An Epidemiological Analysis of Antimicrobial Resistance in *Escherichia coli* of Porcine Agricultural Origin

A thesis submitted for the degree of

Doctor of Philosophy

by

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BSc. (Hons)

Certificate of Authorship

I, Ethan Robert Wyrsch, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

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Ethan Robert Wyrsch

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Conference Presentations

- The Genomic Characterisation of Two Australian Enterotoxigenic *Escherichia coli* O157 Isolates

  New Horizons: Research and Education for Optimal Health
  Oral presentation
  Kolling Building, Royal North Shore Hospital, November 2013

- Characterising Genomic Diversity in Pathogenic and Commensal *E. coli* from Australian Pigs

  BacPath 13: Molecular Analysis of Bacterial Pathogens
  Poster presentation
  Silverwater Resort, Phillip Island, September 2015

- Whole Genome Sequencing and Genomic Epidemiology of Australian, Porcine-Sourced *Escherichia coli*

  International Conference on Plasmid Biology
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  Clare College, Cambridge, September 2016
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- Mobilome of *Escherichia coli* O157 SvETEC: a multiple-drug resistant porcine pathogen

  Mobile Genetic Element: Transposable Elements, Integrative and Plasmids
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List of Abbreviations Used

AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
bp	Base pair
CRL	Complex Resistance Locus
CDS	Calibrated Dichotomous Susceptibility
e	Prefix - *in silico*, electronic
ESBL	Extended-spectrum beta-lactamase
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
HUS	Haemolytic Uremic Syndrome
IS	Insertion Sequence
LCBs	Locally Colinear Blocks
LGT	Lateral Gene Transfer
MDR	Multiple-Drug (Antibiotic) Resistance
MLST	Multi-locus Sequence Typing
PWD	Post-weaning diarrhoea
ST	Sequence Type
SNP	Single Nucleotide Polymorphism
WGS	Whole Genome Sequence

Abstract

The bacterial mobilome, a collection of plasmids, bacteriophages, transposons, and insertion sequences that capture, replicate, and share genes, is responsible for hosting and distributing antimicrobial resistance and virulence genes. Selective pressures generated by antimicrobial use promotes the acquisition of antimicrobial resistance mechanisms. Animal agriculture is one of the primary consumers of antimicrobials globally for the treatment of infection.

The impacts of antimicrobial pressures are apparent through the analysis of *Escherichia coli.* Porcine agriculture is especially pertinent, as enterotoxigenic *E. coli* represent a significant burden of disease in weaning piglets.

For this project, a collection of one hundred and sixty-three *E. coli* isolates were sourced from a commercial piggery in 2007, and a selection of forty-eight multiple-drug resistant strains from this underwent short-read whole genome sequencing. Additionally, four enterotoxigenic *E. coli* pathogens were also sequenced. Genomes were assembled, and data characterised to determine the nature of these strains, including comparisons to isolates from a second facility in NSW during the same period. Whole genome sequence analysis provides a universal toolset to mine information from genomic data, including identifying genes and assessing diversity.

Phylogenetics revealed antimicrobial resistance hosted in representatives across the known subclades of *E. coli*. Sequence type 10 was most prevalent, and numerous subclades belonging to this clonal complex were identified. Plasmid replicons were identified amongst the strains, with IncF, IncI, IncY, IncHI2 and IncX most prominent.

Genes encoding antimicrobial resistance matched known antibiotic use, including tetracycline, ampicillin, aminoglycosides, and sulphonamides. Resistance genes were frequently associated with class 1 integrons and the sulphonamide resistance genes *sul1, sul2* and *sul3*. Numerous strains appeared to host IS*26* transposon Tn*6026* (*sul2* associated) at a known insertion site. Identification of *sul3* was novel in Australian porcine data, associated with two novel IS*26* mediated *mefB* truncations.

Two pathogens sequenced were serotype O157:H19 - related strains evolutionarily distinct from known O157 pathogens. Belonging to clonal complex 23, these strains hosted various resistance and porcine virulence factors, particularly O157 SvETEC which encoded the three

sul genes and numerous large extrachromosomal elements. The remaining pathogens were of known ETEC serotype and had homologues among strains identified in the commensal populations.

Long-read sequencing revealed that *mefB*$_{111bp}$ was hosted on IncHI2:ST3 plasmids with evolutionary origins in Asia. These plasmids were identified in *Salmonella* Typhimurium of Australian porcine origin in 2013, and comparative analyses revealed evolutionary events that shaped the plasmids and complex resistance structures.

Chapter 1: General Introduction

1.1 An anti-microbial world

The ability to stop infection from claiming a life has been one of the major achievements of the modern era, and it is a facet of medicine that we are desperate to keep. Due in large part to a broad range of antimicrobials discovered and developed in the last century (Figure 1.1), our ability to curtail the spread of disease and target infectious microorganisms with precision treatments has increased dramatically. Unfortunately, burgeoning populations and increased international traffic have altered the ways in which we interact with and influence the global microbiome, causing an apparent shift in bacterial behaviour driven by the harsh selective pressures generated using antimicrobials (1). These pressures have placed a high value on the development and retention of bacterial genes encoding antimicrobial resistance (AMR) mechanisms, and there are serious concerns over the effects this is having on bacterial evolution as strains are forced to acquire and maintain these genes at an ever-increasing rate (2). These pressures are so prevalent now that detecting multiple resistance genes in any single strain is commonplace in certain environments.

The influences of these antimicrobials are proportional to scale and duration, and issues arise when these compounds are heavily employed to stop disease in large human or animal populations (3). Globally, usage depends on a variety of health factors and conditions, particularly population density and sanitation levels, which lead to more antimicrobial use if not managed correctly. Developing countries are often the most affected for this reason. In agriculture, too, large factory-style operations are of serious concern due to close-quarters holding conditions for the animals, combined with regular antimicrobial use.

Fortunately, an early intervention strategy termed antimicrobial stewardship has helped avert a more serious resistance crisis in many countries (4, 5). Specific antibiotics are restricted to human use under this scheme and this has stopped, or at least slowed, the development of resistance against drugs of critical human importance in livestock. Unfortunately, this does not preclude the development of resistance to the antimicrobials used in livestock, nor does it eliminate the development of resistance within humans in hospital environments or through community antibiotic use. Nonetheless, antimicrobial stewardship has aided in reducing the effects leading to antimicrobial resistance prevalence. It has also in some ways split the field of antimicrobial resistance epidemiology into human-critical and not, to the fields detriment, as a one health approach is proving critical in tracking the development of multiple-drug resistance across environments (6).

Selective pressures put on bacterial survival go beyond the known antimicrobial usage during treatment. Ingested antimicrobials can pass through a patient's digestive tract unmetabolized, with the compounds then excreted and discharged to the environment. The release of these residues into the environment expands the potential sources of resistance development. Hospital sewage contamination is of serious concern for the environmental development and persistence of antimicrobial resistance, with severe implications for human health (7, 8). In agriculture, too, animal manure is often used as fertilizer, leading to direct contamination of pastures with resistances developed in livestock (9-12). The worst-case scenario is cross-contamination of both antimicrobial residues and bacteria hosting antimicrobial resistance, especially multiple-drug resistance, leading to the formation of pan-resistant zoonotic pathogens.

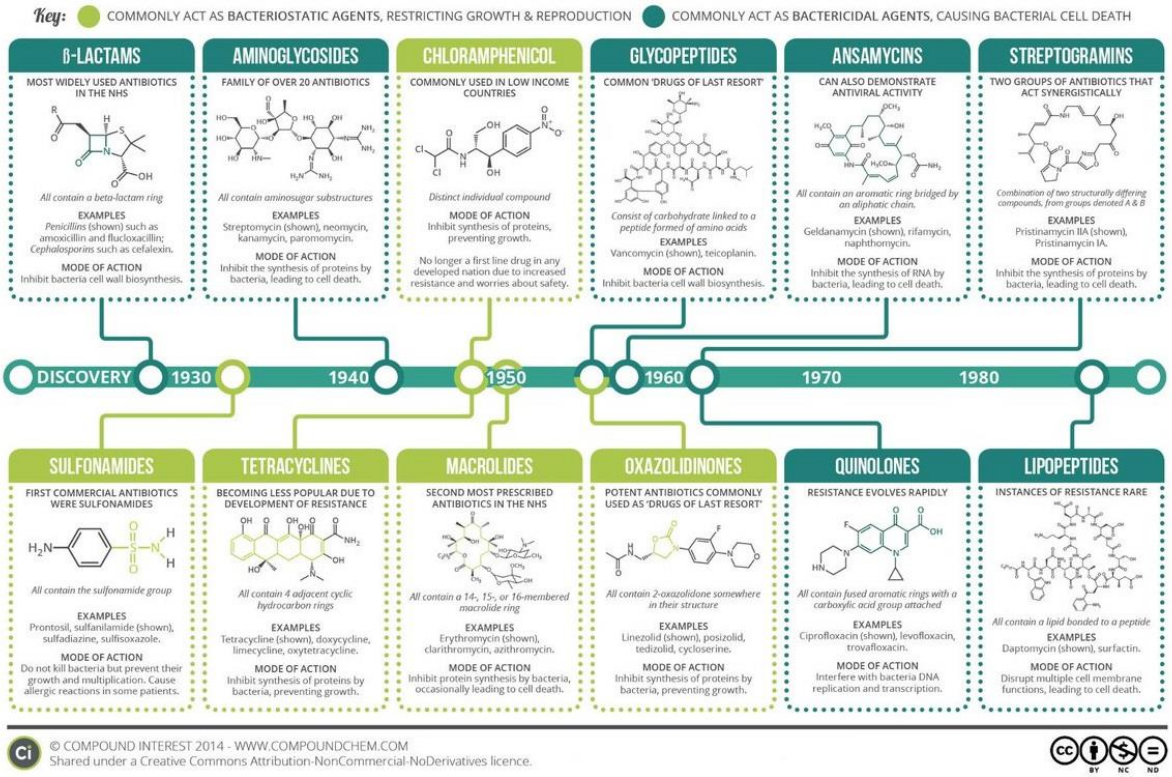

Figure 1.1: Timeline of antibiotic development, with details of the various classes of drugs, including mode of action. As a guide, most compounds developed after chloramphenicol and tetracycline are now reserved for human use under antimicrobial stewardship, where it is applied. Figure from http://www.compoundchem.com.

