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Complete Sequences of Multiple-Drug Resistant IncHI2 ST3 Plasmids in *Escherichia coli* of Porcine Origin in Australia

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IncHI2 ST3 plasmids are known carriers of multiple antimicrobial resistance genes. Complete plasmid sequences from multiple drug resistant *Escherichia coli* circulating in Australian swine is however limited. Here we sequenced two related IncHI2 ST3 plasmids, pSDE-SvHI2, and pSDC-F2_12BHI2, from phylogenetically unrelated multiple-drug resistant *Escherichia coli* strains SvETEC (CC23:O157:H19) and F2_12B (ST93:O7:H4) from geographically disparate pig production operations in New South Wales, Australia. Unicycler was used to co-assemble short read (Illumina) and long read (PacBio SMRT) nucleotide sequence data. The plasmids encoded three drug-resistance loci, two of which carried class 1 integrons. One integron, hosting *drfA12-orfF-aadA2,* was within a hybrid Tn*1721*/Tn*21*, with the second residing within a copper/silver resistance transposon, comprising part of an atypical *sul3*-associated structure. The third resistance locus was flanked by IS*15DI* and encoded neomycin resistance (*neoR*). An *oqx*-encoding transposon (quinolone resistance), similar in structure to Tn*6010*, was identified only in pSDC-F2_12BHI2. Both plasmids showed high sequence identity to plasmid pSTM6-275, recently described in *Salmonella enterica* serotype 1,4,[5],12:i:- that has risen to prominence and become endemic in Australia. IncHI2 ST3 plasmids circulating in commensal and pathogenic *E. coli* from Australian swine belong to a lineage of plasmids often in association with *sul3* and host multiple complex antibiotic and metal resistance structures, formed in part by IS*26*.

Keywords: *Escherichia coli*, antimicrobial resistance (AMR), plasmid, genomics, epidemiology

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INTRODUCTION

In Australia, restrictions on live animal imports, geographic isolation, and sound antibiotic stewardship have limited the incorporation and spread of genes encoding resistance to antibiotics used to treat serious human infections among *Enterobacteriaceae* circulating in food animals (Turner, 2011; Abraham et al., 2015; Reid et al., 2017; Kidsley et al., 2018). Australian porcine *Escherichia coli* are, albeit at low frequency, known to carry bla_{CMY-2}, $bla_{CTX-M-14}$, and $bla_{CTX-M-9}$, possibly because of the use of ceftiofur as an off-label, last line antibiotic to treat serious disease (Abraham et al., 2015). First generation antibiotics; commonly tetracyclines,

penicillins, and sulphonamides are otherwise used for the therapeutic treatment of bacterial infections in swine (Jordan et al., 2009). Both pathogenic and commensal *E. coli* sourced from the feces of pig carry class 1 integrons and are often multiple drug resistant (MDR) (Abraham et al., 2015; Wyrsch et al., 2015; Reid et al., 2017). Notably, the insertion element IS*26* has infiltrated the genomes of commensal *E. coli* of porcine origin, where it has played a role in altering the genetic context of clinical class 1 integrons and facilitated the acquisition of further resistances (Reid et al., 2017). Furthermore, it is important to identify and characterize the genetic vehicles that can carry class 1 integrons and genes encoding resistance to first generation antibiotics which are widespread in commensal *E. coli* populations in Australian pigs (Abraham et al., 2015; Reid et al., 2017; Kidsley et al., 2018) because they are likely to acquire genes encoding resistance to clinically relevant antibiotics when resident in commensal enterobacterial populations in the gut of humans and companion animals.

Plasmids of the incompatibility group HI2 (IncHI2) have been linked with the carriage of tellurium resistance, plus a broad range of antimicrobial resistance genes, including bla_{IMP}, bla_{NDM}, and bla_{VIM} (carbapenemase resistance) (Abraham et al., 2016; Dolejska et al., 2016; Falgenhauer et al., 2017), $bla_{\text{CTX-M}}$ (Garcia Fernandez et al., 2007), *mcr-1* (plasmid-mediated colistin resistance) (Gilrane et al., 2017; Li et al., 2017; Zheng et al., 2017), *oqxAB* and *aac(6')-Ib-cr* (plasmid-mediated quinolone resistance) (Fang et al., 2016), and resistance genes effective against first generation antibiotics and disinfectants (Cain et al., 2010; Cain and Hall, 2012; Abraham et al., 2016; Dolejska et al., 2016; Gilrane et al., 2017). Notably, clinically-important resistance genes have been associated with IncHI2 plasmids that also encode resistance to copper, zinc and, arsenic residues (Fang et al., 2016). In Australia, IncHI2 plasmids carrying a diverse range of antimicrobial resistance genes have been found in MDR *E. coli* (Dolejska et al., 2016; Saputra et al., 2017), *Salmonella enterica* serovar Typhimurium (Cain et al., 2010; Cain and Hall, 2012; Billman-Jacobe et al., 2018), *Enterobacter cloacae* (Sidjabat et al., 2015), and *Enterobacter hormaechei* susbp. *Oharae* (Monahan et al., 2019).

