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Multidrug resistance in *Salmonella* isolates of swine origin: mobile genetic elements and plasmids associated with cephalosporin resistance with potential transmission to humans

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ABSTRACT

The emergence of foodborne *Salmonella* strains carrying antimicrobial-resistance (AMR) in mobile genetic elements (MGE) is a significant public health threat in a One Health context that requires continuous surveillance. Among them, resistance to ciprofloxacin and cephalosporins are of particular concern. Since pigs and pig products are a relevant source of foodborne *Salmonella* for human beings, we studied transmissible AMR genes and MGE in a collection of 83 *Salmonella* strains showing 9 different serovars and 15 patterns of multidrug-resistant (MDR) previously isolated from pigs raised in the conventional breeding system of Northern Spain. All isolates were susceptible to ciprofloxacin and 3 isolates carried bla_{CMY-2} or $bla_{CTX-M-9}$ genes responsible for cefotaxime resistance. Filter mating experiments showed that the two plasmids carrying $bla_{CTX-M-9}$ were conjugative, while that carrying bla_{CMY-2} was self-transmissible by transformation. Whole-genome sequencing and comparative analyses were performed on the isolates and plasmids. The IncC plasmid pSB109, carrying bla_{CMY-2}, was similar to one found in *S*. Reading from cattle, indicating potential horizontal transfer between serovars and animal sources. The IncHI2 plasmids pSH102 and pSTM45, carrying $bla_{CTX-M-9}$, shared similar backbones and two novel "complex class 1 integrons" containing different AMR and heavy metals genes. This study reports an IncHI2 plasmid carrying $bla_{CTX-M-9}$ in both *S*. Heidelberg and *S*. Typhimurium ST34 strains. Our findings emphasize the importance of sequencing techniques to identify emerging AMR regions in conjugative and stable plasmids from livestock production. The presence of MGE carrying clinically relevant AMR genes raises public health concerns, requiring monitoring to mitigate the emergence of bacteria carrying AMR genes and subsequent spread through animals and food.

1. Introduction

Salmonellosis is one of the main foodborne zoonosis worldwide, whose incidence in many countries has decreased through the implementation of control programs in poultry and laying hens (1). However, pigs and pork products are one of the most common sources of *Salmonella* infection for humans (2). Generally, *Salmonella* infection in humans is self-limited and does not require antimicrobial treatment; however, in more susceptible or immune-compromised patients, the recommended treatments are fluoroquinolones and cephalosporins (3). Unfortunately, one of the side effects of the use of antimicrobials in human and veterinary medicine is the increasing emergence of antimicrobial resistance (AMR) of both, pathogenic and non-pathogenic bacteria (4). According to the World Health Organization 22nd model list of essential medicines, ciprofloxacin (2nd generation fluoroquinolone) and cefotaxime (3rd generation cephalosporin) are classified within the "*watch group*" of surveillance antibiotics (5), indicating that these drugs have a relatively high risk of becoming a target for the acquisition of AMR genes and should be prioritized as key targets for monitoring programs. In fact, an increase in the incidence of AMR to these antibiotics in nontyphoidal *Salmonella* has been observed in several countries over the last decades (6-10). Therefore, the detection and control of multidrug resistant (MDR) *Salmonella* is a priority in the One Health context (https://www.onehealthcommission.org/).

The most efficient method to transfer AMR genes in *Salmonella* is mediated by conjugative plasmids (11). In fact, plasmids from different incompatibility groups including IncC, IncI1, IncHI2 and some mosaic plasmids, have been identified in *Salmonella* harboring AMR genes for fluoroquinolones and cephalosporins (12-15). In addition, AMR genes are commonly associated with other mobile genetic elements (MGE) such as transposons, integrons and insertion sequences, being Integrons Class 1 (IC-1) frequently detected in MDR *Salmonella* of swine origin (16). Consequently, AMR genes can be inserted into new MGE through different

recombination mechanisms and contribute to the spread of the resistome in different environments (17).

Horizontal gene transmission in foodborne zoonotic bacteria is a key mechanism to spread AMR genes, individually or in MDR cassettes, with dramatic consequences in animal and human health. Understanding the epidemiology and the mechanisms involved in horizontal transmission of resistance genes in *Salmonella* could help to design intervention strategies directed to reduce their incidence.

The aim of this study was to address and characterize the presence of MDR determinants including ciprofloxacin and cephalosporin resistance genes in *Salmonella* isolates collected from pigs intended for human consumption, and their mobilization capability through integrons and plasmids, as a source of emerging AMR bacteria of clinical and epidemiological relevance. After a phenotypic and genotypic screening, we did not find genes conferring resistance to ciprofloxacin, but three *Salmonella* isolates exhibited *bla* genes conferring resistance to cephalosporins. Combining Illumina and Nanopore sequencing technologies, we found three conjugative plasmids, two (pSB109 and pSH102) sharing homology with previously described plasmids, and a novel plasmid (pSTM45) harboring $bla_{\text{CTX-M-9}}$ and $bla_{\text{TEM-1B}}$ genes.

2. Material and Methods

2.1. Experimental design

We selected a total of 83 *Salmonella* isolates of swine origin (31 from mesenteric lymph nodes and 52 from intestinal content) showing AMR to ≥3 drugs (MDR) in the disk-diffusion test with antibiotics of 6 different families (Table S1). All strains were stored under lyophilization at the IdAB-CSIC bacterial collection. As shown in Table S1, these isolates were originally obtained from a total of 2,407 samples of mesenteric lymph nodes (n=989) or intestinal content (n=1,418) of pigs intended for human consumption, sampled at abattoirs of Navarra (Spain), and processed following the ISO 6579:2002/Am. 1:2007 (18) as previously detailed (19, 20). Each

selected colony was serotyped at the National Reference Centre for Animal Salmonellosis (Algete, Madrid) according to the Kauffmann-White-Le Minor scheme (21), and those selected for this study showed MDR in the standard disk diffusion test (22) to three or more of the antimicrobial families represented by the following drugs: ampicillin (A; ß-lactams); chloramphenicol (C; phenicols); streptomycin (S; aminoglycosides); sulfisoxazole (Su; sulfonamides); tetracycline (T; tetracyclines); and nalidixic acid (Nx; 1st generation quinolones).

These 83 strains were analyzed by PCR (23) to detect Class 1 integrons (IC-1), as the main MGE associated with horizontal transmission of MDR genes in *Salmonella* (16). Next, the 52 strains showing IC-1 (Table 1) were analyzed by sequencing the integron-borne gene cassette and by PCRs to detect a selection of AMR genes (Table S2). Thereafter, the three isolates containing $bla_{\text{CTX-M}}$ (n=2) and bla_{CMY} (n=1) conferring resistance to cephalosporins (*i.e.* strains IdAB109, IdAB102 and IdAB45; Table 2) were further characterized for: (i) susceptibility to cephalosporins by disk diffusion and minimal inhibitory concentration (MIC) to cefotaxime and other antimicrobial drugs; (ii) production of ESBL by a double disk-diffusion synergy test; (iii) production of serin-β-lactamases AmpC type by disk-diffusion test using different cephalosporins with cloxacilline, as an anti- β-lactam agent; (iv) conjugation of plasmids and plasmid characterization, and (v) whole genome sequencing (WGS).

2.2. Detection of IC-1 and individual AMR genes

The detection of IC-1 and AMR genes *sul1*, *sul2*, *sul3*, *aac(3)IV aad*A, *str*A/*str*B, *aad*B, *aph*A1, *aph*A2, *tet*(A), *tet*(B), *tet*(C), *oxa*, t*em, shv,* $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, *aac(6')-Ib-cr*, *qnr*A, *qnr*B, *qnr*C and *qnr*D were performed by PCR as summarized in Table S2. In general, total bacterial DNA samples were obtained by boiling (24) and the amplified DNA products were visualized by gel electrophoresis in 1% agarose and Midori Green (NIPPON Genetics Europe, xxx), using the adequate DNA molecular weight ladder (Promega Biotech Ibérica, Madrid, Spain) for each amplification product.