1.1.1 Flexible genomes and the issue of shared genetic material

1.1.1.1 All for one

Bacterial genomes, especially Gram-negative pathogens like the *Enterobacteriaceae*, can be highly variable, with many able to obtain genes beneficial to their survival from the local

environment. It is this process that allows similar bacterial strains to thrive in different locales and conditions and is in many ways at the heart of rapid bacterial evolution. At origination, these bacterial cells will receive a full copy of the genome from the parent cell, the process of vertical gene transfer. This generally includes both the bacterial chromosome, and any genetic material acquired by the parent cell. Any DNA then acquired outside this process is said to be acquired through lateral gene transfer (LGT). LGT is used by many bacteria to acquire niche adaptations upon entering a new environment, allowing them a greater genetic flexibility without the associated metabolic burden of maintaining the extra genes long term. The genes shared often relate to metabolism, virulence or protection from environmental antimicrobials like heavy metals. Over time, genes particularly useful in any given environment persist (13). With the introduction of so many antimicrobials to so many different environments, including the mammalian gut, avian agriculture, aquaculture and even horticulture, gene acquisition can now be a major influencing factor in bacterial survivability (14), and the need for so many resistance genes to be maintained at once has led to the development of gene loci encoding multiple-drug resistance within a single easily transferrable scaffold.

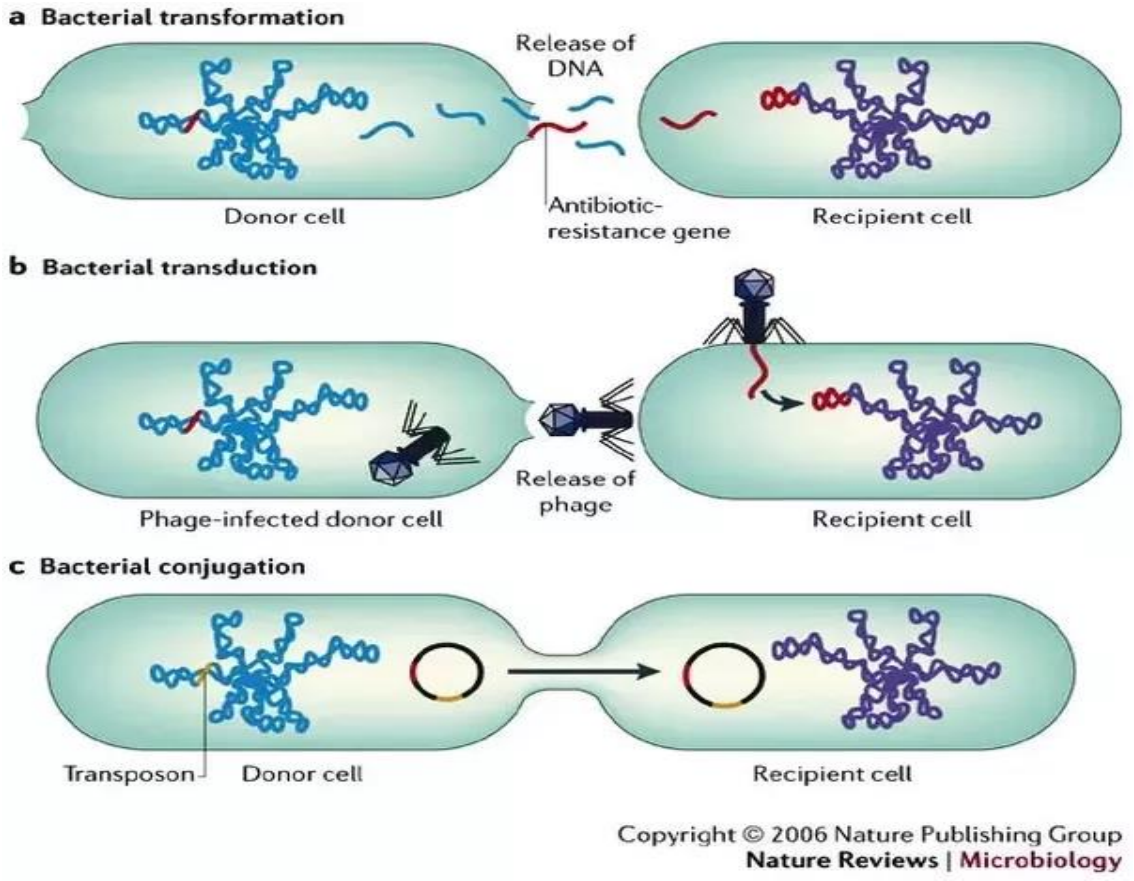

Figure 1.2: Methods of lateral gene transfer. Image from Furuya and Lowy, 2006 (15).

There are three primary mechanisms that facilitate LGT: conjugation, transformation and transduction (16) (Figure 1.2). While each method achieves the same goal, they are facilitated by different systems. Once inside a cell, a DNA molecule may then either act as a functional extrachromosomal molecule, or it may be integrated into the bacterial chromosome, becoming a permanent addition to the strain. Firstly, conjugation involves a circular DNA molecule known as a plasmid, which is transferred through cell-to-cell contact. This is facilitated by an extracellular pilus that bridges from the donor to the recipient cell. Conjugation often involves the replication of the plasmid pre-transfer, allowing the donor to retain a copy of the plasmid. The transfer pilus mechanism, amongst others assisting in plasmid survival, is encoded on the plasmid, allowing for continued self-propagation to any viable hosts in a bacterial community. The second process, transduction, is mediated by bacteriophages infecting cells with genetic material. Transformation is the uptake of free DNA (including plasmids) from the environment, from lysed neighbouring cells for example. This DNA may need to be integrated into the genome via some form of recombination to be functional.

A broad range of bacteriophages and plasmids are responsible for the mobilisation and reintegration of shared genes between many classes of prokaryotes, and with modern influences taking hold, they are now host to a multitude of drug resistance genes alongside their original cargo, such as host-specific virulence factors. Certain biological constraints do control the host ranges of many mobile elements. Plasmids are often typed by their incompatibility group, a characteristic of the plasmids, replication system that prevents other plasmids of the same group from also being hosted by the cell. This prevents interference from similar plasmids during replication and conjugation. Phages, too, acquired bacterial host immunity, limiting the range of new phages that can target host cells.

These larger mobile elements are not responsible for the initial acquisition of the resistance genes from their environmental sources. Nor do they lead directly to the rapid construction of genetic structures conferring multiple antimicrobial resistance. These roles are performed by smaller mobile elements, shown in Figure 1.3, that act in concert with the larger constituents of the bacterial mobilome that serve as both a platform for evolution and a route of dissemination.

Mobile elements share two important traits that have made them successful. Firstly, like the larger mobile elements, they encode the proteins that allow for the movement of the element. This may be a single open reading frame (ORF), or an operon that facilitates the transfer. Further, some mobile elements catalyse the full transfer of a single copy of the element, excising it from its current location in a larger DNA molecule, or they may replicate as part of the process, allowing conservative dissemination into a population. The second trait, which is common to most small mobile elements, is the use of inverted repeats, genetic sequences that determine the boundaries of the mobile element and act as recognition sites for transposition. These repeat systems, particularly, have allowed mobile elements to become facilitators of gene capture and transfer.

One of the most common sets of mobile genetic elements observed mobilising antimicrobial resistance are transposons. In transposons, mobilisation is accomplished through a small operon, often including a transposase, a resolvase, and a modulator protein, with delineating repeat sequences. There are a variety of transposon families, and certain of these have become commonly associated with specific antimicrobial resistances. Recombination and insertion events have led to the acquisition of gene cargo within the delineating repeats, which is then disseminated alongside the transposon. By providing bacterial hosts with

beneficial traits, certain transposons gain a selective advantage. This has led to the emergence of globally successful transposons disseminating heavy metal resistance, and more recently antibiotic resistance, amongst prokaryotes. Insertion sequences (IS) are like transposons, though are essentially only a single transposase gene flanked by inverted repeats. An interesting secondary characteristic of many IS elements is the formation of direct sequence repeats upon insertion of a new DNA molecule, leaving insertion signatures useful in epidemiological analysis. IS elements contribute to the mobilisation of genes via a composite transposition system, forming transposable units between compatible IS elements, where the initial inverted repeat of one element and the terminal repeat of the second element is recognised by the transposase, mobilising the intervening sequence (17), The formation of a composite transposon occurs only when the two IS elements are in the same orientation (Figure 1.3). If the IS elements are in opposite orientations, inversions of the intervening sequence may occur instead.

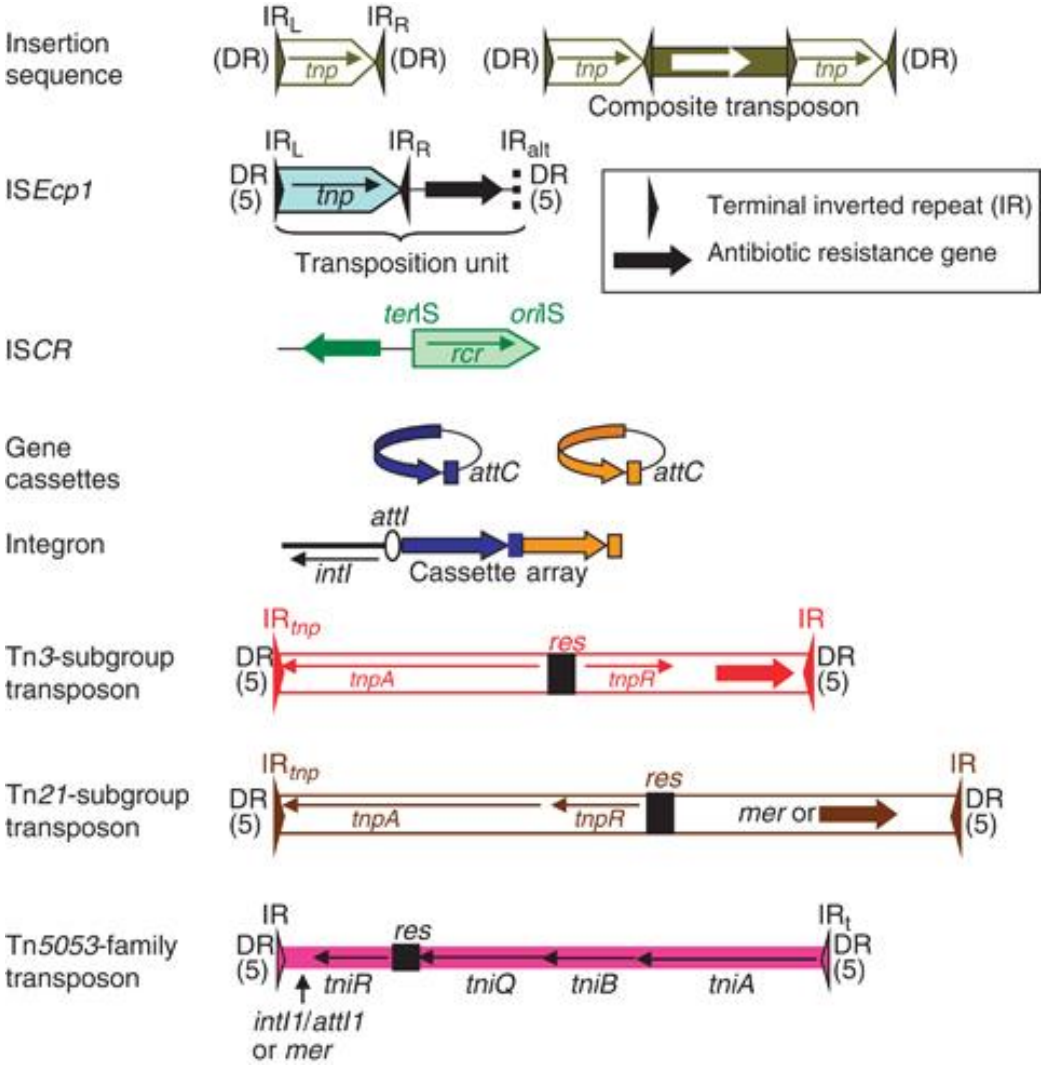

Figure 1.3: Mobile genetic elements associated with antibiotic resistance. The genes *tnp* and *tni* are transposition genes, IRs are inverted repeats, DRs are direct repeats and arrows indicate gene direction. *intI* is an integrase gene, *attI* is the insertion point for gene cassettes in an integron, and *attC* is the recombination point of the gene cassette (17). Image from Partridge *et al.* (2011).