The insertion sequence IS*26* of the IS*6* family plays a significant role in the capture, assembly and mobilization of drug resistance genes found on plasmids (Dionisi et al., 2009; Cain et al., 2010; Shahid, 2010; Venturini et al., 2010, 2013; Lai et al., 2013; Abbo and Hooton, 2014; Chavda et al., 2015; Reid et al., 2015; Garcia et al., 2016) and in the chromosome (Roy Chowdhury et al., 2015, 2018). IS*26* may promote plasmid stability and persistence by mediating deletions of plasmid backbone sequence, the expression of which incurs a burden to the host (Porse et al., 2016). IS*26* can also facilitate the generation of hybrid virulence/resistance plasmids, formed by co-integration of plasmids separately carrying resistance and virulence gene cargo (Mangat et al., 2017; Wong et al., 2017). Although IS*26* has no target site specificity (Harmer et al., 2014), it is often observed localizing next to or within class 1 integrons in multiple drug resistant *E. coli* recovered from the feces of both healthy pigs, poultry and cattle with gastrointestinal disease (Dawes et al., 2010; Reid et al., 2017). IS*26* can shape the structure of class 1 integrons by facilitating the addition of foreign DNA flanked by IS*26*, and by generating inversion and deletions of sequence within complex resistance structures. Consequently, many class 1 integrons have lost genes that reside within the often observed 3′-conserved sequence (3′-CS), particularly the sulphonamide resistance gene *sul1* (Reid et al., 2017). However, sulphonamide resistance persists globally (Grape et al., 2003; Bean et al., 2005; Suhartono et al., 2017), despite restrictions on its use (Enne et al., 2001), with *sul2* (Bean et al., 2005) and *sul3* genes (Grape et al., 2003; Perreten and Boerlin, 2003; Zhou et al., 2014; Reid et al., 2017) found frequently in close association with class 1 integrons. A *sul4* gene associated with a chromosomal locus containing the folate synthesis gene *folK* and a copy of IS*CR20* has recently been described (Razavi et al., 2017). Emerging trends show that genes encoding resistance to last line drugs, such as *mcr-1,* $bla_{\text{CTX-M}}$, and carbapenemases are captured on plasmids already carrying genes encoding resistances to first generation antibiotics (Alonso et al., 2017; Botts et al., 2017; Delannoy et al., 2017; Poirel et al., 2017). Any one of a number of selection pressures including heavy metals and biocides may be sufficient to then facilitate the persistence and spread of multiple drug resistance plasmids (Argudín et al., 2019). This is further compounded by reports that agrichemicals can alter selection for drug resistant bacteria (Kurenbach et al., 2015, 2018). The potential for complex resistance structures to persist under multiple different selective pressures and across diverse environments underpin the importance of adapting a One Health approach to antimicrobial resistance gene surveillance in humans, food and companion animals, agriculture, effluent (municipal, hospital, and agricultural), and the environments impacted by effluent from diverse sources (Djordjevic et al., 2013; Wyrsch et al., 2016; Huang et al., 2017; Mir et al., 2018).

Here we used a combination of Illumina and PacBio SMRT sequencing to completely close two IncHI2:ST3 MDR plasmids from *E. coli* recovered from the feces of Australian swine. One of these, pSDE-SvHI2 is from a severe ETEC/ExPEC pathogen (*E. coli* O157 SvETEC) (Wyrsch et al., 2015), and the other, pSDC-F2_12BHI2, is from *Escherichia coli* F2_12B, a commensal *E. coli* ST93 (Reid et al., 2017). Phylogenetic and genomic comparisons were undertaken between these two plasmid sequences and pSTM6-275, recently isolated from a monophasic variant of *Salmonella enterica* in Australian pigs (Dyall-Smith et al., 2017), as well as all available IncHI2 ST3 plasmids on GenBank.

MATERIALS AND METHODS

Strains and DNA Preparation

Plasmid pSDE-SvHI2 was resolved from *Escherichia* coli O157 SvETEC (CC23:O157:H19), a severe pathogen that caused intractable disease within an Australian commercial piggery in 2007 (Wyrsch et al., 2015). Likewise, pSDC-F2_12BHI2 was resolved from *Escherichia coli* F2_12B, a commensal ST93:O7:H4 strain isolated from a rectal swab of a commercially raised pig, also sourced in 2007 (Reid et al., 2017). Full descriptions of these strains plus information on Illumina

short read sequencing and assembly have been published previously (Wyrsch et al., 2015; Reid et al., 2017). Un-sheared genomic DNA suitable for SMRT sequencing was prepared from mid-log phase sub-cultures of strains grown overnight in LB broth using a gentle phenol-chloroform extraction protocol. Genomic DNA samples were checked for appropriate DNA concentrations and integrity using a Qubit dsDNA HS (high sensitivity, 0.2 to 100 ng) Assay Kit on Qubit 2.0 fluorometer (Life Technologies) and for shearing by agarose gel electrophoresis.

