) as well as epidemiologically connected (same travel location). This finding
439 therefore provides further evidence of either transduction of the AMR-carrying phage-
440 plasmid between these two isolates, or of maintenance of this phage-plasmid during clonal
441 expansion (while likely under the same selective pressures). Importantly, our previously
442 described antimicrobial susceptibility testing indicated phenotypic ESBL resistance,
443 confirming the functionality of the AMR genes present on phage-plasmids. The flanking
444 regions and associated IS elements responsible for mobilisation of bla_{CTX-M} gene variants are
445 widely discussed in the literature (53,54). The two *S.* Typhi phage-plasmids described here
446 possess $bla_{CTX-M-15}$ flanked by IS*Ec9*/IS*Ecp1*, which are also implicated in many plasmids
447 carrying various ESBL resistance genes from multiple species (55,56); further evidence that
448 phage-plasmids can horizontally acquire AMR genes from diverse sources (52).

449 The presence of AMR-conferring genes in phage-plasmids lends itself to a straightforward
450 evolutionary explanation, whereby latent phage-plasmids would increase the fitness of their
451 host when antibiotics are present in the environment. However, many of the phage-
452 plasmids we identified lack genes with an explicit or known AMR function, and mostly
453 contain unannotated genes. The accessory content of temperate phages within *Salmonella*
454 and other bacterial genera is increasingly being revealed to provide important effects for
455 phages as well as their hosts; such as self-defence systems that protect against infection by
456 other phages (57,58), increased pathogenicity (59–62), and improved fitness, biofilm
457 formation and stress responses (59,63,64). Active surveillance of their appearance and
458 distribution might be a valuable tool to understand how bacteria harbouring phage-
459 plasmids will evolve in the future. The unknown genes carried by phage-plasmids might act
460 as a reservoir of foreign material, allowing a rapid response to selective environmental
461 pressures such as antimicrobials, host defence mechanisms or available substrates. Our
462 previously described spontaneous loss of the $bla_{CTX-M-15}$ module from the *S.* Typhi phage-
463 plasmid (25) may be evidence of such a dynamic nature. It is also possible that P1-like
464 phage-plasmids are able to be maintained at a relatively low fitness cost to the bacterium
465 due to their low copy number and restricted size. Furthermore, as prophages have been
466 implicated in driving the evolution of *Salmonella* via re-assortment of virulence and fitness
467 factors to form new pathogenic variants (65), it is possible that phage-plasmids too may play
468 a role in this process. Other recent work has also interestingly highlighted phage-plasmids –
469 in particular P1-like phage-plasmids – as mediating gene flow between phages and plasmids,
470 resulting in the creation of novel elements (48).

471 Our results would not be possible without the creation of a robust and scalable workflow
472 that allowed us to reprocess in one hour short-read sequencing data from hundreds of
473 isolates on a single high performance computing (HPC) node. Our method allows an
474 arbitrary number of genes to be detected, with high sensitivity and accuracy, and, given a
475 sufficient computational budget, it can be scaled up to an essentially arbitrary number of
476 samples. The *repL* gene has previously been used as a target for screening using both *in vitro*
477 (12,24,26) and *in silico* (19,26) based methods. We build on these to prove that *repL*
478 presence is a valid detection method to quickly screen very large genomic datasets for the
479 presence of P1-like phage-plasmids. A previous study did however detect an atypical P1-like
480 phage-plasmid that was missing its *repL* gene (19), suggesting that additionally targeting the
481 IncY/p0111 replication genes may enhance the robustness of the method.

482 The P1-like family of phage-plasmids was the target of this work due to its proven links with
483 AMR gene carriage, though we are determined to expand the scope of our surveillance to
484 include screening other phage-plasmid types including SSU5 (66,67) that are known to
485 encode AMR genes (13,68), as well as other enteric pathogens received by the UK reference
486 laboratory such as *E. coli* and *Shigella* spp.. Active surveillance of plasmids, plasmid replicon
487 types and AMR gene presence is already part of our routine bioinformatics pipeline, and so
488 the implementation of screening for phage-plasmids would be a valuable complement to
489 this service. Additionally, this method could be used to rapidly screen publicly available
490 genome collections, for example on the Sequence Read Archive (69) or Enterobase (70), to
491 get a more accurate idea of global P1-like phage-plasmid prevalence within *Salmonella* and
492 other species. Finally, future studies of phage-plasmids will benefit from the application of
493 techniques, such as long-read sequencing, that are able to capture much more easily the
494 structure and genomic architecture of complex plasmid-like elements, including those
495 carrying AMR modules.