2.3. Characterization of IC-1 borne gene-cassettes

The IC-1 borne gene-cassettes were analyzed by Sanger sequencing (Secugen, Universidad Complutense de Madrid, Spain). DNA fragments amplified by the IC-1 PCR (23) were purified using a commercial kit (ATP^{TM} Biotech, Taipei, Taiwan). DNA sequences were compared using public databases including Pubmed and http://www.lahey.org/Studies.

2.4. Phenotypic screening of ESBL and AmpC isolates

Disk diffusion test was performed by using disks impregnated in cefotaxime 30 µg (BD BBL™ Sensi-Disc™, Becton Dickinson, Canada), as detailed (25). Minimal inhibitory concentration (MICs) for 14 antimicrobial agents were determined using the broth microdilution method (Vet Mic-mo; Swedish Veterinary Institute), with the following antimicrobial agents: ampicillin (A); ciprofloxacin (Cip); nalidixic acid (Na); gentamicin (G); streptomycin (S); tetracycline (T); florfenicol (F); colistin (Co); sulfamethoxazole (Su); trimethoprim (Tm); chloramphenicol (C); kanamycin (K); cefotaxime (Ctx) and ceftazidime (Caz). *E. coli* ATCC 25922 was used as control of susceptibility. Epidemiological cut-off values were determined according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/).

The production of ESBL was confirmed by double disk diffusion synergy test (26). Briefly, bacterial suspensions containing ≈10^5 CFU were spread on BBL Muller-Hinton (MH; BD Shannon, Ireland) agar plates. cefotaxime and amoxicillin-clavulanic acid disks (BD BBL™ Sensi-Disc™, Becton Dickinson, Canada) were placed 20 mm apart; one disk of cefotaxime alone was used as plate control; *S*. Typhimurium strain ATCC 14028 and *Klebsiella pneumoniae* showing ESBL positive (27) were used as controls of susceptibility and synergy. Synergy was defined as an increase in zone diameter ≥5 mm (26).

ESBL-negative strains were further analyzed to detect AmpC serin-β-lactamases production by disk diffusion MH vs. MH supplemented with cloxacillin (200 mg/L; Sigma Laboratorios, Madrid, Spain) and disks (30 µg/disk all from BD BBL™ Sensi-Disc™, Becton Dickinson, Canada) of different cephalosporines (cefepime, ceftriaxone, ceftazidime, cefotetan,

cefoxitin and amoxicillin clavulanic). *Enterobacter cloacae* was used as an AmpC control. Bacteria showing an inhibition zone of ≥5 mm larger in MH-cloxacillin than in MH were considered AmpC producers (28).

2.5 Transferability of plasmids carrying ESBL and AmpC genes.

The horizontal transferability of ESBL or AmpC genes as well as the presence and stability of conjugative plasmids were carried out with the three isolates showing resistance to cephalosporin. For this, filter mating experiments were performed by conjugation and transformation as previously described (29, 30). Briefly, for the conjugation experiments, 500 µL of the donors and recipient strains in exponential growth phase were mixed and placed in a 0.2µm paper filter on a blood agar plate. After overnight incubation, the filter was resuspended in 4mL of saline and 100 µL inoculated onto Brain Heart Infusion agar plates containing rifampicin (100 mg/L) and ceftriaxone (1 mg/L). For the transformation experiments, plasmid DNA was extracted with the Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) and electroporated to electrocompetent plasmid-free *E. coli* strain HB101. Transformants were selected in Luria-Bertani agar supplemented with rifampicin (100 mg/L) and ceftriaxone (1 mg/L).

XbaI-PFGE (31) was carried out to verify the identity of donors, transconjugants and transformant. The transfer of a unique plasmid mediating resistance to cephalosporin as well as the plasmid size was determined by S1-PFGE performed on transconjugants and transformants (32). Additionally, the presence of cephalosporin resistant genes was confirmed phenotypically and genotypically by MIC and PCR, respectively.

2.6 PCR based replicon typing.

Plasmids were classified according to their incompatibility group using the PCR replicon-typing scheme using primers for FIA, FIB, FIC, HI1, HI2, I1-IY, L/M, N, P, W, T, A/C,K, B/O, X, Y, F, and FIIA, as described by Carattoli (33).

2.7 Stability of plasmids carrying cephalosporin resistant genes.

Strains carrying plasmids with cephalosporin resistant genes were grown in plates of TSA supplemented with cefotaxime 1 mg/L (TSA+CTX). Thereafter, one colony was transferred simultaneously onto TSA and TSA+CTX. Plates were incubated at 37 ˚C for 18h. Passages were repeated eight times. Bacteria obtained in the last passage were used to prepare individual inoculum (OD_{600nm}=0.170) followed by serial dilutions and plating. Thus, the plasmid stability was determined by comparing the number of CFU in TSA vs TSA+CTX. The presence of the plasmid was confirmed by *bla*$_{CTX\text{-}M}$ or *bla*$_{CMY}$ PCR of over-colony DNA extracted from at least 50 well-isolated colonies. First passage was used as internal control.

2.8. Whole Genome Sequencing (WGS) and Bioinformatic Analysis

The three *Salmonella* isolates showing AMR to cephalosporins were sequenced by combining Illumina (short-reads) and Nanopore (long-reads) technologies. Briefly, genomic DNA was extracted and purified using the NucleoSpin Tissue DNA purification kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's instructions. Sequencing was performed on the Illumina NovaSeq platform using Nextera XT DNA library preparation kit at Eurofins Genomics (Ebersberg, Germany) and the Oxford Nanopore GridION platform at Nano1Health (University of Barcelona, Spain). Complete genomes were obtained by hybrid assembly using Unicycler v0.4.8.0 (34) and then annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (35). The whole genomes and plasmids carrying ESBL sequences were deposited at the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers CP101854, CP101856, and CP102198.

Thereafter, different *in silico* bioinformatic analysis were performed (all accessed on 2022/07/25) to: (i) verify the serotypes by SeqSero v1.2 (https://cge.food.dtu.dk/services/SeqSero/); (ii) determine the MLST and core genome MLST (cgMSLT) profiles by using MLST v2.0 (and cgMLSTFinder 1.2 (https://cge.food.dtu.dk/services/cgMLSTFinder/), respectively, which were based on the

PubMLST database typing (7 genes) and *Salmonella* Enterobase (3,002 genes) schemes, respectively; (iii) detect *Salmonella* Pathogenicity and Genomic Islands, integrons, and AMR genes by SPIFinder v2.0 (https://cge.food.dtu.dk/services/SPIFinder/), IntegronFinder v2.0.2 (http://dx.doi.org/10.3390/microorganisms10040700), and ResFinder v4.1 (https://cge.food.dtu.dk/services/ResFinder/) programs, respectively; (iv) identify multi-AMR plasmid incompatibility groups by PlasmidFinder v2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/); and (v) compare the plasmids DNA sequences by BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligning them by EasyFig with the highest homology (36).

3. Results

Overall, the 83 *Salmonella* isolates selected for this study belonged to 9 different serovars and exhibited 15 different MDR patterns (Table 1). Of them, 52 isolates carried IC-1 of 3 different sizes (*i.e.*, 1,000, 2,000 and 1,000+1,200 bp) with 7 different IC-1 borne gene-cassettes. Whereas 34 out 40 *S.* Typhimurium isolates contained some type of IC-1, this MGE was absent in the 14 monophasic variants. Besides other AMR genes, 3 of the 52 isolates (*i.e.*, IdAB109, IdAB102, and IdAB45) harbored bla_{CMY-2} (n=1) and $bla_{CTX-M-9}$ (n=2) genes coding for AmpC and ESBL respectively. These three isolates showed phenotypic resistance to cefotaxime in both disk diffusion and MIC tests (Table 2), as well as resistance to other antimicrobial families (Table 3).