Genome Sequencing and Assembly

Long read sequencing was performed by the Ramaciotti Center for Genomics using a Pacific Biosciences RSII sequencer with P6-C4 chemistry. One Single Molecule Real-Time (SMRT) Cell was used for each strain. Plasmid sequences were identified from whole-genome assemblies produced from hybrid read sets (both Illumina and SMRT reads) using the Unicycler pipeline v0.3.1 (Wick et al., 2017), which internally relied upon SPAdes v3.10.1 (Nurk et al., 2013), Bowtie2 v2.3.0 (Langmead and Salzberg, 2012), samtools v1.4.1 (Li et al., 2009), and Pilon v1.22 (Walker et al., 2014).

Annotated sequence for pSDE-SvHI2 and pSDC-F2_12BHI2 have been deposited in GenBank under accession numbers MH287084 and MH287085, respectively.

Plasmid Typing

Plasmids, particularly those that spread amongst the *Enterobacteriaceae*, have been typed by their *in vivo* incompatibility and cell exclusion patterns. Many of these incompatibility groups can now be subtyped by allelic variations in select conserved genes, forming plasmid multi-locus sequence typing schemes. Incompatibility typing and plasmid multi-locus sequence typing was performed through the Center of Genomic Epidemiology website (http://www.genomicepidemiology.org/) using PlasmidFinder (Carattoli et al., 2014) and the IncHI2 pDLST scheme (Garcia-Fernandez and Carattoli, 2010).

Phylogeny and Alignment Analyses

Analyses of conserved core single nucleotide polymorphisms (SNPs) was performed using the Harvest suite (Parsnp v1.2, utilizing the Phipack recombination filter, and gingr v1.2) (Treangen et al., 2014). An analysis was run utilizing all available IncHI2 ST3 plasmids from GenBank (**Supplementary Table 1**), plus two IncHI2 ST1 plasmids, reference pR478 (NC_005211) and pIMP4-SEM1 (KX810825). A second tree was then generated with the ST3 plasmids only, with pSDC_F2_12BHI2 as reference. Gene identification and genomic comparisons were performed using a combination of BLASTn (Camacho et al., 2009) and progressiveMauve (Darling et al., 2010) alignments. Figures were generated from sequence data using SnapGene v3.3.4, BRIG v0.95 (Alikhan et al., 2011), and Easyfig v2.2.2 (Sullivan et al., 2011).

Annotations were managed using SnapGene v3.3.4. Automated annotations were generated by RASTtk (Brettin et al., 2015). Insertion sequences were identified and annotated manually with the aid of ISfinder (Siguier et al., 2006). Remaining annotations were performed manually utilizing BLASTn and publicly available databases, including The Repository of Antibiotic-Resistance Cassettes (Tsafnat et al., 2011) (http://rac.aihi.mq.edu.au/rac/), The Comprehensive Antibiotic Resistance Database (Jia et al., 2017) (https://card.mcmaster.ca/), and the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore/).

RESULTS

Unicycler hybrid assemblies resolved pSDE-SvHI2 as a 275,402 bp circular sequence and pSDC-F2_12BHI2 as a 288,288 bp circular sequence with 344 (140 hypotheticals) and 357 (134 hypotheticals) coding sequences (CDS) respectively. Both sequences typed as incompatibility group HI2, sequence type 3 (IncHI2 ST3) plasmids. The full map of each plasmid is presented in **Figure 1**, along with content comparisons to the most related ST3 plasmids, most of which are from Australia and China. The plasmids had a typical IncHI2 structure, including the RepHIA and RepHI2 replication operons, *trh* and *tra* transfer operons and tellurite resistance (*ter* operon) (Gilmour et al., 2004), plus each carried three variants of complex resistance loci, two of which are class 1 integron associated, one encoding *sul1* in a Tn*1721*/Tn*21* hybrid transposon, and one encoding *sul3* from within a Tn*7*-like copper/silver resistance transposon.

Single nucleotide polymorphism analysis was performed on the set of all ST3 plasmids (**Figure 2**, **Supplementary Table 2**), and on this same set plus IncHI2 ST1 plasmids as reference to confirm tree topology (**Supplementary Figure 1**). Forty-three plasmid sequences available from 2006 to 2017 were included in the ST3-only analysis. Based on the availability of metadata, plasmid sequences were from *Enterobacteriaceae* of different sources in the pacific region, including Australian porcine production operations and multiple human, agricultural and environmental sources in China. The ST3 sequences formed a single clade with one exception, MH715960 from Taiwan, which separated with 125 core SNPs compared to the Australian reference sequence. Of these 125 core SNPs, 101 are within an ∼1.5 kb region of the *ter* operon. The remaining sequences formed four major subclades, with closest relatives to the Australian reference ranging from two to 14 SNPs. One subclade, highlighted red in **Figure 2**, demonstrates relatedness between three Australian plasmids from the feces of pigs, pSDE-SvHI2, pSDC-F2_12BHI2, and pSTM-275, an apparently separate Australian plasmid lineage (pIncHI2-MU3) also from pig feces, and six other plasmid sequences from diverse sources in China. Content comparisons of this clade can be seen in **Figure 1**. Of the remaining plasmids, the most distant relative had 25 SNPs identified from conserved core sequence, suggesting a close evolutionary relationship between plasmid sequences reported from Australia and China.

The ST3 plasmids carried multiple class 1 integron structures with different *sul* genes including *sul1, sul2, sul3,* and a *sul3*-associated *mefB*, encoding a macrolide efflux pump (**Figure 2**).