496 ## Conclusions

497 We developed and applied a robust, high-throughput method to perform the first large-
498 scale genomic surveillance of P1-like phage-plasmids in a national collection of *Salmonella*
499 isolates. Although the prevalence is lower than what has been reported for other species of
500 Enterobacterales, the phage-plasmids we identified are present across a number of
501 serovars. They carry genes conferring not only AMR, but also several so far unknown and
502 potentially relevant traits. The apparent ease of transmission across serovars, species and
503 continents, and potential to integrate into the chromosome, highlight the importance to
504 routinely monitor the presence of such phage-plasmids. We have expanded the knowledge
505 of P1-like phage-plasmids by linking their sequences back to host genomic diversity and
506 supplementing them with epidemiological metadata, showing that while such phage-
507 plasmids are understood to be ancient features, their acquisition by bacterial strains
508 appears to be a dynamic process driven by recent evolutionary pressure and so it is
509 important to examine them in the context of their host strains. This study highlights the
510 necessity of regular sequence data curation for the detection, characterisation and tracking
511 of novel mobile elements involved in AMR transmission and other factors that can change
512 the biological properties of bacterial pathogens.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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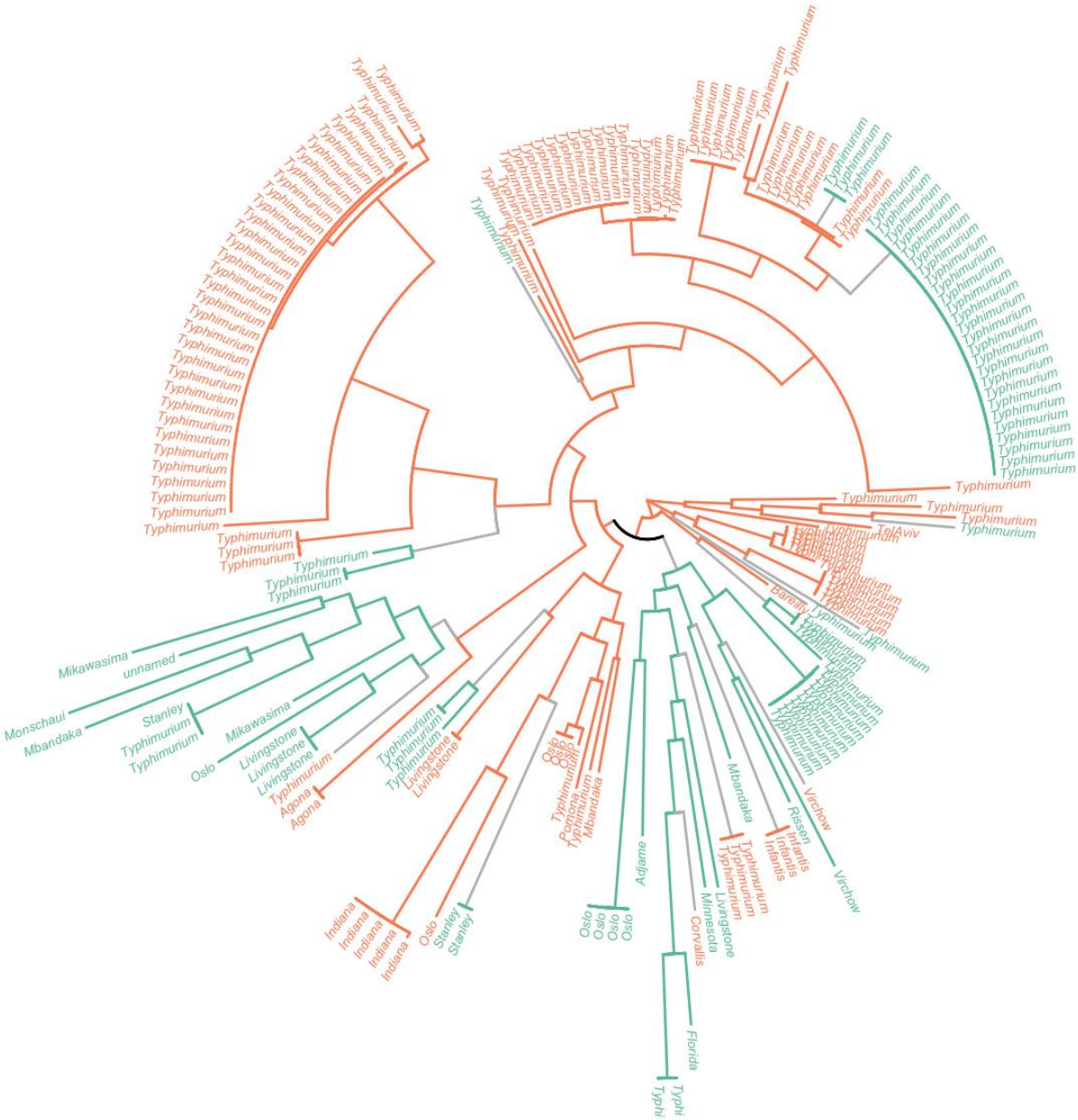
716