Both plasmids carrying $bla_{CTX-M-9}$ were conjugative. S1-PFGE of *E. coli* transconjugants obtained from these *Salmonella* donors (IdAB45 and IdA102) showed just one plasmid each of ≈ 300 Kb (IdAB45) and ≈ 280 Kb (IdAB-102), respectively. In contrast, isolate yielding bla_{CMY-2} IdAB109 did not conjugate, but S1-PFGE analyses demonstrated the presence of two plasmids of ≈250Kb and ≈145 Kb each in this isolate (Figure S2). The bla_{CMY-2} gene was confirmed to be located in the ≈ 145 Kb plasmid observed by S1-PFGE in the transformant (Figure S1, Table 2).

Moreover, plasmids carrying cephalosporin resistant genes were stable after 8 passages in the absence of antibiotic pressure, with similar bacterial counts between passage 1 and 8 and 100% of colonies testing positive by PCR for $bla_{CTX-M-9}$ or bla_{CMY-2} genes (data not shown).

Whole genome sequencing of isolates and plasmids carrying cephalosporin resistant genes.

The WGS data of the three isolates were uploaded to the GenBank and the accession numbers assigned, plasmid names assigned and sizes, AMR genes found, and bioinformatics results obtained by PubMLST, cgMLSTFinder, SPIFinder, IntegronFinder, ResFinder, and PlasmidFinder are presented in Table 2. According to MLST and cgMLST, strain *S*. Typhimurium IdAB45 was classified as ST34 and cgST186136, strain *S*. Heidelberg IdAB-102 as ST15 and cgST194237, and strain *S*. Bredeney IdAB-109 as ST306 and cgST255665. SPIFinder, Blast and IntegronFinder detected different *Salmonella* Pathogenicity Islands (SPI), the presence of IC-1 in the sequences of the three isolates, and the presence of *Salmonella* Genomic Island 1 (SGI-1) in IdAB-109 and SGI-4 in IdAB45. PlasmidFinder and ResFinder detected the IC-1 and different AMR genes located in the plasmids, confirming the presence of bla_{CMY-2} gene in plasmid pSB109 and $bla_{CTX-M-9}$ in pSTM45 and pSH102 (Table 2).

Plasmid pSB109 consisted of 151,740 bp, had a mean GC content of 52.7%, belonged to IncC incompatibility group, and was non-conjugative in the filter mating experiments (Table 2). The annotation analysis evidenced that plasmid pSB109 had the transcriptional activator complex AcaCD, disrupted by an IS3-like element ISEc52 family transposase, which is essential for IncC plasmid transfer. The presence of several AMR genes was detected including bla_{CMY-2}, *floR*, *tet*(A)*, aph(6)-Id, aph(3'')-Ib, sul2, aad*A7*, qac*E and *sul1.* As shown in Figure 1, the AmpC-type β-lactamase gene bla_{CMY-2} was located next to the ISEcp1 of the IS1380 family transposase. Downstream ≈ 50 Kb of this IS, an IC-1 containing AMR genes including *intI*-*aad*A7-*qac*E-*sul1* was detected. Besides, this plasmid showed 95 % coverage and 100 % identity with plasmid pSA20025921.1 (accession no. CP030215.1) recovered from a *S.* Reading of bovine origin. The non-matching fragments corresponded to three copies of the ISEc52, one copy of IS110, and

one copy of IS66. Additionally, pSB109 showed 62% coverage and 100% identity with p24358-2 (accession no. CP051443.1) from a *S*. Bredeney which also belonged to the IncC group and contained the bla_{CMY-2} gene. However, the latest was smaller (96,127 bp) and IC-1 was absent.

On the other hand, the two conjugative plasmids recovered from *S.* Heidelberg (pSH102) and *S.* Typhimurium (pSTM45) showed similar size (286,802 bp and 306,822 bp, respectively), similar average GC content (46.6 % and 46.8 %, respectively), high degree of sequence homology (97 % coverage and 100 % identity), and a conserved IncHI2 plasmid backbone of 247,783 bp and 247,766 bp, respectively (Figure 2A). Moreover, both plasmid backbones had 100 % coverage and 99.98 % identity between them, similar genetic AMR regions (≈40 kb in pSH102 and ≈60 kb in pSTM45), and both contained the replicon IncHI2, the partition system, the conjugative genes, the telluric resistance cluster and other hypothetical proteins. Additionally, pSH102 and pSTM45 backbones showed 97 % coverage and 99.99 % identity with plasmids of many Enterobacterales, including *Klebsiella pneumoniae* plasmid pZ0117KP0017-1 (accession no. CP098155.1), *Enterobacter hormaechei* plasmid pGENC200 (accession no. CP061495.1), *E. coli* plasmid pEc21617-310 (accession no. MG878867.1) and *S. enterica* plasmid pKST313 (accession no. LN794248.1). However, pSH102 and pSTM45 differed between them on the number of AMR genes, with pSH102 yielding four additional genes conferring resistance to aminoglycosides (*aph(3')-Ia, aph(3'')-Ib, aph(6)-Id*) and sulfonamides (*sul2*) (Table 2). On the contrary, pSTM45 contained genes coffering resistance to heavy metals (Figure 2B).

Blast search of the ≈40 Kb AMR region of plasmid pSH102 retrieved plasmid pRCS54 (accession: LT985263.1) of *E. coli* (79 % coverage and 99.97 % identity) from incQ1 incompatibility group. Linear alignment of these regions (pSH102 vs. pRCS54, Figure 1B) revealed that pSH102 contained the putative colistin resistance gene *mcr-9* and several insertion sequences and a Tn3 transposase family located downstream of the region which is absent in pRCS54. For the pSTM45 ≈ 60 Kb region, the highest homology was obtained for pEhoE1-1 (accession no. CP066095.1) of *Enterobacter hormaechei* (54 % coverage and 99.99 % identity)

from IncHI2 incompatibility group. The alignment of these AMR regions (pSTM45 vs. pEhoE1-1, Figure 4B) revealed the presence of *bla*$_{CTX-M-9}$, *bla*$_{TEM-1B}$, *tet*(A), *aadA2b* and some transposons of pSTM45 that were not present in pEhoE1-1, as well as different genetic organization of both plasmids (Figure 1C).

BLAST comparison of the complete plasmids pSH102 and pSTM45 with all available *Salmonella* spp. genomes at NCBI, including the genomes of the specific serovars *S*. Heidelberg and *S*. Typhimurium, respectively, revealed no identical plasmids described in the NCBI database to date. With respect to all genomes described for *Salmonella* spp., pSH102 showed the highest homology with the 324,175 bp *S*. Senftenberg plasmid pCFSAN004025.1 (accession no. CP039271.1; 84 % coverage and 99.99 % identity) and, among serovar Heidelberg, with a 232,341 bp unnamed plasmid (accession no. CP027411.1, 62 % coverage and 98.40 % identity). Neither of these two *Salmonella* plasmids contained IC-1 or genes encoding ESBL. Likewise, the plasmid showing the highest homology with pSTM45 was pKST313 (accession no. LN794248. 1; 86 % coverage and 99.99 % identity) recovered from a *S*. Typhimurium ST313 human clinical strain. However, the plasmid pSTM45 containing a novel AMR region showed relevant differences with respect to pKST313 such as (i) 41 % coverage of their AMR regions, and (ii) different ESBL genes (i.e. *bla*$_{CTX-M-9}$ and *bla*$_{TEM-1B}$ in pSTM45 vs. *bla*$_{CTX-M-15}$ gene in pKST313).

4. Discussion

The recommended antibiotic treatment for invasive salmonellosis in risk population are fluoroquinolones and 3rd generation cephalosporins. Several studies have demonstrated the presence of multidrug resistant (MDR) *Salmonella* of pig origin with resistance genes associated with MGE (26). A total of 83 (33.6 %) MDR *Salmonella* strains from pigs of the conventional production of Spain were selected for this study. None of them exhibited resistance to ciprofloxacin and only three strains of three different serovars showed resistance to 3rd

generation cephalosporins, all of them carrying the resistance genes in large plasmids of the IncC and IncHI2 incompatibility groups.