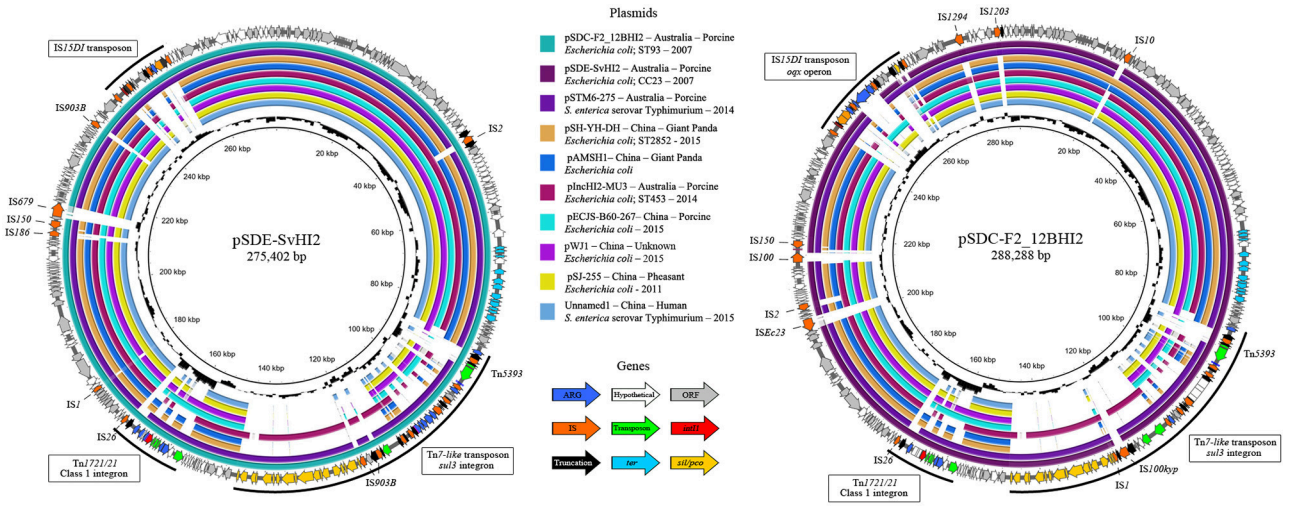

FIGURE 1 | Plasmid maps and alignments of pSDE-SvHI2 **(Left)** and pSDC-F2_12BHI2 **(Right)**. Maps display plasmid annotations, with genes color coded by function. Annotations highlight important complex resistance structures and insertions on each plasmid. Inner rings show BLASTn alignments against related IncHI2 ST3 plasmid sequences. Center histograms show GC content.