Utilising these characteristics, mobile elements play an active role in the capture and distribution of genes. While captured randomly through the normal activity of these elements, selective pressures maintain those that mobilise useful genes, propagating alongside successful hosts. This is the mechanism from which the antimicrobial resistance crisis has truly arisen.

1.1.1.2 One for all

A prokaryotic strain can really acquire a resistance mechanism in one of two ways. The first is to generate the resistance internally. That is, successful random mutation in a population can develop proteins less susceptible to the antimicrobial targeting them, or that target the

antimicrobials. The second is to acquire such mechanisms on laterally transferred elements. As discussed, these resistance genes are captured from their chromosomal origins by small mobile elements, which then tend to find their way to plasmids and phages.

Combined, these mobile genetic elements provide the tools for the construction of complex antimicrobial resistance loci (CRLs). Under antibiotic pressure, any transposon hosting relevant AMR genes is sustained within bacterial populations due to the selective advantage they provide. This allows time for further mobilisation and integration into these structures, leading to the clustering of mobile and antimicrobial resistance genes. It has also been observed that some IS elements are more likely to insert near other elements, increasing the chance of gene clustering. This assembly of a CRL, which often eventually encodes multiple antimicrobial resistances, leads to the existence of structures encoding multiple resistances, that can be maintained under the pressure of a single antimicrobial treatment. Over time, consolidation leads to MDR that can transfer in a single event, be maintained by multiple different pressures, and that serves as a platform for further mobile element activity.

Many genes contributing antimicrobial resistance have provided such selective advantages they are now disseminated throughout most environments and on a multitude of different host structures around the world. One of the major systems that has become distributed in this manner is the integron. Integrons are gene capture and expression systems that have the capacity to incorporate gene cassettes (Figure 1.3), small DNA elements encoding usually a single ORF (18). In MDR isolates, these cassettes generally encode antibiotic resistance genes (19, 20), particularly in clinically derived bacterial isolates. The true utility of these integron systems is their ability to allow rapid adaptation at the single gene level, giving a variable resistance profile depending on the specific integron structure present. While there are numerous types of integrons used in the prokaryotic world, two have emerged as primary contributors to drug resistance.

Integrons are a broad family of chromosomal mechanisms, however those facilitating drug resistance are separated into classes 1, 2 and 3 based on differences in the integrase gene *intI*. These integrons otherwise share the same structure: an integrase gene encoding a tyrosine recombinase, an *intI* promoter, and a promoter for the gene cassettes, and an *attI* cassette insertion site. The gene cassettes captured have a corresponding *attC* site that allows the integration process to take place.

These systems have been captured and dispersed by different transposons, though by far it is the class 1 integrase that is dominant due to its capture by a series of mobile elements. This successful structure is known as the clinical class 1 integron, due to its presence in clinical MDR infections. The clinical class 1 integron was captured first by a Tn*402* transposon. Tn*402* is part of a family of *res* hunter transposons and mobilised the integron into a series of other backbones, particularly the mercury resistance transposon Tn*21*. Tn*21* is a prevalent example of a mobile genetic structure carrying a heavy metal resistance operon, in this case to mercuric ions. Figure 1.4 shows the combination of mobile genes that form the transposon, including the mercury resistance operon (*mer*), transposition genes (*tnp*), a *res* site, and inverted repeats (21). Heavy metals represent both an environmental and anthropogenic selective pressure on the maintenance of these structures in a population.

The clinical class 1 integron, shown in the upper part of Figure 1.4, is divided into sections as follows: a 5′-conserved sequence, encoding the integrase gene and its associated integrase and cassette promoters; a variable region which may contain a gene cassette, or cassette array, inserted at the *attI* site; a 3′-conserved sequence encoding a truncated quaternary ammonium compound resistance *qacE* gene and the sulphonamide resistance gene *sul1* (once former gene cassettes, now permanent additions to the structure); and a remnant of the Tn*402* transposition module, encoding a truncated *tniB* and a full *tniA* gene. This is bordered by initial (IRi) and terminal (IRt) Tn*402* inverted repeats (22). In Figure 1.4, this integron subtype also hosts IS*1326* and IS*1353.*

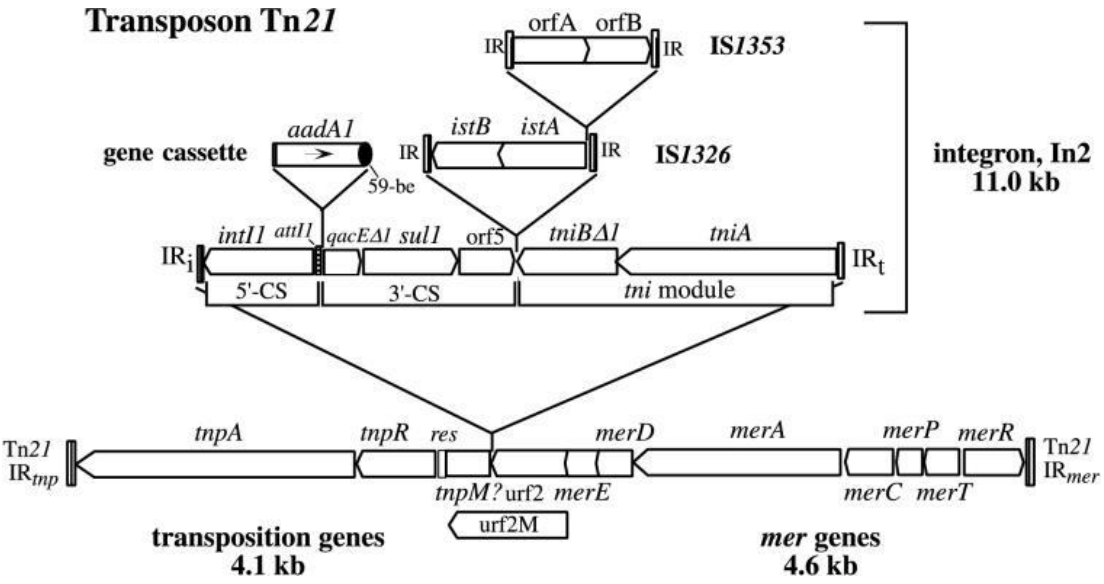

Figure 1.4: Transposon Tn*21* with its components. Includes a transposition module (*tnp*), mercury resistance (*mer*), inverted repeats (IR; initial and terminal), the Tn*402*-mobilised integron, comprised of the 5′-CS encoding the integrase *intI1* and the *attI* gene cassette insertion site, the 3′-CS encoding sulphonamide resistance (*sul1*) (21), and IS*1353* and IS*1326* insertion sequences. Converging lines show the insertion points and arrow direction indicate gene orientation. Image from Liebert *et al* (1999).

There are a multitude of different families of transposons described, as well as several clinically important integron variants, hosting a myriad of gene cassettes, all of which can be assembled randomly together via multiple gene acquisitions and lateral gene transfer (23). What then shapes these processes and influences the development of multiple-antimicrobial resistance is the selective pressures applied.

1.1.2 Does agriculture pose a threat to public health?

1.1.2.1 Healthy animals feed healthy people

In the world of livestock, antimicrobials are used primarily to treat individual infections (24, 25). If an outbreak occurs, healthy animals can be treated prophylactically to prevent disease from developing. Food additives like copper salts are provided to animals to promote healthy growth, and at-risk animals can be further supplemented with antibiotics and extra heavy metal supplements such as zinc oxide. This is a primary source of antimicrobial selective pressures on these animals. There is a strategy banned in many countries, including Australia, known as antibiotic growth promotion, a phenomenon whereby animals treated with low doses of antimicrobials are healthier and grow larger, faster (26). The growth promoting effects of antimicrobials are still poorly understood, however there are several explanations

that have been developed (26), including a reduction in bacterial load and modifications to the gut microbiome. Certainly not all facilities engage in using low-dose antimicrobials in this fashion, however the uninhibited use of antimicrobials in general has led to widespread antimicrobial resistance in agricultural animals (5). As mentioned earlier, antibiotic stewardship in many countries has limited the development of resistance to drugs used for critical human healthcare, however it is far from a perfect system and the development of MDR, and the activity of mobile elements that it tends to generate, presents a threat to the health of all.

The choice to make use of an antibiotic in the treatment of infection is determined by several criteria, relating to the target and the host (5, 27, 28). Certain antibiotics are more effective against either Gram-positive or Gram-negative bacteria; some species have intrinsic resistances or are now notorious for harbouring certain antimicrobial resistance genes. In Australian porcine agriculture, a survey in 2006 showed *Escherichia coli, Lawsonia* spp. and *Mycoplasma hyopneumoniae* infections attracted the most antimicrobial usage for treatment (29). The use of non-restricted antimicrobials, penicillins, tetracyclines, sulphonamides, olaquindox, macrolides, neomycin, lincomycin and spectinomycin were reported, as well as some use of human-relevant drugs ceftiofur and dimetridazole. Similar usage profiles have been reported in the major pig producers of the world, particularly China and the United States of America (30). The influence this has had on the microbiomes of the livestock is clear, and very apparent in the Gram-negative bacterium *Escherichia coli*.

1.1.2.2 *Escherichia coli*: The Enterobacteriaceae we know and have

Escherichia coli are Gram-negative, motile bacteria of the *Enterobacteriaceae* family. They are facultative anaerobes, ubiquitous in nature and are a prominent member of most mammalian microflora, employing a commensal lifestyle within the gastrointestinal tract (GIT). *E. coli* may also be pathogenic, capable of causing disease through opportunistic infections in wounds and the urinary tract as extraintestinal pathogenic *E. coli* (ExPEC), or through direct infection from within the GIT (31). The various pathogenic strategies utilized by *E. coli* are encoded by key virulence genes generally hosted on mobile scaffolds. Modern *E. coli* pathogens are grouped based on modes of pathogenesis, with specific virulence factors causative in the pathogenesis (32). These classes are enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and even avian pathogens (APEC) (33).