IncC plasmids have a wide range of hosts and are mostly conjugative plasmids, therefore they play a key role in the dissemination of AMR genes (37). Moreover, IncC plasmids have been previously described in *Salmonella* harboring up to ten AMR genes for more than five classes of antibiotics (17). Herein, we describe an IncC plasmid, named pSB109 detected on *S*. Bredeney, which contained the bla_{CMY-2} gene next to the ISEcp1 transposon-like element. ISEcp1 transposons play an important role in the spread of bla_{CMY-2} genes in Enterobacterales. In fact, the genetic organization with structure ISEcp1-bla_{CMY-2}-blc-sugE is highly conserved in different plasmid backbones (38-40). In particular, the bla_{CMY-2} gene has been frequently reported in IncC plasmids from different *Salmonella* serovars such as Typhimurium, Choleraesuis, Heidelberg, Bredeney, Newport, among others, and different origins including humans, pigs and poultry (15, 41, 42), but so far no plasmids containing bla_{CMY-2} gene have been reported in *S*. Bredeney strains of sequence type ST306.

Conjugation experiments revealed the absence of conjugative capability in pSB109. This finding can be attributed to the disruption of the transcriptional activator complex AcaCD by the insertion sequence found; thus, indicating plasmid compatibility with SGI1 inside strain *S*. Bredeney. The conjugation experiments evidenced that pSB109 lacks conjugative capability. Previous studies have shown that IncC conjugative plasmids trigger SGI1 excision from the chromosome and transfer to a new host (44). However, for stable maintenance in the cell, SGI1 destabilizes the plasmid, resulting in reported incompatibility between SGI1 and its IncC mobilizing plasmids (45, 46). The plasmid AcaCD complex, activates the expression of *xis*, *traNS*, and *traHGS* genes of SGI1, initiating the excision of SGI1 (47, 48, 49). Therefore, in plasmid pSB109, the disruption of AcaCD by an insertion sequence would hinder its ability to activate SGI1 excision. Additionally, S1 PFGE studies, revealed the presence of a 250 kb plasmid in this isolate that was not possible to reconstruct through sequencing technology. Presumably, this

plasmid has been lost during passage when culturing the isolate for sequencing, since the isolate was grown in media supplemented with ceftriaxone to select for the cephalosporin resistance phenotype. Instability of plasmids have been described before for *Salmonella*, especially when subjected to high environmental selective pressure.

In this study, we have also described two conjugative plasmids of the incHI2 incompatibility group. According to different studies, IncHI2 is the most predominant incompatibility group found in MDR *Salmonella* (13, 14). In general, IncHI2 plasmids have a well-conserved backbone structure with variable regions (17). Here, we described two conjugative plasmids that have the conserved IncHI2 backbone and novel antimicrobial resistance regions containing *bla*$_{CTX-M-9}$. The two AMR regions of plasmids pSH102 and pSTM45 had less than 86% similarity to any of the plasmids available in the public databases. To our knowledge, IncHI2 plasmid containing the *bla*$_{CTX-M-9}$ gene has not been previously reported in the *S*. Heidelberg serovar nor in S. Typhimurium ST34 strains. So far, plasmids of the IncHI2 group with the *bla*$_{CTX-M-9}$ gene have been described in strains of the monophasic variant of *S*. Typhimurium ST34 (50) but not specifically in biphasic *S*. Typhimurium strains with this sequence type.

Comparative analysis revealed that pSH102 and pSTM45 plasmids contained two previously unreported types of incomplete “complex class 1 integrons”. Previous studies have shown that this structure is comprised by a 5′ conserved segment (*intI1-attI*), an intermediate variable region (VR1) and a 3′ conserved segment (Δ*qacE-sul1*), followed by an IS*CR1* element, an IS*CR1*-linked resistance gene region (VR2) and the same 3′ conserved segment (51, 52). However, the last repetition of the 3′ conserved segment (Δ*qacE-sul1*) was not present in neither of the two conjugative plasmids described in this study and an IS5 was detected at its position. The deletion of the 3′ conserved segment could be due to the insertion of this IS*5*, since these type of IS are MGE with the capacity to move in both, the chromosome and the plasmids (53). Within the ISCR1-linked resistance gene (VR2) regions of both plasmids, the extended-spectrum β-lactamase *bla*$_{CTX-M-9}$ gene and the colistin resistance *mcr-9* gene were detected. It is interesting

to notice, that although the presence of *mcr-9* was confirmed by sequencing, the two isolates were phenotypically susceptible to colistin, as well as the transconjugants. As reported in other studies, *mcr-9* encodes an acquired phospho-ethanolamine transferase not conferring by itself clinical resistance to colistin (54, 55). Still, there were two considerable differences between AMR regions of the two conjugative IncHI2 plasmids described herein, i) the pSH102 contained aminoglycoside and sulfonamide resistance genes flanked by two IS*26* sequences that were not present in pSTM45 and ii) pSTM45 had a structure of 30 Kb within the VR2 region yielding genes conferring resistance to mercury, cadmium, zinc and silver that were not present in pSH102.

Overall, in this study we present two IncHI2 conjugative plasmids and one IncC non-conjugative plasmid harboring cephalosporin resistance genes, with two novel AMR regions from three MDR *Salmonella* of different serotypes. In these regions, we found a previously unreported "complex class 1 integrons" where the *bla*$_{CTX-M-9}$ gene was located together with AMR genes belonging to different antimicrobial classes including colistin, and/or genes that confer resistance to heavy metals. Conjugation is a significant factor driving the rapid evolution of bacterial genomes as it facilitates the adaptation of bacterial strains through the transmission of advantageous metabolic properties, including resistance to antibiotics and heavy metals (56). Hence, the performance of conjugation and transformation assays in this study was essential for comprehending the mobility of the described plasmids. Insertions and rearrangements of antibiotic resistance genes by MGE represent a mechanism for evolution and shaping different incompatibility families of plasmids within *Salmonella* serovars. Therefore, the presence of MGE is of public health concern, as clinically relevant AMR genes together with genes conferring resistance to heavy metals are being incorporated into new transferrable mega plasmids that could provide bacteria the ability to survive under extreme selective pressure. Hence, the low frequency of cefotaxime and fluoroquinolones resistance underlines the success of the initiatives implemented at food animal agriculture to reduce the use of antimicrobials in order to prevent the spread of resistance.

5. Conclusions

This scientific paper provides valuable insights into the AMR patterns of *Salmonella* isolates from pigs sampled at abattoir and intended to human consumption. Despite of a significant percentage of strains showing AMR to three or more antibiotic families (MDR), only three isolates of different serovars showed AMR to third-generation cephalosporins, carrying the genes responsible in large plasmids of the IncC and IncHI2 incompatibility groups. The mobile nature of these plasmids spotlights the risk of pig products as possible source of MDR *Salmonella* to human beings representing a public health concern. However, the low frequency of *Salmonella* strains from animal origin carrying AMR to cefotaxime suggests the success of antimicrobial use reduction initiatives in food animal to prevent the spread of AMR. These findings emphasize the need for continued surveillance and intervention strategies from the farm to mitigate the potential impacts on human health to combat AMR in foodborne *Salmonella*. Also, our study underscores the relevance of sequencing techniques to identify novel AMR regions in plasmids from livestock production.

AUTHOR CONTRIBUTIONS

MJG, VG and LMG designed and performed the experiments; LMG and LL gave conceptual advice; AAG and LL performed the genome assembly and bioinformatic analysis; LMG and MJG funded the experiments; VG and AAG wrote the draft; MJG analyzed all data and wrote the manuscript; all authors have corrected and approved the final manuscript.