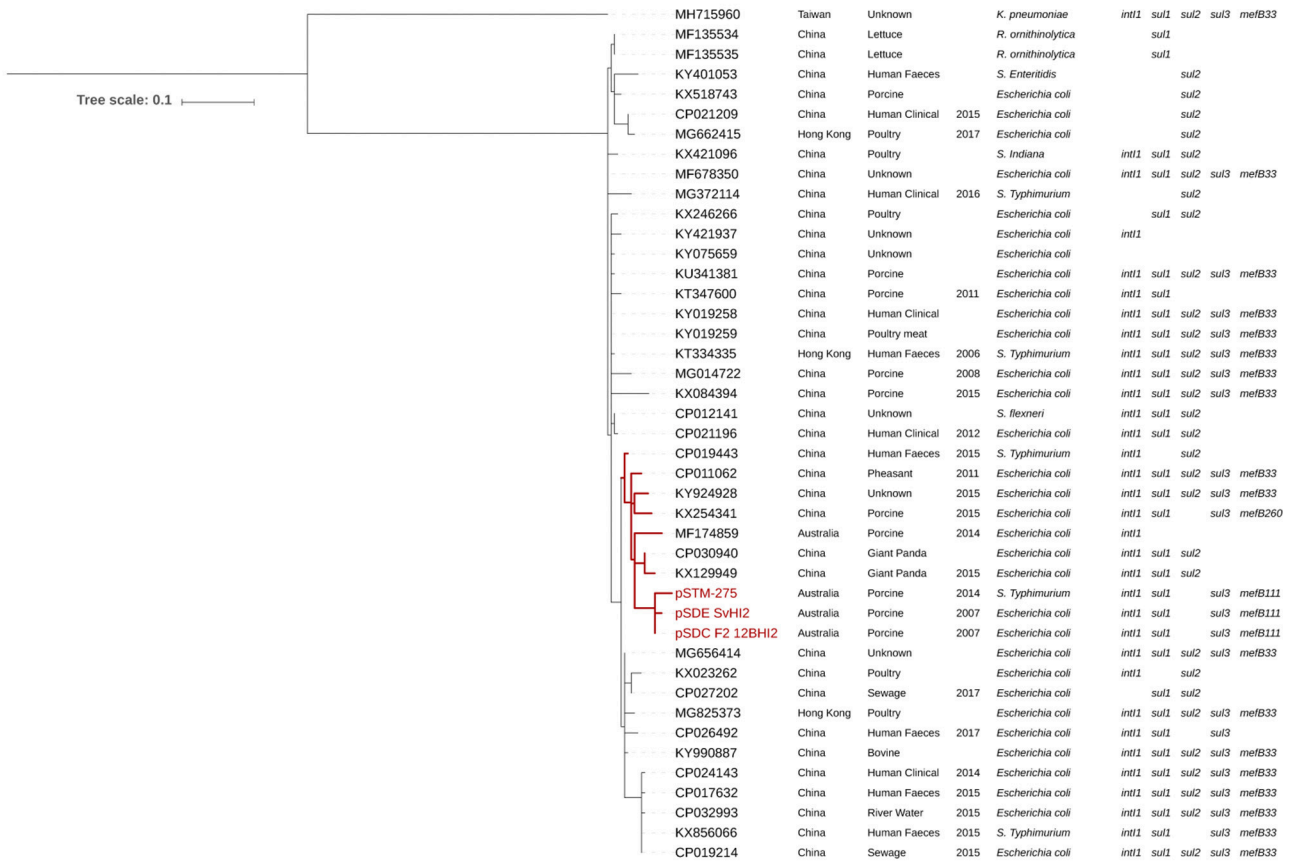

FIGURE 2 | Core phylogeny of IncHI2:ST3 plasmid sequences. Mid-point rooted phylogram constructed using Parsnp, using a conserved core SNP alignment of IncHI2 ST3 sequences, with pSDC-F2_12BHI2 as reference. Sequence metadata and the presence of integron marker genes is also included. Scale shows number of SNPs per site.

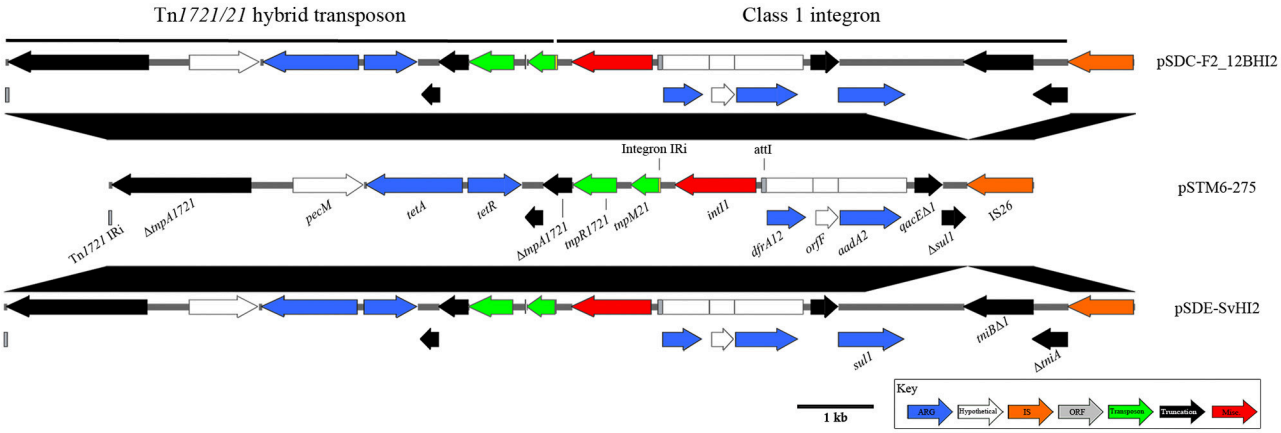

FIGURE 3 | Diagrams and comparisons of Tn*1721*/Tn*21* hybrid transposons carrying a class 1 integron. Clinical class 1 integron-based locus, with comparisons to the highly similar structure found in pSTM6-275 (center). Genes are colored by function, with black connecting regions indicating homology between loci.

One sequence (KX254341) of Chinese origin was observed with only 260 bp remaining of *mefB* ($\Delta mefB_{260}$). The Australian IncHI2 ST3 plasmids explored here carry $\Delta mefB_{111}$. The remaining *sul3*-positive plasmids carry a $\Delta mefB_{33}$ signature, aside from CP026492 from China which completely lacks *mefB*. This includes both the pseudo-phylogenetically distant MH715960 from Taiwan and the earliest Chinese plasmid sourced from 2006. Interestingly, one single subclade of sequences from China and Hong Kong was universally negative for *intI1* and *sul3*, but not for either *sul1* or *sul2*. Many of these plasmids are also associated with various globally important resistance genes, including bla_{CTX-M}, *oqxAB* and *mcr-1* (**Supplementary Table 3**).

pSDE-SvHI2, pSDC-F2_12BHI2, and pSTM-275 host a class 1 integron located within a Tn*1721*/Tn*21* hybrid tetracycline resistance transposon (**Figure 3**). This integron structure is host to the only complete *intI1* gene on the plasmids, and has acquired trimethoprim (*dfrA12*), *orfF* and streptomycin/spectinomycin (*aadA2*) resistance gene cassettes. Comparisons to similar Tn*1721*/Tn*21* hybrid transposons show identical sequence across the hybridization point between $tnpR_{1721}$and $tnpM_{21}$, however these sequences also show a modified transposition module, with a loss of Tn*1721 tnpA* sequence between two homologous 8 bp regions (CCAGGGCG), between the second $\Delta tnpA_{1721}$ and its neighboring predicted relaxase. The In2-like class 1 integron *tniA* gene is truncated to 436 bp by insertion of an IS*26* element with no observable associated repeats, suggesting a complex evolutionary path to this final structure. As part of the class 1 integron 3′-CS, the structure encodes sulphonamide resistance (*sul1*), however in pSTM6-275 the terminal IS*26* truncation lies within the *sul1* gene, giving a distinguishable gene marker.