The extent of diversity observed within *E. coli* necessitates being able to characterise isolates to track the evolution of pathogenicity and antimicrobial resistance, and particularly identify commensal strains with the capacity to become MDR pathogens. Classically, *E. coli* strains are grouped into serotypes by the identification of specific surface antigens such as the lipopolysaccharide (O-antigen) (34), flagellin (H-antigen) (35) and fimbrial adhesins (K-antigen) (36). Common and epidemic pathogenic serotypes that are known to cause disease in humans include *E. coli* O157:H7 , O111:H11 and O104:H4 (37) (38). More recently, DNA sequencing has provided new data and tools for the tracking of bacterial evolution, allowing direct sequence comparisons and the development of more reliable datasets. Initially, phylogrouping was developed (39) separating strains into groups A and B1 (classically considered commensal), and B2 and D (the pathogens). With access to more sequence data, Multi-locus Sequence Typing (MLST) (40) schemes were developed to type strains (i.e. give them a number) based on, in the case of the *E. coli* Achtman scheme used here, seven core genes sequences. With whole genome sequence data, full genome variant phylogenetics can determine close evolutionary relationships. Sequence based phylogenetics coupled with serotyping data can now be employed to fully classify *E. coli* and can improve the details known of pandemic strains such as EHEC O157 (41, 42) and *E. coli* ST131 (43-45).

1.1.2.3 Enterotoxins…it's always enterotoxins…

What separates pathogenic *E. coli* from commensal *E. coli* are a set of genes encoding virulence factors. These genes include toxins, adhesins, autotransporters, invasins and more (46). The classes of pathogenic *E. coli,* and often their associated serotypes, are commonly isolated hosting specific virulence genes. In most cases, a strain will acquire a virulence-mobilising element, become successful and disseminate, however in recent years various strains of pathogenic *E. coli* have been described that encode virulence genes associated with multiple pathogenic types (47), developing into the cross-class pathogens. Highlighting the seriousness of these novel pathogens, an enteroaggregative *E. coli* O104:H4 laterally obtained the ability to produce shiga toxins, which are classically a phage-encoded enterohaemorrhagic *E. coli* virulence factor seen in strains of the serogroups O157 and O55, among others (48). The result of this combination of virulence genes was the largest known outbreak of an enteropathogenic *E. coli* strain in a human population (49). How antimicrobial selective pressures and the existence of hybrid virulence-resistance elements will influence the rate of pathogen development is still in question.

Globally, enterotoxigenic *E. coli* are one of several causes of severe diarrhoeal disease in a diverse array of organisms. ETEC-related diarrhoea affects millions of children, particularly in developing countries with little to no sanitation, and is the leading cause of bacterial-derived Traveller's Diarrhoea (50). Agriculturally, enterotoxigenic *E. coli* lead to high morbidity and mortality rates in piglets. Enterotoxigenic *E. coli* are the causative agents of three distinct diseases in young pigs categorised by the age at which the pig develops clinical signs: neonatal (ND), pre-weaning and post-weaning diarrhoea (PWD). Neonatal diarrhoea affects piglets aged 0-4 days old and is the most severe of the three, with pre- and post-weaning diarrhoea seen in pigs aged 4-15 days and >15 days to 12 weeks old, respectively (46). Various methods have been employed to protect young pigs from ETEC infection and any subsequent diarrhoeal disease. These methods include, but are not limited to, treatment with antimicrobials, vaccination, and feed modulation (Fairbrother et al., 2005). While these protective steps have helped reduce levels of ETEC infection, particularly in neonatal and pre-weaning colibacillosis, antimicrobial resistance is eliminating the last resort treatment options. The ETEC O-antigen types most commonly associated with diarrhoeal disease in Australian piglets are O8, O45, O138, O141, O147, O149 and O157 (51).

Porcine ETEC pathogenesis is defined by a set of virulence factors targeting the pig GIT. The diarrhoea is a result of cell leakage through the action of enterotoxins; Heat-stable (ST) and Heat-labile (LT), both of which can cause diarrhoea through different molecular pathways within the pig GIT (31). Heat stable enterotoxins are small proteins that bind to intestinal epithelial cells, leading to a secretion of fluids out of the cell (31). Heat stable enterotoxins come in two forms, STa (*estA*) and STb (*estB*) (52). Heat-labile toxins are a close relative of the cholera toxin produced by *Vibrio cholerae* and have several major sub-groupings similar to the different heat stable enterotoxins. LT-I (*eltAB*) is the porcine ETEC equivalent of a similar human-targeting toxin, while LTIIa and LTIIb are two versions of the type II heat labile enterotoxin. Type I LT bind to the surface of small intestine endothelial cells, where they target the ganglioside GM_1. LTI are then endocytosed into the cell, where they have a similar effect to STa, forcing fluid out of the endothelial cell by increasing Cl^- secretion (31). Interestingly, improved sequence data has aided in the characterisation of an STb gene (*estB*) allele that has been identified in multiple *E. coli* isolates that demonstrates no virulence *in vitro* (53).

To effectively deliver toxins to the host epithelium, ETEC must first bind to the host cells. Various adhesins are found in ETEC that promote adherence to the pig GIT. The K88 (F4,

fae), K99 (F5), 987P (F6), F18, F41 fimbriae all provide host intestinal cell binding, with K88, K99 and F18 fimbriae being particularly prevalent (Nataro and Kaper, 1998;Nagy and Fekete, 1999;Koh et al., 2008). Interestingly, the K88 and F18 adhesins are often associated with pre- and post-weaning diarrhoea, whilst the K99 adhesin is associated with neonatal diarrhoea and multi-species colonisation (DebRoy and Maddox, 2001). These binding effects are also thought to be supplemented by a range of herd factors, like age and feed supplementation, plus host of other highly prevalent adhesins such as *fimH* (Bouckaert et al., 2006) and *eaeH* (Sheikh et al., 2014), which have been shown to provide additional binding post-attachment.

A multitude of additional genes found in *E. coli* are known to play a role in pathogenicity. Iron acquisition genes (54), serum survival genes such as *iss* (55) and haemolysins, particularly the alpha-haemolysin *hlyA* (56), are often observed in diarrhoeagenic and extra-intestinal pathogenic *E. coli* isolates. These virulence factors have been identified in porcine ETEC worldwide, with examples coming from South Korea (57), Europe (58), the Americas (59, 60), Vietnam (47), and a number of studies within Australia (40, 46, 47, 51) to name a few. Various plasmid vectors for ETEC virulence genes have additionally been identified mobilising these genes (61), and the association of these virulence plasmids with certain antimicrobial resistances has been observed both historically (62) and in recent times (63).

Identifying potential pathogens and antimicrobial resistant strains within food animals is one of the primary issues to tackle in agricultural research, as infection can be the largest source of financial loss in production (61). Early identification allows a facility to take precautionary measures and be aware of treatment options should disease develop and allows for the screening of resistances and pathogenesis factors with any relevance to human health.

1.2 Approaches and experimental design for the epidemiological analysis of porcine agricultural samples

In this project, a collection of commensal and pathogenic *Escherichia coli* from agricultural porcine samples hosting antimicrobial resistance underwent whole genome sequencing and analysis. The data was then split into various sub-studies and combined with both national and international datasets to generate inferences on the influence of mobile elements on the distribution of antimicrobial resistance in NSW piggeries.

All strains were provided by the New South Wales Department of Primary Industries Elizabeth Macarthur Agricultural Institute (EMAI), as part of the Australian Centre for Genomic Epidemiological Microbiology (ausgem) initiative. The base collection included 163 *E. coli* isolates sourced from two sets of pigs in a facility in New South Wales, Australia circa 2007. Also provided by the EMAI were four enterotoxigenic pathogens to undergo similar characterisation.

The collection presents an opportunity to observe an interesting dichotomy generated by antimicrobial treatment. Approximately half of the larger single-site collection, 85 isolates, was sourced from 17 different animals during an enterotoxigenic diarrhoea outbreak, with the other half (78 isolates) sourced from 16 different, similarly aged animals in the same location. The second cohort was isolated 4 weeks post-outbreak, after consistent treatment with neomycin had been administered through in-feed antibiotic supplementation and diarrhoea in all piglets had ceased in the first cohort. This chance opportunity may provide insight into the difference in sampled *E. coli* both during and post-outbreak.

When this collection was provided to us by the New South Wales Department of Primary Industries, they came characterised by PCR (single gene identification), RAPD barcode typing, and phenotypic antibiotic sensitivity testing (46). The isolates were screened for relevant genes, including 32 virulence-associated genes, 25 genes related to antimicrobial resistance, 16 bacteriocin-encoding genes and genes used for basic phylogrouping (39). Phenotypic testing data were generated for each isolate against the following antibiotics: ampicillin (25 μg), augmentin (60 μg), timentin (85 μg), cephalexin (100 μg), cefoxitin (30 μg), cefotaxime (5 μg), cefepime (10 μg), nalidixic acid (30 μg), ciprofloxacin (2.5 μg), imipenem (10 μg), sulphafurazole (300 μg), trimethoprim (5 μg), tetracycline (10 μg), apramycin (15 μg), neomycin (30 μg), gentamicin (10 μg), azithromycin (15 μg) and chloramphenicol (30 μg).

Table 1.1: Virulence and antimicrobial resistance genes and their function, identified within the "Outbreak" and "Post-treatment" cohorts of the *E. coli* collection via PCR.

	Gene Target	Outbreak Prevalence (%)	Treatment Prevalence (%)	Gene Function/Resistance
Toxins	*estA*	7	9.3	Heat stable Enterotoxin a
	estB	45.9	19.2	Heat stable Enterotoxin b
	eltAB	31.8	12.8	Heat labile Enterotoxin
	hlyA	5.9	3.8	Alpha haemolysin
Adhesins	*fae*	28.2	14.1	K88 (F4) fimbrial Adhesin
	fed	5.9	1.2	F18 fimbrial Adhesin
	fimH	57.6	46.2	Conserved Adhesin
	east1	45.9	30.8	Enteroaggregative Adhesin
Integrons	*intI1*	64.7	79.5	Class 1 Integron
	intI2	12.9	30.8	Class 2 Integron
AMR	*aphA1*	7	57.7	Neomycin/Kanamycin resistance
	bla_{TEM}	42.4	64.1	Penicillin resistance
	cmlA	14.1	67.9	Chloramphenicol/Neomycin resistance
	*strAB**	46.7	41.4	Streptomycin resistance
	*sul2**	5.3	5.7	Sulphonamide resistance
	*merA**	26.7	40	Mercury resistance
IS	IS*26**	96	92.8	IS*26* Insertion Sequence

* Indicates AMR-related data produced as part of this project. The remainder is data provided with the collection.