Data availability

WGS data are available in public databases indicated in the text and in Table 2; other data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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TABLE S1 *Salmonella* strains of swine origin and previous information of selected the IdAB-CSIC collection for this study.

Original swine samples		*Salmonella* strains at IdAB-CSIC collection	
Type	**No. samples analyzed**	**No. strains/ serotypes** [a]	**No. strains with AMR / multi-AMR** [b]
Mesenteric lymph nodes	989	77/ 12	41/ 31
Intestinal content	1,418	170/ 21	124/ 52
Total	**2,407**	**247/ 27**	**165/ 83**

[a] Serotyped at the National Reference Center of Animal Salmonellosis (Algete, Madrid, Spain); [b] AMR: antimicrobial resistance by standard disk diffusion method; multi-AMR: AMR to ≥3 antimicrobial families.

TABLE S2 PCR used to detect IC-1 and individual AMR genes in the *Salmonella* strains selected for this study.

MGE/ gene	Oligonucleotides	Control strain used [source]	AMR family/agent encoded	Conditions and primer reference
5CS 3CS	F: 5´ GGCATCCAAGCAGCAAG 3´ R: 5´ AAGCAGACTTGACCTGA 3´	*S.* Typhymurium DT104	IC-1 conserved regions	[23]
sul1	F: 5´ CGGCGTGGGCTACCTGAACG 3´ R: 5´ GCCGATCGCGTGAAGTTCCG 3´	*S.* Branderburg E3G2V1C30 [30]	Sulfonamides	[57]
sul2	F: 5´ CGGCATCGTCAACATAACCT 3´ R: 5´ TGTGCGGATGAAGTCAGCTC 3´	*S.* Typhimurium monophasic E3G3V2C59 [30]		
sul3	F: 5´ CAACGGAAGTGGGCGTTGTGGA 3´ R: 5´ GCTGCACCAATTCGCTGAACG 3´	*S.* Branderburg E3G2V1C30 [30]		
StrA/ StrB	F: 5´ ATGGTGGACCCTAAAACTCT 3´ R: 5´ CGTCTAGGATCGAGACAAAG 3´		Aminoglycosides • Streptomycin	
aac(3)IV	F: 5´ TGCTGGTCCACAGCTCCTTC 3´ R: 5´ CGGATGCAGGAAGATCAA 3´	*S.* Rissen E3G5V4C41 [30]	• Aminoglycoside acetyltransferases	
*aad*A	F: 5´ GTGGATGGCGGCCTGAAGCC 3´ R: 5´ AATGCCCAGTCGGCAGCG 3´			
*aad*B	F: 5´ GAGGAGTTGGACTATGGATT 3´ R: 5´ CTTCATCGGCATAGTAAAAG 3´			
*aph*A1	F: 5´ ATGGGCTCGCGATAATGTC 3´ R: 5´ CTCACCGAGGCAGTTCCAT 3´	*S.* Panama E3G3V4C16 [30]	• Kanamycin	
*aph*A2	F: 5´ GATTGAACAAGATGGATTGC 3´ R: 5´ CCATGATGGATACTTTCTCG 3´			
tet(A)	F: 5´ GGCGGTCTTCTTCATCATGC 3´ R: 5´ CGGCAGGCAGAGCAAGTAGA 3´	*S.* Branderburg E3G2V1C30 [30]	Tetracyclines	
tet(B)	F: 5´ CGCCCAGTGCTGTTGTTGTC 3´ R: 5´ CGCGTTGAGAAGCTGAGGTG 3´	*S.* Panama E3G3V4C16 [30]		
tet(C)	F: 5´ GCTGTAGGCATAGGCTTGGT 3´ R: 5´ GCCGGAAGCGAGAAGAATCA 3´	*E. coli* GN-3021 [58]		
bla_{OXA}	F: 5´ ATATCTCTACTGTTGCATCTCC 3´ F: 5´AAACCCTTCAAACCATCC 3´	*E. coli* [27]	Plasmid mediated β-lactamases	[59]
bla_{SHV}	F: 5´ AGGATTGACTGCCTTTTTG 3´ F: 5´ ATTTGCTGATTTCGCTCG	*E. coli* E1V1C80a [26]		
bla_{TEM}	F: 5´ATCAGCAATAAACCAGC 3´ F: 5´ CCCCGAAGAACGTTTTC 3´	*E. coli* E1V1C63a [26]		[60]
$bla_{CTX\text{-}M\ universal}$	F: 5' CGATGTGCAGTACCAGTAA 3´ R: 5' TTAGTGACCAGAATCAGCGG 3'		Class A ESBL	[61]
$bla_{CTX\text{-}M\text{-}9}$	F: 5' GTGACAAAGAGAGTGCAACGG 3´ R: 5' ATGATTCTCGCCGCGAAGCC 3´			[29]
$bla_{CMY\text{-}2}$	F: 5' GCACTTAGCCACCTATACGGCAG 3' R: 5' GCTTTTCAAGAATGCGCCAGG 3'	*E. cloacae*	AmpC serin-β-lactamases	
aac(6')-Ib	F:5' TTGCGATGCTCTATGAGTGGCTA 3' R: 5' CTCGAATGCCTGGCGTGTTT 3'	*E. coli* VO162_S12		[62]
qnrA	F: 5' GGATGCCAGTTTCGAGGA 3' R: 5' TGCCAGGCACAGATCTTG 3'	*S.* Branderburg E3G2V1C30 [30]	Plasmid mediated fluoroquinolone resistance	[63]
qnrB	F: 5' GGMATHGAAATTCGCCACTG 3' R: 5' TTTGCYGYYCGCCAGTCGAA 3'			[64]
qnrC	F: 5' GGGTTGTACATTTATTGAATCG 3' R: 5' CACCTACCCATTTATTTTCA 3'	*E. coli* GN2221 [66]		[65]
qnrD	F: 5' CGAGATCAATTTACGGGGAATA 3' R: 5' AACAAGCTGAAGCGCCTG 3'			[67]
qnrS	F: 5' TCGACGTGCTAACTTGCG ´3 R: 5' GATCTAAACCGTCGAGTTCGG ´3			[68]

MGE: Mobile Genetic Element

TABLE S3 Minimal inhibitory concentration (µg mL-1) of *Salmonella* strains showing ESBL or AmpC by using the broth microdilution test with a panel of 14 antimicrobial agents.

Strain	**Minimal inhibitory concentration (µg mL-1) of antimicrobial agents a**													
	Amp	Cip	Na	G	S	T	F	Co	Su	Tm	Cm	K	Cfx/[b]	Caz
IdAB-45	>128	0.06	8	1	256	32	8	<0.5	>1024	>16	4	<8	>2/32	>2
IdAB-102	>128	0.12	4	5	>256	32	<4	<0.5	>1024	>16	4	>16	>2/16	>4
IdAB-109	>128	0.06	8	1	>256	64	>32	<0.5	>1024	>16	>64	>16	>2/36	>16

[a] Antimicrobial agents: ampicillin (A); Ciprofloxacin (Cip); Nalidixic acid (Na); Gentamicin (G); Streptomycin (S); Tetracycline (T); Florfenicol (F); Colistin (Co); S sulfamethoxazole (Su); Trimethoprim (Tm); Chloramphenicol (C); Kanamycin (K); Cefotaxime (Cfx); Ceftazidime (Caz). Epidemiological cut off values were determined according to recommendations of the European Committee on Antimicrobial susceptibility testing (EUCAST); [b] MIC was also analyzed using higher concentration of cefotaxime (from 256 to 1 µg/mL): MIC ≤ 1 µg/mL (sensitive), = 2 µg/mL (intermediate), ≥ 4 µg/mL (resistant).

TABLE 1 Characteristics of the 83 MDR *Salmonella* strains of swine origin selected for this study from the IdAB-CSIC collection, and genetic characterization of their Integron Class 1 (IC-1) and other AMR genes determined in strains carrying IC-1.