A large (58,759 bp), mosaic combination of a globally observed Tn*7*-like transposon (copper and silver resistance, *sil*/*pco*), and transposons mobilizing antimicrobial resistance genes (**Figure 1**) was also observed in this Australian plasmid lineage. An IS*26*-associated, class 1 integron-encoding structure has inserted near the Tn*7-like* transposition module (**Figure 4**). From the pSTM6-275 sequence, it appears this insertion was in a Tn*3* associated gene, however in pSDE-SvHI2 and pSDC-F2_12BHI2 IS*26* activity has altered sequences around the insertion site. The integron is a variant of a *sul3*-associated structure that has been described previously in Australian plasmid pCERC3 (KR827684), now including signature IS*26*-meditated deletions in *intI1* ($\Delta intI1_{705}$) and *mefB* ($\Delta mefB_{111}$). The integron cassette array encodes *estX, psp, aadA2, cmlA,* and *aadA1*. The crossover site described near *qacH* reported in pCERC3 was identical, confirming it is a derivative of the same integron sequence encoding *sul3-qacH-mefB*, previously seen within a Tn*21* background (Moran et al., 2016). We also noted an IS*1203** insertion into the *qacH* ORF, which may prove epidemiologically useful. The integron structure is followed by a short intermediate sequence encoding *rop*, then by a Tn*5393* variant (encoding streptomycin resistance) with a novel IS*903B** insertion into IS*1133*.

From these sequences it is difficult to ascertain what originally mobilized the $\Delta intI1$ module into the Tn*7-like* transposon. While IS*26* flanks the captured sequence ($\Delta intI1$ to *sul3*), no repeats have been identified to indicate a clean insertion location near the Tn*7-like* transposon, nor the bordering sequence near *rop*.

Of the 1,030 bp sequence by *rop*, 257 bases nearest the Tn*5393* repeat are associated with the tetracycline resistance transposon Tn*1721* [AJ634602.1] while the remaining 773 bp nearest the IS*26*/*sul3* end matched IncN plasmid backbone [HF545433.1]. These remnant sequences may give indications as to the sources of these structures should more pertinent references become available. Further, the structures we have described have undergone individual insertions and inversion events. In pSDE-SvHI2, an IS*26* insertion into *tnsA* has led to the generation of new direct repeats (DRβ) and has subsequently led to an IS*26* mediated inversion. In pSDC-F2_12BHI2, an IS*26* insertion near the *tnsA* ORF has generated DRγ, and an inversion event has again occurred, flipping the structure

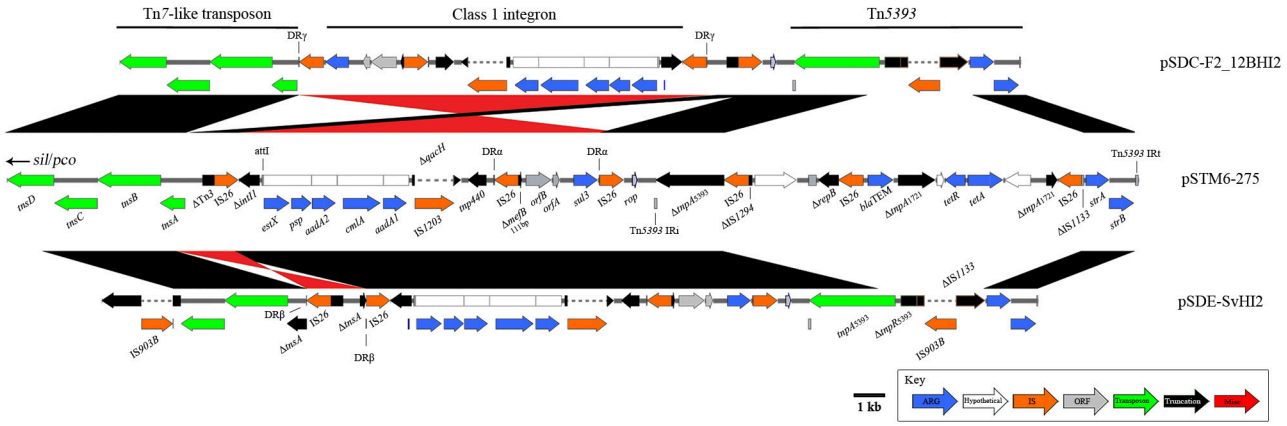

FIGURE 4 | Diagrams and comparisons of *sul3*-associated resistance structures carrying an IS*26* truncated *intI1* Structural comparisons between related *sul3* encoding regions, comprising a putative transposon, *sul3*-CS integron and Tn*5393*. Alignments in black show homology, with inversions indicated in red. Plasmids pSDE-SvHI2 (bottom) and pSDC-F2_12BHI2 (top) both show further rearrangement compared to the more recent pSTM6-275 structure.