Utilising available data, a subset of 48 genetically diverse isolates positive for the class 1 integrase *intI1* were selected for short-read whole genome sequencing, alongside the four enterotoxigenic strains. Half (24) were selected from each cohort. One of the five pathogens, *E. coli* O157 SvETEC, was a particularly severe pathogen and additionally underwent long read whole genome sequencing to generate complete structures for analysis.

The methodologies utilized here fall under two categories. Molecular strategies involve the direct experimentation on DNA using various techniques and technologies; manipulating and characterising DNA isolated from bacterial culture. Bioinformatic strategies use software *in*

silico to align and analyse the genome sequence data generated by various molecular technologies.

1.2.1 Molecular strategies

The molecular strategies used were performed to obtain DNA from isolated strains, screen potential sequencing candidates for marker genes, and obtain data complementary to the sequence data generated.

DNA Isolation and Whole Genome Sequencing - Many molecular techniques and sequencing technologies require isolated DNA, but the amount and quality of DNA required for success varies. Genomic DNA can generally be extracted using either harsher, faster methods suitable for single gene identification and short read sequencing, or gentler methods capable of extracting long DNA strands, conserving sequence context for long-read sequencing. For this project, DNA was extracted from cultured *E. coli* isolates for PCR and short read sequencing using a Bioline Isolate II Genomic DNA kit. Genomic DNA suitable for long read sequencing was extracted by phenol-chloroform, avoiding actions that would shear the DNA. Short read whole genome sequencing (WGS) was performed using Illumina sequencing platforms, both MiSeq and HiSeq, utilising massively paralleled sequence-by-synthesis reactions. Long read sequencing was performed on a PacBio RS II sequencer with Single Molecule Real Time (SMRT) sequencing.

Polymerase Chain Reaction (PCR) – PCR uses oligonucleotides, short DNA molecules, to target a region of isolated DNA and perform exponential amplification of that sequence using a thermostable DNA polymerase. The mass of amplified DNA is then separated using agarose gel electrophoresis and visualised using a fluorescent stain. PCR is particularly useful for initial screening protocols, identifying isolates that host key genes associated with AMR. It is also a useful tool for characterising complex genomic regions with clustered antimicrobial resistance genes and mobile elements that are difficult to assemble using short-read WGS technology.

Fosmid Library - A fosmid library is a collection of *E. coli* isolates hosting dispersed, unique regions of a source genomic DNA extraction. Each region is approximately 40 kb, hosted on plasmids within the *E. coli* hosts. The CopyControl Fosmid Library Production Kit from Epicentre was used to generate this library for *E. coli* O157 SvETEC. Using this kit, unique 40 kb regions of insert gDNA extract were ligated into plasmid backbones, which were packaged into phages. These phages were then added to EPI300 *E. coli* and the unique

gDNA derived plasmids are inserted into the bacteria, which were grown on an antibiotic-selective media to produce a library of isolates, each hosting a single plasmid. This process was performed to separate out sections of genomic DNA into new hosts, so that these regions could be studied using molecular strategies without the interference of other, similar genomic regions. This was particularly useful for the study of AMR as these regions often contain similar sets of genes, which can interfere with the PCR primer targeting if a strain hosts multiple integrons, for example.

1.2.2 Bioinformatic strategies

Software utilised in molecular analyses perform a range of functions - genome assembly, visualisation of sequence comparisons, detection of polymorphisms and calculating phylogenetic divergence. Different packages are deployed across various platforms (Linux, Windows PC and Mac) depending on what the platform can offer in utility. Software on Linux systems can be deployed on High Performance Computing (HPC) clusters for parallel processing of large datasets, while Windows and Mac applications are supported by user interfaces (Java, etc) that enables graphical exploration of the data. Note that many of the following pipelines rely on multiple software components that perform alignments and generate maximum-likelihood trees, including pplacer, HMMER (64), bowtie2 (65), SPAdes (66), bwa aligner (67, 68), Canu (69) and FastTree 2 (70). Many software packages were accessed using the UTS HPC and using Bioconda. The databases EnteroBase and GenBank are primary hosting sites for genomic data and were used heavily throughout this project.

FastQC – a tool for the quality control of sequence data generated by high throughput sequence technology. FastQC gives graphical and numerical representations of read quality, ensuring the read sets for each isolate should proceed to draft genome sequence assembly.

Aaron And Andrews Awesome Assembly pipeline (A5-miseq) – a whole genome sequence assembly pipeline optimised to handle the 250 bp paired-end reads generated by Illumina platforms (71). It assembles a *de novo* draft whole genome sequence of the isolate from raw reads and generates a range of supporting files giving details of the assembly. Short read d*e novo* assemblies are termed draft due to gaps in the read alignments. These gaps are caused either by low sequencing coverage or an assembly conflict due to repeated sequences greater than the 250 bp read length. This is a limitation of short-read sequencing, and leaves genomes split into contiguous sections that were possible to assemble, that can range from over 500 kb down to 1 kb or less.

Hierarchical Genome Assembly Process (HGAP) – a whole genome sequence *de novo* assembler designed for the reads generated by PacBio SMRT sequencing (72). It also provides detailed assembly statistics. Ideally, long read assemblies generate completed sequences. That is, the reads are long enough to bridge any gaps in assembly that short read sequences fail to resolve and should produce circularised sequences that represent each DNA molecule within a strain, including the chromosome. An assembly issue with HGAP involves those circles with a repeated beginning/end to the sequence. The pipelines circulator and minimus2 can be used to resolve these structures.

Unicycler – Unicycler is a hybrid assembly pipeline that generates complete, circular assemblies using the combination of short- and long-read sequences (73). It is designed to capitalise on the sequencing depth provided by short-read sequencing and couple it with the long-read contextual data.

Basic Local Alignment Search Tool (BLASTn) – BLASTn is a sequence alignment algorithm that is used to align nucleotide sequences, generating a visualisation of that alignment as well as relevant statistics (74). In practice, BLASTn alignments can be used to match reference sequences, often ORFs of known genes or specific genetic signatures, to whole genome sequence data. The GenBank nucleotide database (75) also hosts the global repertoire of bacterial sequences, and can be accessed through a BLASTn server to characterise sequence data.

Open Reading Frame Finder (ORF Finder) – ORF finder is a tool that allows the identification of open reading frames putatively representing the coding regions of genes within an input sequence. Useful for the annotation of unknown sequences. It was accessed at http://www.ncbi.nlm.nih.gov/gorf/gorf.html.

IS Finder – IS Finder is a BLASTn-based alignment tool, that matches an input sequence to a curated database of insertion sequences and transposons, allowing the identification of specific mobile elements. It was accessed at https://www-is.biotoul.fr/.

Mauve – Mauve is software package designed for the graphical representation of whole genome sequence data and has utilities to arrange scaffolded whole genome sequences in comparison to a reference (movecontigs) and generate whole genome comparisons (progressiveMauve) (76, 77).

Rapid Annotation using Subsystems Technology (RAST) – The RAST server performs automated prokaryotic WGS annotation, identifying open reading frames and providing a protein family identification based on sequence homology (78). This pipeline provides a useful way to quickly characterise a full genome, but automated annotations generally need curation.

The Comprehensive Antibiotic Resistance Database (CARD) – CARD is a database containing sequences for antimicrobial resistance genes (79). The database is linked to a Resistance Gene Identifier tool that can provide antimicrobial resistance gene annotation to whole genome sequence inputs, identifying individual antimicrobial resistance and efflux pump genes.

Phage Search Tool (PHAST/PHASTER) – PHAST, and its enhanced release PHASTER, are automated bacteriophage identification and annotation servers (80, 81). They identify and annotate any phage-associated sequences from whole genome sequence data and provide scores for phages that are potentially functional.

Multi-locus Sequence Typing (MLST) – Multi-locus sequence typing (MLST) uses the sequences of a small number of specific genes to classify and organise bacteria into a sequence type, providing some basic phylogenetic distribution data (82). The Achtman *E. coli* MLST scheme, which is based on the sequences of seven housekeeping genes, is generally used. In MLST, the specific alleles of each gene are assigned a number (e.g. *adk6, fumC5*), and each profile is then also assigned a number (e.g. sequence type 131 or ST131), and similar sequence types are grouped into clonal complexes (e.g. CC23). This process has also been applied to core plasmid sequence, allowing the subtyping of several plasmid incompatibility groups.

Serotyping and phylogrouping – Detection of the alleles encoding specific cell surface antigens (O- and H-types) allows for *in silico* serotyping. The same applies for identifying *E. coli* phylogroups.

PhyloSift – PhyloSift is a pipeline that extracts, translates, and aligns input sequences against a reference database of phylogenetically relevant genes; RNA sequences and the protein sequences of 37 conserved prokaryotic genes (83). The alignment data are used to extrapolate phylogenetic relationships. Advantages to using a limited but selective sequence pool is to reduce the effects of recombination.

Harvest Suite: parsnp and gingr – The Harvest suite is a set of tools for determining phylogenetic relationships from single nucleotide polymorphisms (SNPs) and visualising these data (84). Parsnp performs polymorphism identification compared to a reference sequence, and only on sequences conserved between each sequence included. Combined with the PhiPack recombination filter, Parsnp excels at removing the influence of mobile elements and recombination from SNP-based phylogenetic reconstruction. Parsnp also generates tables of variant calls between input sequences. Gingr creates visual outputs to observe SNP clustering and sequence exclusion.

FigTree – FigTree is a free Windows application for rendering phylogenetic trees, with numerous options to alter the visual characteristics.

EasyFig – EasyFig takes multiple input sequences and generates a visual representation of BLASTn alignments between sequences (85).

Blast Ring Image Generator (BRIG) – Software based on CGView that generates circular representations of BLASTn alignment data against a reference sequence (86). It can also graph GC content and skew for the reference sequence and show annotations if required.

SnapGene – SnapGene is a genome annotation and management package for Windows, useful for both annotation of sequence data and the generation of figures from those annotations.