717 ## Supplementary Figures



718
719 **Fig. S1** Phylogenetic tree based on the 51,260bp alignment of 54 core genes of the 193 *repL*-
720 positive phage-plasmid contigs (Fig. 1) coloured by plasmid replication type, as observed at
721 the tips and inferred via ancestral state reconstruction along the branches (IncY = green,
722 p0111 = orange), showing 17 changes in replication type state (IncY>p0111 (5/17) or
723 p0111>IncY (12/17) = grey).

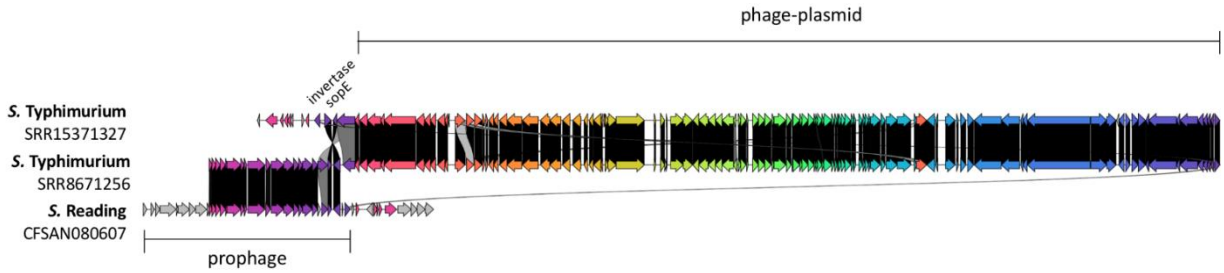


Fig. S2 Phage-plasmids in *S.* Typhimurium putatively integrated in chromosome at site of prophage.

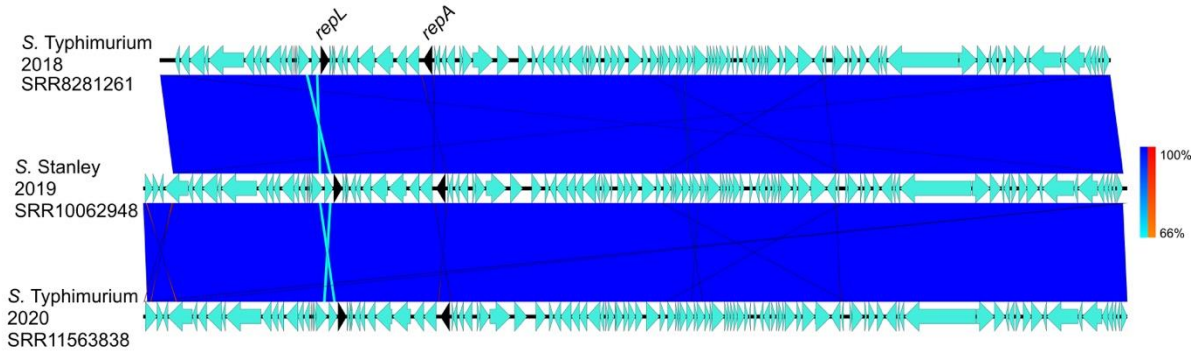


Fig. S3 Highly similar phage-plasmid sequences in different serovars: *S.* Stanley from 2019, and *S.* Typhimurium from 2018 & 2020. Arrows indicate gene direction and scale bars indicate level of similarity for forward (blue) and reverse (red) sequences.