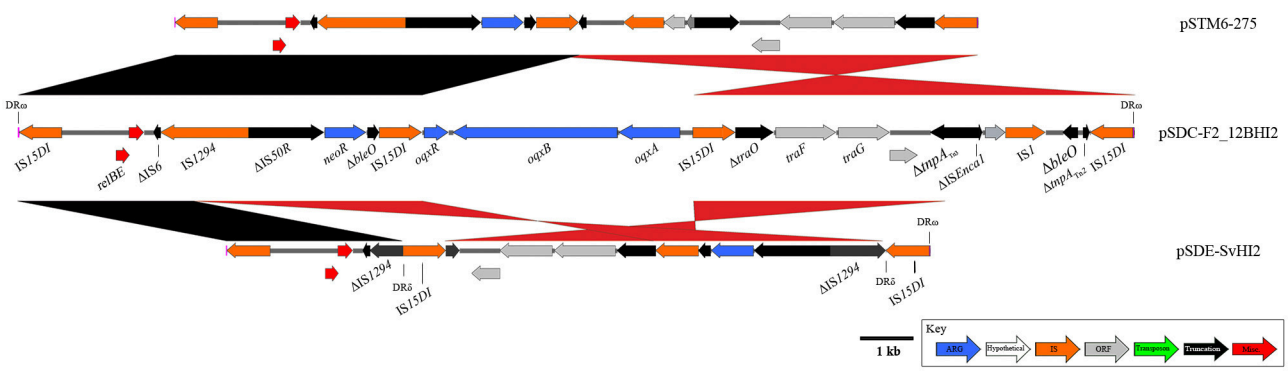

FIGURE 5 | Diagrams and comparisons of an IS*15DI*-mobilized composite transposon. Composite transposon captured by IS*26* variant *IS15DI*, mobilizing the neomycin resistance gene *neoR*. pSDC-F2_12BHI2 (center) also hosts a Tn*6010*-like element, inserted at the central IS*15DI*.

between Δ*intI1* and *sul3*. A separate inversion event has then occurred between the IS*26* elements nearest to *rop*, re-inverting this sequence to match the original orientation. Importantly, the insertion of a complex Tn*1721*/Tn*2*/IS*26* structure into Tn*5393* within pSTM6-275 demonstrates further resistance consolidation occurring in the 7-year gap between pSDE-SvHI2/pSDC-F2_12BHI2 and pSTM-275 isolations. This insert encoded *tetAB* and bla_{TEM}. Unfortunately, this insertion has removed most of IS*1133*, and we cannot determine if pSTM6-275 carried the ΔIS*1133*-IS*903B** insertion described above. The other Australian plasmid, pIncHI2-MU3, encodes the Tn*7-like sil/pco* heavy metal resistance transposon and is positive for Tn*3* and *intI1*, but lacks any *sul* gene.

Finally, an IS*15DI* (IS*6*-family element; 3 SNPs compared to IS*26*) mobilized resistance region with a highly recombined and varied structure (**Figure 5**) has also been identified in pSDE-SvHI2 and pSDC-F2_12BHI2. The original IS*15DI* insertion into the plasmid backbone has generated 8 bp direct repeats (AACAGCGT) that remain flanking the structure. This composite transposon appears to mobilize neomycin resistance (*neoR*), and a truncated bleomycin resistance (*ble*) gene. It has also acquired IncN backbone (*tra* genes) and the *relBE* toxin/anti-toxin system, alongside various other whole and truncated IS elements. An internal IS*15DI* element is present in both pSDE-SvHI2 and pSTM6-275 and is replaced by a Tn*6010*-like element (similar to KT716391.1, mobilized by IS*15DI*) carrying *oqxABR* in pSDC-F2_12BHI2. there is an IS*15DI* mediated inversion of the *tra* associated region in pSDC-F2_12BHI2. Plasmid pSDE-SvHI2 has an IS*15DI* insertion into IS*1294*, generating DRδ. This has been followed by a rearrangement event leading to the movement of DRδ and *neoR* toward the terminal IS element, including an inversion, and a loss of sequence through to the $\Delta tnpA_{Tn3}$.

DISCUSSION

Plasmids belonging to the incompatibility group HI2 are carriers of antimicrobial resistance genes globally. With the increased

availability of IncHI2 sequences, a di-locus sequence typing scheme was established (Garcia-Fernandez and Carattoli, 2010) to aid in tracking plasmid lineage development and dissemination. In Australia, most IncHI2 plasmids have been observed mobilizing bla_{IMP-4}, the predominant carbapenemase-encoding gene within *Enterobacteriaceae* on the eastern seaboard of Australia (Sidjabat et al., 2015). Here we report a comprehensive analysis of two Australian IncHI2 ST3 plasmids from *E. coli*, and make comparisons to plasmid pSTM6-275 from *S. enterica* serotype 1,4,[5],12:i:- (Dyall-Smith et al., 2017). Phylogenetic analyses of the current available IncHI2 ST3 dataset showed these three Australian porcine IncHI2 ST3 plasmids are nested within a subclade alongside another Australian porcine ST3 plasmid sequence, pIncHI2-MU3 (Abraham et al., 2018), which hosts neither of the class 1 integrons identified here, but does host the metal resistance genes as part of the Tn*7*-like transposon. This suggests various lineages of ST3 plasmid may be circulating within Australian porcine agricultural operations.