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Chapter 2: Genomic microbial epidemiology is needed to comprehend the global problem of antibiotic resistance and to improve pathogen diagnosis

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Genomic Microbial Epidemiology Is Needed to Comprehend the Global Problem of Antibiotic Resistance and to Improve Pathogen Diagnosis

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Contamination of waste effluent from hospitals and intensive food animal production with antimicrobial residues is an immense global problem. Antimicrobial residues exert selection pressures that influence the acquisition of antimicrobial resistance and virulence genes in diverse microbial populations. Despite these concerns there is only a limited understanding of how antimicrobial residues contribute to the global problem of antimicrobial resistance. Furthermore, rapid detection of emerging bacterial pathogens and strains with resistance to more than one antibiotic class remains a challenge. A comprehensive, sequence-based genomic epidemiological surveillance model that captures essential microbial metadata is needed, both to improve surveillance for antimicrobial resistance and to monitor pathogen evolution. *Escherichia coli* is an important pathogen causing both intestinal [intestinal pathogenic *E. coli* (IPEC)] and extraintestinal [extraintestinal pathogenic *E. coli* (ExPEC)] disease in humans and food animals. ExPEC are the most frequently isolated Gram negative pathogen affecting human health, linked to food production practices and are often resistant to multiple antibiotics. Cattle are a known reservoir of IPEC but they are not recognized as a source of ExPEC that impact human or animal health. In contrast, poultry are a recognized source of multiple antibiotic resistant ExPEC, while swine have received comparatively less attention in this regard. Here, we review what is known about ExPEC in swine and how pig production contributes to the problem of antibiotic resistance.

Keywords: multiple antibiotic resistance, animal production, environmental pollutants, genomic epidemiology, agriculture, *Escherichia coli*

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ANTIMICROBIAL RESISTANCE IN AGRICULTURAL AND CLINICAL SETTINGS

Meat production farming practices have increased in scale over the past 30 years (Landers et al., 2012) and are predicted to continue to increase in line with rising incomes in low- and middle-income countries (Van Boeckel et al., 2015). This rationalization of farming has led to intensification of animal production and greater reliance on antimicrobials to control infectious disease, improve feed conversion efficiency, and promote animal growth (Levy, 1992; Sarmah et al., 2006; FAO, 2009; Krishnasamy et al., 2015; Van Boeckel et al., 2015). Modeling studies estimate that

global antimicrobial consumption will increase by 67% from an estimated 63,151 tons in 2010 to 105,596 tons in 2030 (Van Boeckel et al., 2015). Much of this rise in consumption is expected to come from China, India, Russia, South Africa, and Brazil; countries where meat consumption is growing due to rising incomes (Van Boeckel et al., 2015). Large scale animal production facilities generate huge volumes of animal waste that is contaminated with veterinary antibiotics. While estimates vary, it is thought that in the USA, 11–14 million kilograms of antibiotics are used annually in the production of animals for food (i.e., livestock; Institute of Medicine; National Research Council; Panel on Animal Health, 1999; Landers et al., 2012). Almost half of these are used for non-therapeutic purposes, which is significantly more than the estimated 1.4 million kilograms of antibiotics used in human medicine (Institute of Medicine; National Research Council; Panel on Animal Health, 1999). Many classes of antibiotics are used in animal production including β-lactams, sulfonamides, tetracyclines, streptogramins, macrolides, lincosamides, polyethers, quinoxalines, elfamycins, glycolipids, arsenicals, and polypeptides (Sarmah et al., 2006). How these compounds are modified within the animal, how they persist in soils and waterways after they are excreted, and their fate when animal slurries are used to fertilize pastures are complex and poorly understood (Sarmah et al., 2006; Zhu et al., 2013). Antimicrobial residues that contaminate groundwater tables, aquatic environments, and land used for crop production exert selection pressure on microbial populations that may promote the lateral transfer of resistance and virulence genes between populations, and contributes to the emergence of novel pathogenic profiles (Venturini et al., 2010, 2013; Baquero and Tobes, 2013; Baquero et al., 2013; Roy Chowdhury et al., 2015). It is perhaps unsurprising that commensal gastrointestinal bacterial populations are commonly resistant to more than one antimicrobial (Bettelheim et al., 2003; Diarrassouba et al., 2007; Bailey et al., 2010) and consequently the frequency of community-acquired infections with resistance to multiple antimicrobials are also increasing (Onken et al., 2015; Paniagua-Contreras et al., 2015; Yahiaoui et al., 2015). Furthermore, surveys have shown the presence of antibiotic resistance genes in the gastrointestinal tracts of humans and animals that have never received antibiotic interventions, specifically in: human feces from remote communities (Bartoloni et al., 2009; Ruppe et al., 2009; Eisenberg et al., 2012), migratory bird populations (Foti et al., 2011; Carroll et al., 2015; Jamborova et al., 2015; Stedt et al., 2015) and various wildlife species (Allen et al., 2010; Baez et al., 2015; Jobbins and Alexander, 2015; Katakweba et al., 2015). This demonstrates that resistance genes in host bacteria can readily spread to naïve microbial populations via waterways, air currents, road incursions into remote areas, animal and bird migration, and land clearance (Tsubokura et al., 1995; Middleton and Ambrose, 2005; Ewers et al., 2009). The application of manure onto pasture from ruminants that have not received recent antibiotic therapy is known to stimulate the expansion of resident flora encoding resistance to β-lactam antibiotics (Udikovic-Kolic et al., 2014). These observations demonstrate our limited understanding of the impact of the application animal waste to agriculture. The mechanisms by which mobile elements flux through microbial populations are not fully understood, but it is clear that efforts must be made to reduce environmental contamination sourced from human and animal effluent.

Antimicrobial resistance is largely an ecological problem (Summers, 2002, 2006; Stokes and Gillings, 2011; Landers et al., 2012; Berendonk et al., 2015; Gillings et al., 2015). In general, studies have focused on the emergence and carriage of resistance genes in certain key microbial populations, without an appreciation for the wider complexity of the problem, or its origins. Hence, there are numerous under-reported microbial sources of resistance genes. Antibiotic resistance genes often traffic in association with transposons that carry mercury resistance operons; these play an important role in transforming mercury to less biologically toxic forms, thereby regulating the availability of toxic mercury compounds in the environment. Mercury-resistance gene operons have evolved as key components of Tn*3*-family transposons and these transposons are globally disseminated. Mercury has been released into the environment via geological processes over millions of years and, although anthropogenic activity over the past two centuries has contributed significantly to the release of mercury into waterways and the atmosphere, it is likely that mercury levels were higher prior to the industrial revolution than they are now as a result of extensive volcanic activity (Osborn et al., 1997; Barkay et al., 2003). Despite this, large scale surveillance studies of antimicrobial resistance fail to screen microbial populations for the presence of mercury-resistance genes (Djordjevic et al., 2013). Resistance genes can also accumulate in microbial populations in soils that have been fertilized with animal waste and treated effluent (Heuer and Smalla, 2007; Chee-Sanford et al., 2009; Heuer et al., 2011a,b; Kristiansson et al., 2011; Baquero et al., 2013; Berendonk et al., 2015). Meat, seafood, and vegetables are also important and under-recognized sources of antimicrobial resistance genes in bacteria (Johnson et al., 2005a; Agerso et al., 2014; Kim and Woo, 2014; Ahmed and Shimamoto, 2015a,b; Ben Said et al., 2015; Le et al., 2015; van Hoek et al., 2015; Zhang et al., 2015; Zurfluh et al., 2015).

Anthropogenic industrial processes generate heavy metals including cadmium, lead, arsenic, copper, silver, and a wide range of synthetic organic compounds that pollute natural ecosystems. As such, heavy metal contamination represents yet another selective pressure for the development of antimicrobial resistance (Baker-Austin et al., 2006; Castillo et al., 2008; Li et al., 2010; Zhu et al., 2013). Genes coding for resistance to silver, copper, arsenic and antimony are associated with complex resistance gene loci in bacteria resistant to more than one antimicrobial and have great significance to human, livestock, and plant health (Berg et al., 2005; Seiler and Berendonk, 2012; Hobman and Crossman, 2015; Bondarczuk et al., 2016). The widespread occurrence of genes coding for resistance to heavy metals has, in part, been demonstrated in studies using information mined from publicly available genome sequences highlighting the value of genomic surveillance studies (Hobman and Crossman, 2015).

Genome-wide studies of genes coding for resistance to antibiotics, biocides and heavy metals have shed light on how these genes co-assemble on mobile genetic elements. In an analysis of 2522 fully sequenced bacterial genomes and 4582

plasmid sequences, Pal et al. (2015) were able to identify examples where antimicrobial resistance genes co-occurred. The authors found that the most likely co-selection scenario occurred in bacterial strains that carried plasmid-borne antibiotic resistance genes and biocide/heavy metal resistance genes on the same chromosome. A plasmid localized gene cluster coding for a cadmium/zinc resistance gene (*cadD*) with aminoglycoside and macrolide resistance genes was also identified (Pal et al., 2015). Resistance to these combinations of antimicrobials would benefit bacterial populations found in intensive animal production systems where cadmium and zinc are both used to promote growth and aminoglycosides and macrolides are frequently administered for the treatment of Gram-negative and Gram-positive infections, respectively (Castillo et al., 2008; Li et al., 2010). It is clear that plasmids can carry combinations of genes coding for resistance to more than one microbial agent alongside genes coding for resistance to biocides and heavy metals. Invariably these plasmids are large, conjugative and commonly found in pathogens from hospital and intensive farming environments (Chen et al., 2007; Woodford et al., 2009; Venturini et al., 2010, 2013; Pal et al., 2015). Sub-lethal levels of antibacterial biocides and heavy metals found in mildly polluted aquatic and land-based ecosystems may be sufficient to maintain these mobile elements carrying multiple antibiotic, biocide and heavy metal resistance genes (Stepanauskas et al., 2006; Gullberg et al., 2014).

Although plasmids can vary in size and coding ability, their size is generally positively correlated with the size of the host chromosome (Smillie et al., 2010). Plasmids conferring multiple-drug resistance are usually large, as they code for a suite of resistance genes, alongside associated integrons, transposition factors, and genes for heavy metal resistance, which can be in excess of 20,000 bp or more in size. Small plasmids that acquire resistance to more than one antimicrobial are, however, not without precedent. Labar et al. (2012) described the sequence of a small plasmid (pASL01A; 27,072 bp) that codes for resistance to ampicillin, streptomycin, sulfonamides, trimethoprim, and mercury (Labar et al., 2012). Most of the plasmid (21,904 bp) is comprised of a Tn*21* derivate mercury resistance transposon that carries a class 1 integron with a trimethoprim resistance *dfrA7* gene cassette and Tn*6029C* (Roy Chowdhury et al., 2015). Tn*6029C* comprises a $bla_{\mathrm{TEM-1}}$-IS*26-repA/C-sul2-strA-strB* gene cluster flanked by direct copies of IS*26* (Labar et al., 2012; Roy Chowdhury et al., 2015). The plasmid backbone (5,168 bp) and related plasmids are widespread in enterobacterial populations in Africa (Labar et al., 2012).

Antimicrobials, including heavy metals, are used widely in animal production to treat clinical disease, to prevent disease outbreaks during critical or vulnerable periods, and to promote growth (McEwen and Fedorka-Cray, 2002; Landers et al., 2012). The use of antibiotics to promote growth is a phenomenon whereby animals receiving very low doses of antimicrobials appear healthier and grow larger (Roy Chowdhury et al., 2014). Although, the mechanism for this growth promoting effect is still poorly understood, the majority of pigs produced in the USA are exposed to tetracyclines and tylosin to prevent disease and promote growth (Apley et al., 2012). While these approaches have proved effective in controlling disease and aiding farms globally, the uninhibited use of antimicrobials also provides the selection pressure that drives antimicrobial resistance (Pulcini and Gyssens, 2013). As such, concerted efforts to reduce the use of antimicrobials in food production should be a priority.