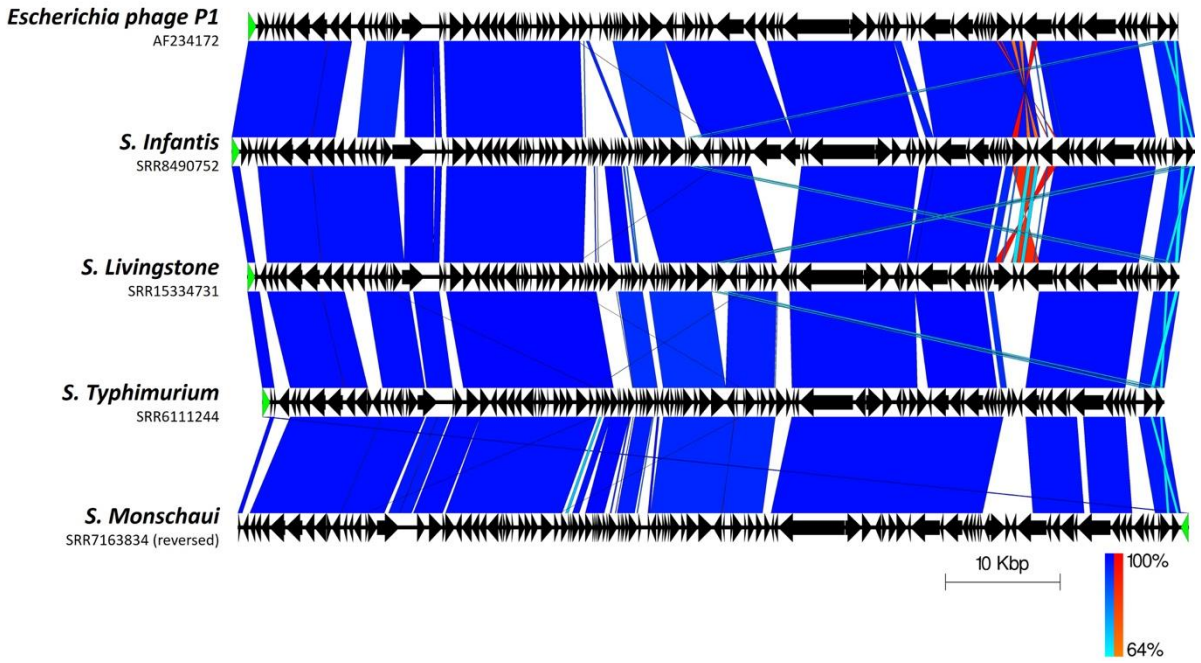


Fig. S4 Variation in selected phage-plasmids from multiple serovars (*S.* Infantis, *S.* Livingstone, *S.* Monschaui and *S.* Typhimurium) compared to P1 bacteriophage. Arrows indicate gene direction and scale bars indicate level of similarity for forward (blue) and reverse (red) sequences. The *repL* gene is highlighted in green.