Selection criteria of *Salmonella* strains		**Genetic characterization of IC-1 and individual AMR genes**		
Serovar (n) [a]	**MDR profiles (n)** [b]	**IC-1 size** [c] **(n)**	**Integron-borne gene cassette** [d]	**AMR genes** [e] **(n)**
Typhimurium (34)	ACSSuT (20)	1,000+1,200 (19);	*aadA2*; *pse1*; *cmlA1* (18)	*aadA*; *aphA2*; *sul1* (19)
			aadA2; *tem; aadA1* (1)	
		2,000 (1)	*bla*-oxa; *aadA1* (1)	*aadA; aphA2*; *sul1*; *tetB* (1)
	ACSSuTNx (12)	2,000 (12)	*bla*-oxa; *aadA1* (12)	*aadA*; *aphA2*; *sul1*; *tetB* (12)
	ASSuTNx (1)	2,000 (1)	*bla*-oxa; *aadA1* (1)	*aadA*; *aac3* IV; *sul1*; *sul3*; *tetB* (1)
	ASSuTNx-Cfx (1)	2,000 (1)	*dfrA16*; *aadA2* (1)	bla_{CTX-M9}; bla_{TEM-1}; *sul1*; *sul2*; *tetA*; *aadA* (1)
Heidelberg (1)	ASSuT-Cfx (1)	2,000(1)	*dfrA16; aadA2* (1)	bla_{CTX-M9}; bla_{TEM-1}; *sul1*; *sul2*; *tetA*; *aphA2* (1)
Bredeney (1)	ACSSuT-Cfx (1)	1,000 (1)	*aadA7* (1)	bla_{CMY-2}*; aadA; strA/strB; sul1; sul2; tetA* (1)
Rissen (1)	ACSSuT (1)	2,000 (1)	*dfrA1*; *aadA1* (1)	*aadA*; *aac3* IV; *sul1*; *sul3*; *tetA* (1)
Derby (15)	SSuT (14)	1,000 (14)	*aadA2* (14)	*aadA*; *sul1*; *tetA* (11); *aadA*; *sul1* (3)
	CSSuT (1)	1,000 (1)	*aadA2* (1)	*aadA*; *sul1; tetA* (1)
Monophasic (14)	ASSuT (11); ASSu (3)			
Bovismorbificans (9)	ASSuT (5); AST (2); ASuT (2)			
Typhimurium (6)	ASSuT (6)	Absence (31)	N.A.	N.A.
Wien (1)	ACSSu (1)			
Anatum (1)	STNx (1)			
9 serovars (83)	**15 AMR profiles (83)**	**3 size IC-1 (52)**	**7 gene-cassettes**	**8 AMR genetic profiles**

[a] n: number of strains, in all columns; [b] multi-AMR: antimicrobial resistance patterns by disk diffusion test with: A: ampicillin (ß-lactam); C: chloramphenicol (Phenicols); S: streptomycin (Aminoglycosides); Su: sulfisoxazole (Sulfonamides); T: tetracycline (Tetracyclines); Nx: Nalidixic acid (1st generation quinolones); and Cfx: cefotaxime (3rd generation cephalosporins), the latter was performed once *bla* genes were identified by PCR. [c] IC-1 amplified by PCR [23]. [d] IC-1 borne genes cassettes identified by sequencing the PCR product. [e] Other AMR genes identified by specific PCRs detailed in Table S2.

TABLE 2 Characterization of the three *Salmonella* strains carrying ESBL (strains IdAB-109 and IdAB-102) or AmpC (strain IdAB-42) genes.

Phenotype of the strains selected			MLST [b]	Strain accession number	Characterization of genetic mobile elements							
Strain code	Serovar	AMR/ MIC Cfx (µg/mL)/ IC-1 [a]	PubMLST/ cgMLST		IC/ SGI [c]	Plasmids						
						Name assigned	Accession number	Size (bp)	Mean GC content	Inc. group [d]	AMR genes [e]	Conjugative capability [f]
IdAB-109	Bredeney	ACSSuT-Cfx/ 32/ 1,000 bp	ST306/ cgST255665	CP101856	IC-1/ SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-13, C63PI, SGI-1	pSB109	CP101857	151,740	52.7%	IncC	*bla*$_{CMY-2}$; *tet*(A); *qac*E; *sul1*; *sul2*; *aph(3'')-Ib*; *aph(6)-Id*; *aad*A7; *floR*	No
IdAB-102	Heidelberg	ASSuT-Cfx/ 16/ 2,000 bp	ST15/ cgST194237	CP101854	IC-1/ SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-12, SPI-13, SPI-14, C63PI, CS54	pSH102	CP101855	286,802	46.6%	IncHI2; IncHI2A	*bla*$_{CTX-M9}$; *bla*$_{TEM-1B}$; *tet*(A); *dfr*A16; *aad*A2b; *qac*E; *sul1*; *sul2*; *mcr-9*; *aph(3')-Ia*; *aph(3'')-Ib*; *aph(6)-Id*	Yes
IdAB-45	Typhimurium	ASSuTNx-Cfx/ 32/ 2,000 bp	ST34/ cgST186136	CP102198	IC-1/ SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-12, SPI-13, SPI-14, C63PI, CS54, SGI-4	pSTM45	CP102199	306,822	46.8%	IncHI2; IncHI2A	*bla*$_{CTX-M9}$; *bla*$_{TEM-1B}$; *tet*(A); *dfr*A16; *aad*A2b; *qac*E; *sul1*; *mcr-9*	Yes

[a] See description in Table 1 footnote; [b] MLST: Multilocus sequence typing based on PubMLST (7 genes) or Enterobase (core genome MSLT - cgMSLT; 3,002 genes) schemes; [c] *in silico* analysis of all *Salmonella* class integrons (IC) and Genetic Islands (SGI) by IntegronFinder and *Salmonella* Pathogenicity Islands (SPI)Finder, respectively; [d] Incomp. group: incompatibility groups of multidrug-resistant plasmid by PCR and *in silico* by PlasmidFinder; [e] AMR genes annotated by ResFinder; [f] by conjugation or transformation with *E. coli* recipient.