Given that the persistence and spread of resistance is intrinsically linked with the presence and activity of antimicrobials, it is significant that 30–90% of ingested antibiotics are excreted in an un-metabolized or only partially metabolized form (Sarmah et al., 2006) into waste treatment plants and animal waste-holding facilities. Antibiotic residues are known to persist in secondary effluent despite the treatment process (Sarmah et al., 2006; Watkinson et al., 2007; Martinez, 2008; Le-Minh et al., 2010; Bondarczuk et al., 2016). Furthermore, the pH, temperature, nutrient concentration, and bacterial loads in waste treatment plants are conducive to the evolution and spread of antimicrobial resistance (and virulence) genes (Andersen, 1993; Dolejska et al., 2011). This is in part evidenced by an increase in the presence of the class 1 integrase gene, a reliable proxy for resistance to more than one antimicrobial in bacterial populations sampled in wastewater treatment plants (LaPara et al., 2011; Ma et al., 2013; Gillings et al., 2015), and an increase in the number of resistance genes in wastewater treatment plants and rural domestic wastewater treatment systems (Gao et al., 2012; Chen and Zhang, 2013; Ju et al., 2015; Mao et al., 2015). This indicates that controlling the use of antimicrobials is a prerequisite for slowing the development and spread of pathogens with multiple antimicrobial resistance mechanisms.

ANTIMICROBIAL RESISTANCE IN *Escherichia coli*

Escherichia coli is a widespread and abundant organism capable of causing a wide range of gastrointestinal and extra-intestinal diseases. It can survive and proliferate in a diversity of terrestrial and aquatic environments (van Elsas et al., 2011). As such, its genetic profile is shaped profoundly by horizontal gene transfer, and it is a useful marker species for understanding how antimicrobial resistance and virulence genes accumulate over evolutionary time. Horizontal gene transfer events are known to generate novel combinations of putative and established virulence factors, and generate novel pathotypes of *E. coli* (Wu et al., 2007; Bielaszewska et al., 2014). Although, *E. coli* is a commensal inhabitant of the gastrointestinal tract of warm-blooded animals, pathotypes have evolved that cause intestinal [intestinal pathogenic *E. coli* (IPEC)] and extra-intestinal [extraintestinal pathogenic *E. coli* (ExPEC)] diseases. Different pathotypes of *E. coli* cause diarrheal disease, hemolytic uremic syndrome (HUS), urinary tract infection (UTI), pyelonephritis, septicemia, meningitis, and respiratory disease (pneumonia) in humans and animals. Known IPEC pathotypes include the attaching and effacing *E. coli* (AEEC), Shiga-toxigenic *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse adhering *E. coli* (DAEC) and the recently described enteroaggregative hemorrhagic *E. coli* (EAHEC; Kaper et al., 2004; Brzuszkiewicz

et al., 2011). EHEC are a subgroup of STEC, which colonizes the terminal regions of the gastrointestinal tract of ruminants. It is notable that different serotypes of *E. coli* preferentially colonize different ruminant hosts (Djordjevic et al., 2001, 2004; Hornitzky et al., 2002) and carry different Shiga toxin gene variants (Ramachandran et al., 2001; Brett et al., 2003a,b). These studies underscore the importance of understanding the ecology of disease-causing organisms and how niche adaptation influences lateral gene transfer. Notably, however, ruminants are not a recognized source of ExPEC (Manges and Johnson, 2015).

Many recent molecular surveillance studies on *E. coli* report the frequency of genes that code for extended-spectrum beta lactamase enzymes (ESBL's), which are indicative of resistance to penicillin, cephalosporin, and other clinically important antibiotics, but fail to screen for resistance to the older, first generation antibiotics (Bean et al., 2009; Ewers et al., 2012; Glasner et al., 2013; Roschanski et al., 2015). Genes that code for resistance to first generation antibiotics are often carried in bacteria containing class 1 integrons (Johnson et al., 2005b; Jakobsen et al., 2011; Hansen et al., 2014). From a diagnostic perspective, recent genomic studies on an outbreak of EAHEC and EAEC strain O104:H4, highlighted this issue (Ahmed et al., 2012). EAHEC is a recently emerged *E. coli* pathotype and EAEC strain O104:H4 has acquired the Shiga toxin gene *stx2a*, a diagnostic marker of EHEC. Outbreak strains were found to be resistant to ampicillin, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline but little effort was given to determining how the genes that coded for these antibiotic resistance phenotypes were assembled (Ahmed et al., 2012). We were subsequently able to show that the genes coding for resistance to these antibiotics were assembled in Tn*6029D*, a transposon that abuts a class 1 integron in a Tn*21*/Tn*1721* hybrid backbone (Roy Chowdhury et al., 2015). The resistance region resided in genomic island 3 which also carried the virulence gene *ag43* encoding a self-associating serine protease auto-transporter, important for biofilm formation. This is an example of how antimicrobial resistance genes and virulence genes co-localize on the same mobile genetic element (Roy Chowdhury et al., 2015). Chromosomal islands are increasingly associated with carriage of class 1 integrons encoding resistance to multiple antibiotics of clinical significance (Roy Chowdhury et al., 2016).

Salmonella genomic island 1 (Boyd et al., 2001; Levings et al., 2005), Salmonella genomic island 2 (Levings et al., 2008) and several large plasmids in EHEC strain O26:H (Venturini et al., 2010), and an atypical EPEC, strain O111 (Venturini et al., 2013) are also examples of virulence and resistance genes being carried on the same mobile element. Strains of *E. coli* that carry genes that are markers for more than one *E. coli* pathotype pose a dilemma for diagnostic laboratories. There are numerous reports describing novel *E. coli* strains with combinations of virulence genes that are diagnostic of more than one *E. coli* pathotype; this suggests that newly emerging strains are increasingly likely to have complex resistance loci facilitated by the clustering of mobile element-associated resistance and virulence genes. In a study of 265 cases of *E. coli* causing UTI, 28 carried virulence genes typical of IPEC (Bielaszewska et al., 2014). Several other examples of hybrid *E. coli* strains have been described and their presence demonstrates the need to have genomic surveillance strategies in place to determine how antibiotic resistance and virulence genes assemble in emerging pathogens (Wallace-Gadsden et al., 2007; Abe et al., 2008; Bielaszewska et al., 2014; Mariani-Kurkdjian et al., 2014).

Extraintestinal pathogenic *E. coli* is a phylogenetically diverse group comprised of a broad range of *E. coli* sequence types (STs). ExPEC are fecal *E. coli* that rarely cause intestinal disease but have acquired a wide variety of virulence gene cargo that facilities their ability to cause disease at extraintestinal sites, particularly in the urinary tract. ExPEC are the most frequently isolated Gram negative bacterial pathogen infecting humans incurring significant mortality and morbidity. Notably, ExPEC are a leading cause of sepsis and are increasingly resistant to multiple antibiotics posing a serious health concern (Manges and Johnson, 2015; Poolman and Wacker, 2016). ExPEC have a fecal origin but rarely cause gastrointestinal disease. The acquisition of a diverse range of virulence factors by lateral gene transfer has armed ExPEC with an ability to cause life-threatening blood-borne, urinary tract, respiratory, skin and soft tissue infections, and meningitis in humans of all ages (Russo and Johnson, 2003; Poolman and Wacker, 2016). ExPEC vary widely in the combinations of putative virulence genes that they carry. Many genes encode for adhesins, iron acquisition systems and other putative virulence attributes and this genetic redundancy complicates efforts to develop broadly applicable molecular diagnostic tests to detect ExPEC (Johnson et al., 2001; Spurbeck et al., 2012). Importantly, livestock are important reservoirs for ExPEC (Ewers et al., 2007, 2009; Manges et al., 2007; Vincent et al., 2010; Nordstrom et al., 2013; Manges and Johnson, 2015). Virulence genes are often shared among *E. coli* isolates from samples collected from humans, and the meat and feces of intensively reared livestock, particularly poultry (Rodriguez-Siek et al., 2005; Vandekerchove et al., 2005; Jakobsen et al., 2010a,b). Consistent with this view, ExPEC carry genes coding for resistance to antimicrobials frequently used in veterinary medicine. Hospital diagnostics laboratories do not screen for most of the antibiotics used in veterinary medicine and so resistance to these is under-reported. In a Chinese study of 315 ExPEC isolates from various pig tissues, more than 63% exhibited resistance to 10 antibiotics including ampicillin, trimethoprim, sulfadimidine, tetracycline, neomycin, streptomycin, and kanamycin (Tan et al., 2012). The genes $bla_{\mathrm{TEM-1}}$ (ampicillin resistance); *strAB* (streptomycin resistance); *sul1* and, *sul2* (sulfonamide resistance); *aphA1* (kanamycin and neomycin resistance); *tetA*, *tetB,* and *tetG* (tetracycline resistance); as well as various *dfrA* genes (trimethoprim resistance) coded for resistance to these antibiotics.

The class 1 integron is widespread in clinical settings. Bacteria that are resistant to more than one antimicrobial often carry class 1 integrons and their presence is a reliable indicator of multiple antimicrobial resistance (Leverstein-van Hall et al., 2003). The class 1 integron structures identified in most clinical samples are thought to be derived from the capture of a chromosomally located class 1 integron, prevalent within environmentally abundant bacterial species belonging

to the broad β-proteobacterium group, by a Tn*402* family transposon (Gillings et al., 2008; Gillings, 2014). Global spread of the class 1 integron is in part a result of the propensity for the Tn*402* family of transposons to target *res* sites found on a wide variety of conjugative and mobile plasmids, including the widespread mercury-resistance transposon, Tn*21*, and the broader Tn*3* family (Liebert et al., 1999; Gillings et al., 2008, 2015). For this and a variety of other reasons described below, resistance genes cluster on mobile elements. Clinical class 1 integrons have subsequently evolved, typically by the loss of most of the Tn*402 tniB* transposition gene, and partial fusion of the *qacE* gene (coding for an efflux pump for disinfectants, Hall et al., 1994; Partridge et al., 2001) with the sulfonamide resistance gene *sul1*. The most commonly observed structure observed in clinical isolates (**Figure 1A**) is comprised of a 5′ conserved segment (5′-CS), a 3′-conserved segment (3′CS) and a variable region inserted in between them. The 5′-CS contains the *intI1* gene encoding the class 1 integrase; the Tn*402* inverted repeat, the two promoters, one required for the transcription of the integrase and the other for the gene cassettes and the *attI1* recombination site. The variable region contains a diverse array of resistance gene cassettes (Partridge et al., 2009) and the associated *attC* sites. The 3′ conserved segment (3′-CS) most frequently contains *qacEΔ1* and *sul1* genes, remnants of the transposition module and a terminal Tn*402* inverted repeat. Most gene cassettes found in clinical class 1 integrons from nosocomial and livestock settings code for resistance to trimethoprim (*dfrA*) and aminoglycosides (*aadA*); despite this over 130 different gene cassettes have been described (Partridge et al., 2009) and, to date, 410 annotated variants are recognized (Tsafnat et al., 2011).