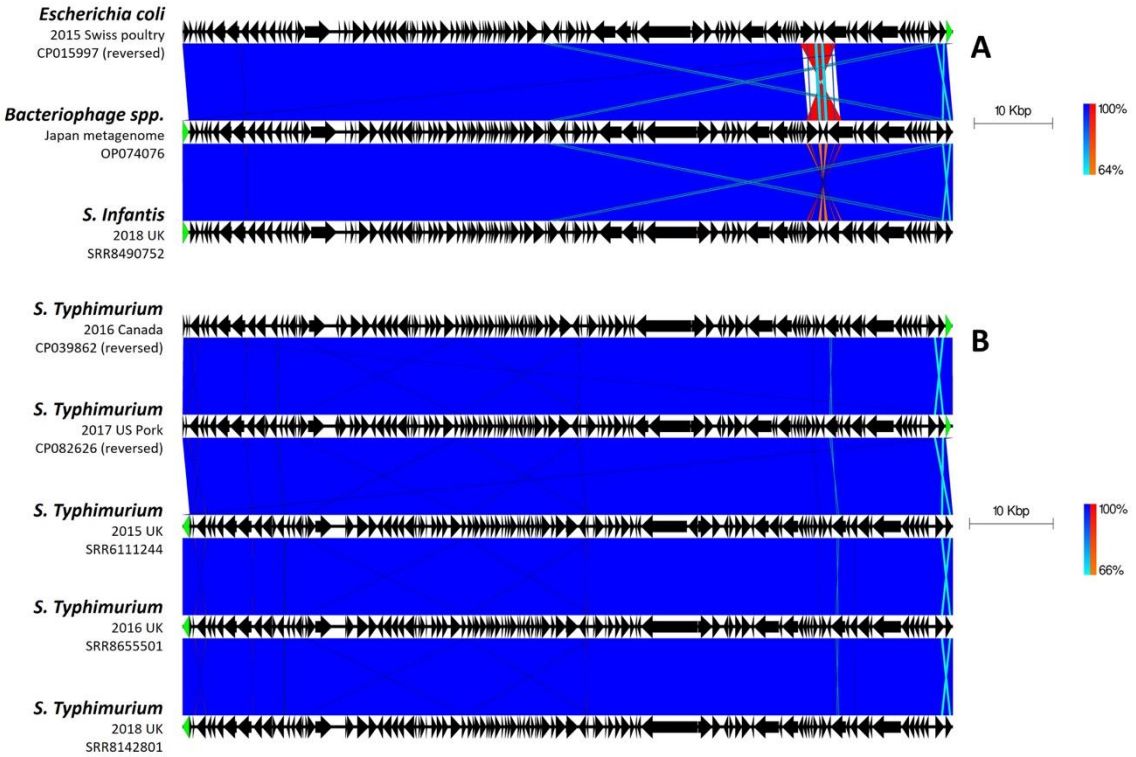


Fig. S5 Highly similar phage-plasmid sequences from public databases showing conserved sequence across: **A)** different species; **B)** isolates of *S.* Typhimurium from different years and countries. Arrows indicate gene direction and scale bars indicate level of similarity for forward (blue) and reverse (red) sequences. The *repL* gene is highlighted in green.

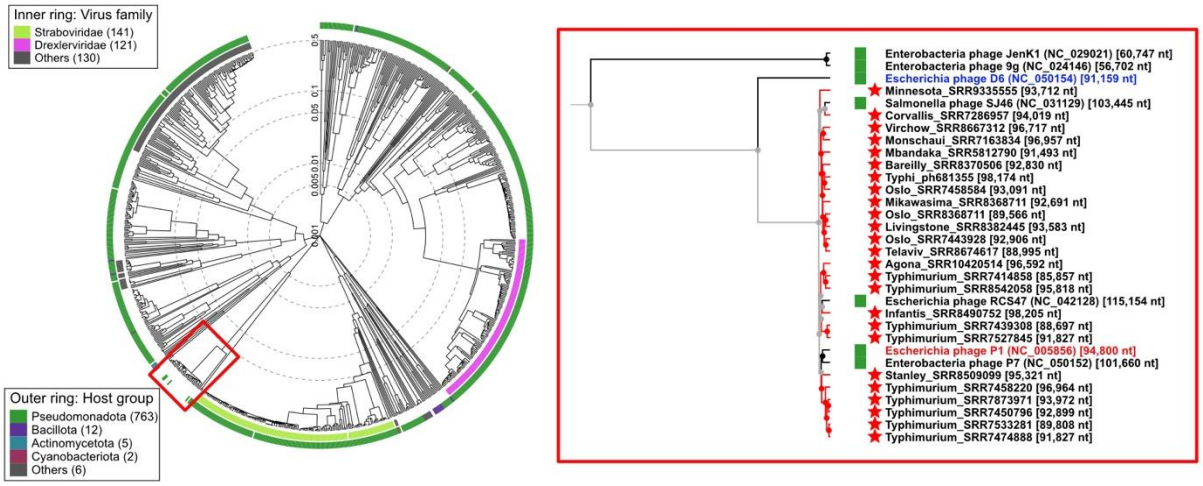


Fig. S6 Detailed output from ViPTree server showing taxonomic context of P1-like phage-plasmids. Left: proteomic tree based on tBLASTx sequence similarity of 825 prokaryotic dsDNA viruses, with location of the P1-like clade highlighted in red; Right: zoomed-in view of highlighted clade, showing 25 representative phage-plasmids from this study and their nearest relatives such as P1 (red), D6 (blue), P7 and SJ46.