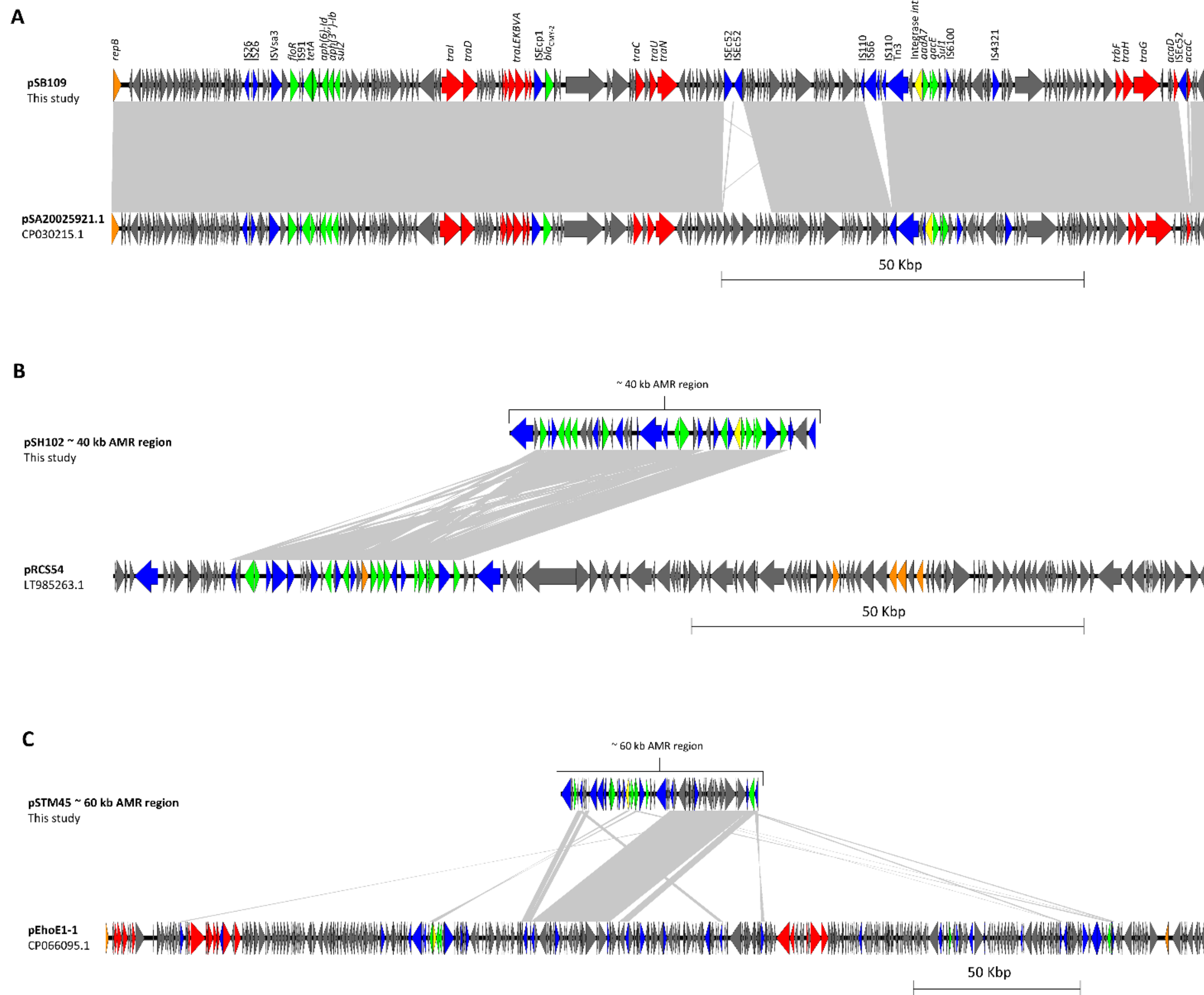

FIGURE 1. Linear alignment of **(A)** complete plasmids pSB109 (this work) vs. pSA20025921.1 from *S.* Reading (NCBI accession no. CP030215.1); **(B)** the ~ 40 kb AMR region of plasmids pSH102 vs. pRCS54 (accession no. LT985263.1); and **(C)** the ~ 60 kb AMR region of plasmids pSTM45 vs. pEhoE1-1 (accession no. CP066095.1). Coding sequences (CDSs) are colored according to their function: green, antimicrobial resistance genes; blue, transposases; yellow, IC-1; orange, plasmid replication; red, conjugative transfer; grey, other roles. Regions with > 99 % identity are shaded in grey. Cross lines represent the position of genes in each plasmid.

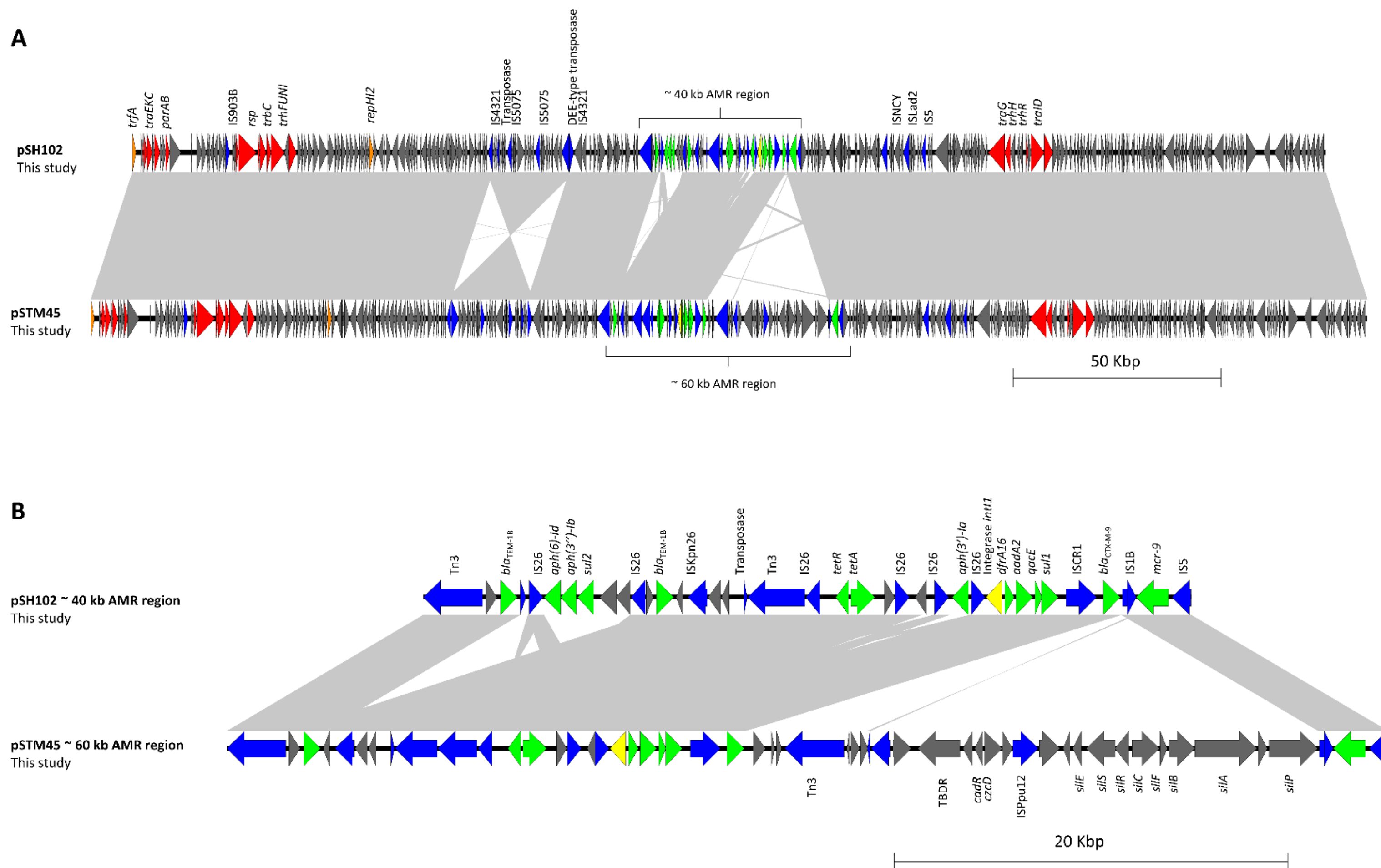

FIGURE 2. Linear alignment of **(A)** complete plasmids pSH102 vs. pSTM45 (both of this work); and **(B)** the AMR regions of plasmids pSH102 vs. pSTM45. Coding sequences (CDSs) are colored according to their function: green, antimicrobial resistance genes; blue, transposases; yellow, IC-1; orange, plasmid replication; red, conjugative transfer; grey, other roles. Regions with > 99 % identity are shaded in grey. Cross lines represent the position of genes in each plasmid.