The IncHI2 ST3 plasmids sequenced to date have been resolved from multiple *Enterobacteriaceae* including *Escherichia coli*, various *Salmonella enterica* serovars, *Shigella flexneri*, *Klebsiella pneumonia,* and *Raoultella ornithinolytica.* Additionally, these *Enterobacteriaceae* isolates were taken from different sources, including pathogens and non-pathogens. As these ST3 plasmids carry diverse resistance gene cargo and may be found in numerous members of the *Enterobacteriaceae* family they will likely be important targets for future antimicrobial resistance surveillance (Fang et al., 2018). Our analyses also demonstrate evidence of differential gene acquisition by these plasmids, continuing to expand and alter the antimicrobial resistance gene repertoire which they are associated with internationally. Particularly, *sul3*-associated integrons carrying different Δ*mefB* fragments were identified here.

The *sul3* gene was first described in pigs (Perreten and Boerlin, 2003) and has since been reported widely in association with *Enterobacteriaceae* in humans, farm animals and their waste—particularly swine (Antunes et al., 2007; Phuong Hoa et al., 2008; Byrne-Bailey et al., 2009; Curiao et al., 2011; Moran et al., 2016). In Australia and elsewhere, *sul3* is identified frequently in commensal *E. coli* from the feces of swine (Guerra et al., 2004; Bischoff et al., 2005; Reid et al., 2017) but less frequently in avian pathogenic *E. coli* (APEC) from diverse poultry production systems in Australia (Cummins et al., 2019). These observations suggest that swine production, and food animal production more broadly, plays a major role in the evolution of IncHI2 ST3 plasmids that carry *sul3*. Plasmid pSTM6-275 was shown to be thermostable at 44°C, and would readily conjugate at 27°C but not at 37°C, highlighting the IncHI2 plasmid families propensity to transfer under environmental conditions (Garcia-Fernandez and Carattoli, 2010; Billman-Jacobe et al., 2018).

Genetic signatures noted in this study; a combination of the IncHI2 ST3 di-locus typing alleles and $mefB_{111}$ have been seen in commensal *E. coli* short read assemblies from both the farm were strain F2_12B was sourced, and a separate Australian porcine operation circa 2007 (Reid et al., 2017). Combined with the isolation of pSTM6-275 in 2014, our data suggests these plasmids are purveyors of drug resistance in porcine agricultural settings, likely aided by the use of copper feed additives and the widespread use of first generation antibiotics (Jordan et al., 2009). It is notable that the IncHI2 plasmids sequenced in our study carry a unique IS*6* family mobilized transposon encoding neomycin resistance, and additional fluoroquinolone resistance in pSDC-F2_12B. Also, they are host to a globally observed Tn*7*-like heavy metal resistance transposon (Fang et al., 2016), that has in turn become host to a variant of a *sul3*-*mefB* class 1 integron, which appears unique to Australian samples at this time. The $\Delta mefB_{111}$-associated class 1 integron was carried only by the Australian plasmids pSDE-SvHI2, pSDC-F2_12BHI2 and pSTM-275, while $\Delta mefB_{33}$ was observed throughout the remainder of the ST3 plasmid clade. Notably, $\Delta mefB_{33}$ variants are not associated with the presence of the Tn*7*-like metal resistance transposon, so characterizing their distribution and methods of mobilization will be critical to monitoring complex resistance locus diversification.

Genomic sequence data has shown that IS*26* is playing an important role in shaping the context of MDR islands in MDR fecal *E. coli* in Australian commercial pigs. Recently, we reported high carriage rates (101/103 isolates; 98%) of IS*26* among commensal *E. coli* carrying class 1 integrons (Reid et al., 2017), however we were unable to determine the genetic context of some class 1 integrons. Nonetheless, the frequency of carriage of *sul3* and IncHI2 plasmids in our earlier study was significant in two separate commercial swine production facilities. Here we show that IS*26* (and the IS*15DI* variant) has played a pivotal role in the evolution of several porcine IncHI2 ST3 plasmids both as a means of creating deletions and inversions and altering antibiotic resistance gene content.

Finally, the epidemiological analysis of *Enterobacteriaceae* has been heavily influenced by the spread of antimicrobial resistance and the development of MDR pathogens, leading to a focus on the detection of clinically-relevant resistance genes (bla_{IMP}, *mcr-1,* and bla_{CTX-M} are examples of this). To further understand the true variety and dissemination of multiple drug resistance, sampling from agricultural and environmental sources impacted by antimicrobials will be important.

DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, MH287084 and MH287085.

AUTHOR CONTRIBUTIONS

EW performed genomic analyses, generated figures, and drafted the manuscript. TC provided curated *E. coli* collections for the study. PRC assisted with data interpretation. ML, EW, and CR prepared sequencing samples. MD performed genome assemblies. SD conceived the study and together with EW and PRC wrote the manuscript. All authors provided edits, read, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2019.00018/full#supplementary-material

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