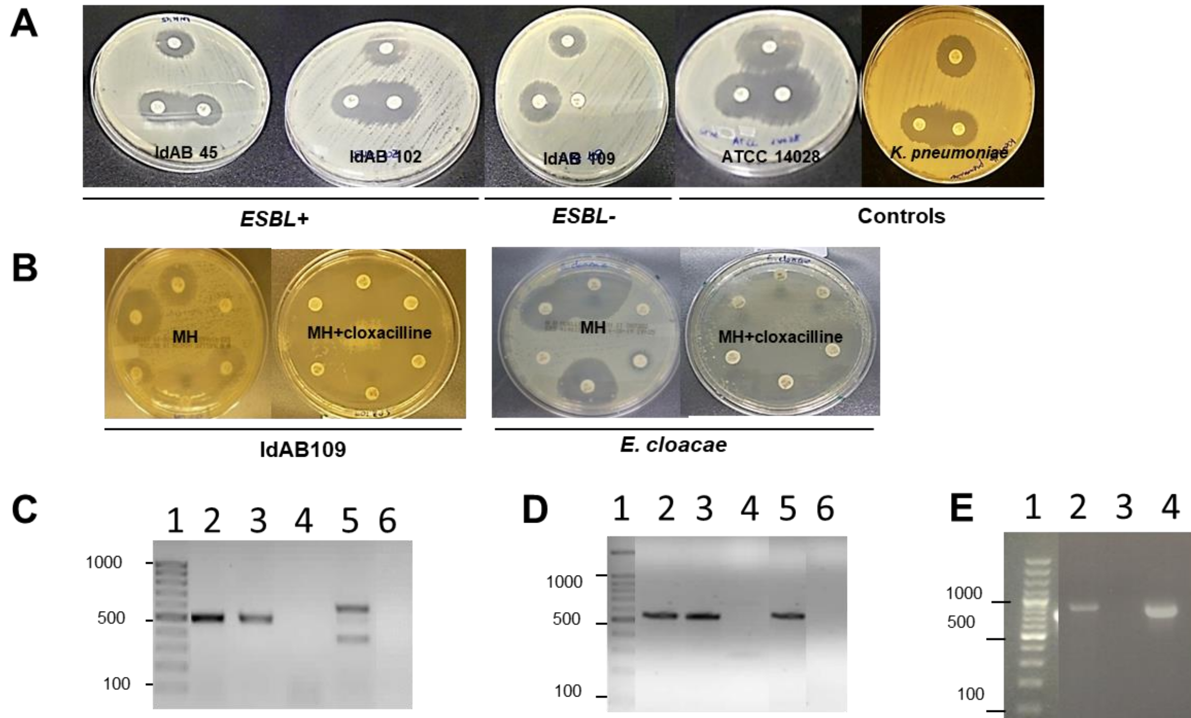

FIG S1. Representatives images of phenotypic and genotypic characterization of ESBL and AmpC production in *Salmonella* strains from swine origin. **(A)** Production of ESBL by a double-disk diffusion synergy test, using disks of cefotaxime (30 µg) and amoxicillin-clavulanic acid (30/10 µg) or cefotaxime (30 µg) alone as control; *Klebsiella pneumoniae* was used as control. **(B)** identification of AmpC plasmid mediated AMR in strain *S.* Bredeney IdAB109 by disk diffusion test in MH vs. MH-Cloxacillin; *Enterobacter cloacae* was used as control. **(C)** Multiplex PCR for detection of *bla*, *oxa* and *tem* β-lactamases by [57]. **(D)** PCR for detection of CTX-M universal primers by [53]. **(E)** PCR of CMY-2 by [59]. Lanes: 1 Molecular weight; 2 IdAB-109; 3 Control negative. Lanes: 1: Molecular Weight; 2: strain IdAB45; 3: strain IdAB102; 4: strain IdAB109; 5: Positive controls: *oxa* (619 bp) *tem* (516 bp) and *shv* (392 bp) in panel C; *E. coli* E1V1C63a (585 bp) in panel D; *E. cloacae* (758 bp) in panel E.

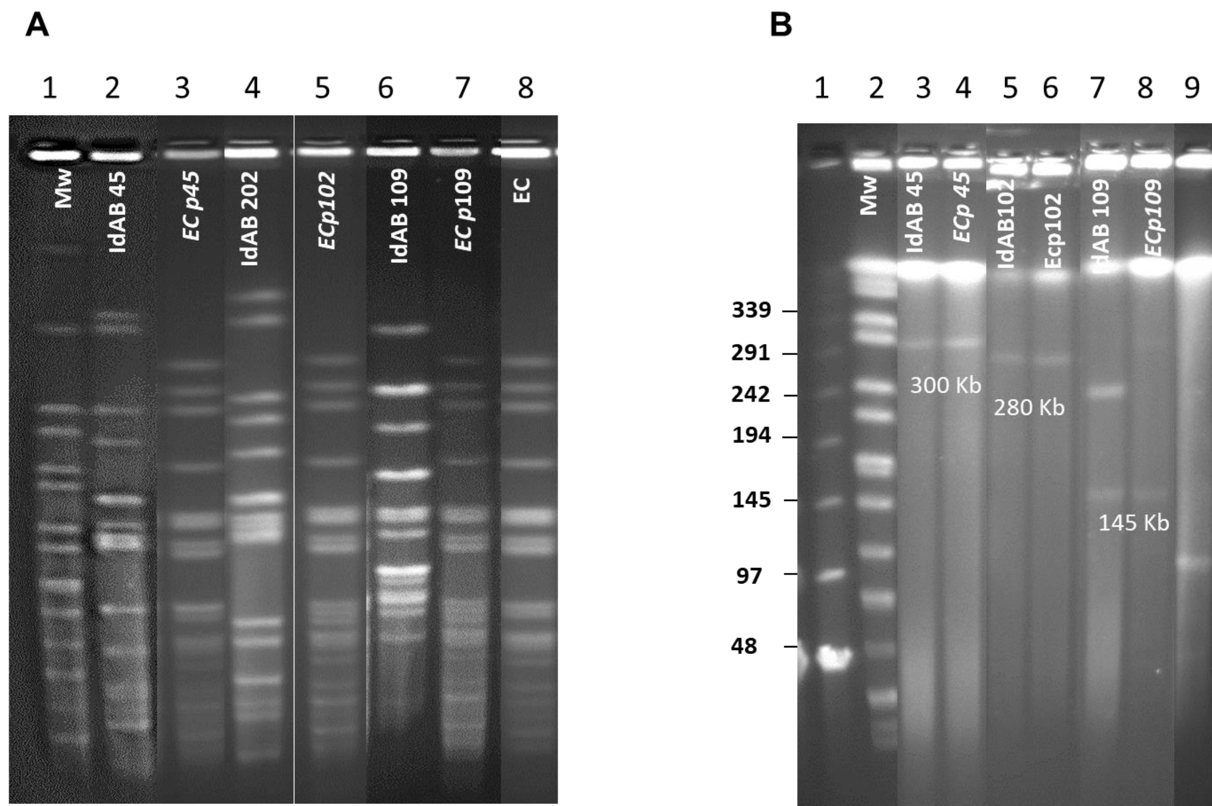

FIG S2. PFGE and S1-PFGE profiles of *Salmonella* donors, *E. coli* receptor and transconjugants carrying ESBL or AmpC genes. **(A)** *Xba*1-PFGE of *Salmonella* and *E. coli* strains. Lanes: MW: *S*. Braenderup strain H9812 used as molecular weight control; IdAB45: *S.* Typhimurium strain IdAB45; ECp45: *E. coli* transconjugant carrying plasmid of IdAB45; IdAB102: *S*. Heidelberg strain IdAB102; ECp102: *E. coli* transconjugant carrying the plasmid from IdAB102; IdAB109: *S*. Bredeney strain IdAB109; ECp109: *E. coli* electroporated carrying the plasmid from IdAB109; EC: E. *coli* K12 receptor of plasmids used as control. **(B)** Plasmid detected by nuclease S1-PFGE of the *Salmonella* donor strains and *E. coli* receptors. Lanes 1: ʎ ladder (Biorad); MW: *S*. Braenderup H9812 digested with *Xba*I; IdAB45: *S.* Typhimurium strain IdAB45 showing a plasmid of ≈300 Kb; ECp45: *E. coli* transconjugant carrying plasmid of IdAB45; IdAB102: *S*. Heidelberg strain IdAB102 showing a plasmid of ≈280 Kb; ECp102: *E. coli* transconjugant carrying the plasmid from IdAB102; IdAB109: *S*. Bredeney strain IdAB109 showing two plasmids; ECp109: *E. coli* electroporated carrying the plasmid from IdAB109 of ≈145 Kb; 9: *Salmonella* control strain E1V1C17a showing a ≈120 Kb plasmid .