Further variation in the structure of the 3′-CS has also been described (Pan et al., 2006; Cain et al., 2010; Dawes et al., 2010; Saenz et al., 2010; Venturini et al., 2010, 2013; Mattiello et al., 2015). In one instance, the 3′-CS was almost completely lost and had been replaced with the compound transposon, Tn*6026* (Venturini et al., 2013). The Tn*6026* structure contains the beta-lactamase bla_{TEM-1} gene; the sulfonamide resistance gene, *sul2*; the streptomycin resistance gene *strAB*; the neomycin/kanamycin resistance gene, *aphA1* and it is flanked by direct copies of IS*26* (**Figure 1B**). Tn*6026* is located between *attI*, the insertion point of the gene cassette, and the defective *tni* module of a clinical class 1 integron, replacing all but the first 24 bp of the 3′-CS (Cain et al., 2010; Dawes et al., 2010; Venturini et al., 2010, 2013). A *sul3* variant structure associated with IS*26* has also emerged over the last decade from human- and animal-sourced isolates (**Figure 1C**). The first report of *sul3* was from Swiss pigs and humans in Grape et al. (2003) and Perreten and Boerlin (2003). Since then, *sul3* has been reported in samples from a number of countries (Pan et al., 2006; Saenz et al., 2010; Tang et al., 2011; Mattiello et al., 2015; Wyrsch et al., 2015; Moran et al., 2016). These atypical class 1 integrons (**Figure 1**) are often components of larger complex resistance regions, hosted on plasmids that also carry virulence genes (Venturini et al., 2010, 2013).

Small mobile elements, including insertion sequences (IS*1*, IS*26*, IS*Ecp1c*, and IS*6100* are of note), Insertion Sequence Common Regions (ISCRs) and composite transposons formed from two or more IS elements, play an important role in reshaping the 3′-CS of clinical class 1 integrons (Daly et al., 2005; Dionisi et al., 2009; Venturini et al., 2010). The genes *blaSHV-11*, and *blaSHV-12* (coding for β-lactam resistance; Ford and Avison, 2004; Dionisi et al., 2009); *qnrB19* (coding for quinolone resistance; Dionisi et al., 2009); and *aphA1* (coding for kanamycin/neomycin resistance; Cain et al., 2010) have been shown to be flanked by direct copies of IS*26*. The mechanism of action of IS*26* has been the subject of several recent studies (Harmer et al., 2014; Harmer and Hall, 2015; He et al., 2015). These studies show that resistance loci containing IS*26* can be hotspots for the capture of further resistance genes and other genetic cargo flanked by IS*26* and demonstrates the important role of IS*26* and other mobile elements in the assembly and re-assembly of novel complex resistance loci.

Currently, genomic epidemiological surveillance relies heavily on whole genome and metagenomic sequence information generated using short-read (<400 bp) sequencing platforms. A fall in the cost of high throughput, next generation sequencing with platforms that generate short-read lengths means they have become the leading technology for characterizing microbial evolution and uncultured microbial consortia, and to catalog

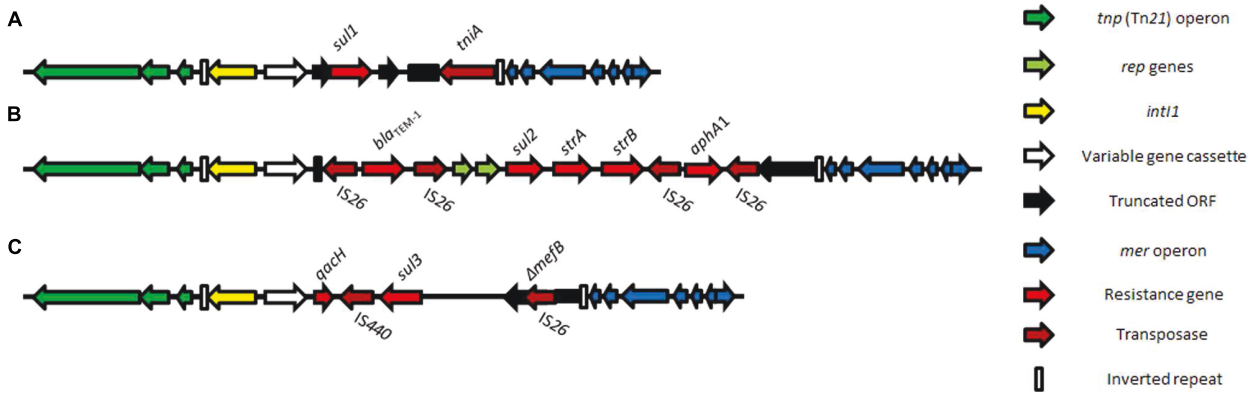

FIGURE 1 | Class 1 integron structures associated with the three sulfonamide resistance gene alleles *sul1* (A), *sul2* (B), and *sul3* (C), shown in a *Tn21* backbone.

antimicrobial resistance and virulence gene carriage. However, short-read sequencing technologies are unable to assemble complex resistance gene loci, or generate complete genome sequences, without which it is difficult to characterize mobile genetic elements rapidly and reliably. These limitations severely restrict efforts to comprehend the role of lateral gene transfer in the evolution of microbial pathogens and antimicrobial resistance, particularly in regards to the microevolutionary events that lead to the generation of novel complex resistance loci. Long-read sequencing technologies have improved significantly over the past 5 years and are beginning to address this shortfall, but are currently either cost prohibitive for large-scale genomic epidemiological surveillance or require further improvements in base call accuracy (Loman and Pallen, 2015).

Genes that code for resistance to older-generation antibiotics are often incorporated within complex resistance regions. In part, this provides a plausible explanation for why these genes persist despite enforced restrictions on prescribing of older-generation antibiotics. In the UK, strict limitations on the prescription of sulfonamide drugs were enforced in Anonymous (1995). However, functional *sul* genes persist in clinically important Gram negative bacteria and their frequency has not declined since the restriction was imposed (Enne et al., 2001; Bean et al., 2005, 2009). A similar scenario unfolded in Finland when trimethoprim was withdrawn from use for the treatment of UTI (Heikkila et al., 1990). In the Enterobacteriaceae, resistance to sulfonamides, trimethoprim, ampicillin, tetracycline, and fluoroquinolones remains high (Enne et al., 2001; Nozarian and Abdollahi, 2015; Paniagua-Contreras et al., 2015; Uzun et al., 2015; Yahiaoui et al., 2015). Carriage rates for *sul1*, *sul2,* or *sul3* genes in *E. coli* isolated from retail meat, livestock, healthy humans, and humans with clinical disease also remains a problem (Enne et al., 2001; Blahna et al., 2006; Frank et al., 2007; Altalhi et al., 2010; Bailey et al., 2010; Saenz et al., 2010; Vogt et al., 2014; Yahiaoui et al., 2015). Carriage of *sul* genes is linked with the presence of both typical and atypical class 1 integrons, including genes that code for resistance to ampicillin, streptomycin, and trimethoprim (Bettelheim et al., 2003; Bean et al., 2009; Bailey et al., 2010; Soufi et al., 2011; Ewers et al., 2014; Yahiaoui et al., 2015). Combinations of genes coding for resistance to first generation antibiotics contribute significantly to the multiple antimicrobial resistance phenotypes observed in commensal *E. coli* (Bettelheim et al., 2003; Vinue et al., 2008, 2010; Bailey et al., 2010). Genetic studies that have examined resistance to a broad spectrum of antibiotics frequently report the presence of $bla_{\mathrm{TEM-1}}$, *sul1*, *sul2*, *strAB*, *tetA(*A), and a range of *aadA* and *dfrA* genes (Soufi et al., 2011; Labar et al., 2012; Ewers et al., 2014; Yamamoto et al., 2014; Yahiaoui et al., 2015). The streptomycin resistance gene, *strAB*, and the *sul2* sulfonamide resistance gene are also spread via small plasmids such as RSF1010 (Yau et al., 2010), and IncQ plasmids that are related to RSF1010 (Tietze et al., 1989). The *strAB* gene and the adjacent inverted repeat IR_R, as seen in RSF1010, probably had their origins in Tn*5393c*, while *sul2* was recruited from a CR2-containing element (Yau et al., 2010). These molecular events describing the evolution of the *sul2-strAB* configuration observed in RSF1010 have been described previously (Yau et al., 2010). The *sul2-strAB* gene cluster and flanking sequences from RSF1010 have been captured onto Tn*21* derivative transposons, on large and small plasmid backbones, and in chromosomal islands; they are often in association with IS*26* and are distributed widely (Daly et al., 2005; Cain et al., 2010; Venturini et al., 2010, 2013; Labar et al., 2012; Reid et al., 2015; Roy Chowdhury et al., 2015).

E. coli IN INTENSIVE PRODUCTION ANIMALS

Porcine Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) are the causative agents of three distinct diseases in young pigs: neonatal, pre-weaning, and post-weaning diarrhea (Fairbrother et al., 1988; Noamani et al., 2003). These diseases vary in severity and are categorized by the age at which the pig develops symptoms. Diarrhea caused by ETEC strains, generally known as enteric colibacillosis, is a problem in all countries involved in pig production, and various methods have been employed to protect pigs from ETEC infection and any subsequent diarrheal disease. These methods include, but are not limited to, treatment with antibiotics, vaccination, and feed modulation (Fairbrother et al., 2005; Virdi et al., 2013). While these protective steps have helped reduce levels of ETEC infection, particularly in neonatal and pre-weaning colibacillosis, antimicrobial resistance has become a global challenge that is severely limiting antimicrobials as a treatment option.

Pathogenic *E. coli* express a wide variety of functional molecules including toxins, adhesins, autotransporters, and invasins (Nataro and Kaper, 1998; Kaper et al., 2004), which enable the *E. coli* cells to damage host tissues. In porcine ETEC, these molecules, referred to as virulence factors, include heat-stable (ST) and heat-labile (LT) enterotoxins, both of which can cause diarrhea via different molecular pathways within the pig's gastrointestinal tract (Nataro and Kaper, 1998). Various adhesins have evolved in ETEC and are expressed on the bacterial cell surface, allowing adhesion to the host's gastrointestinal tract. In pigs, the K88 (F4), K99 (F5), 987P (F6), F18 and F41 fimbrial adhesins all provide host intestinal cell binding, with K88, K99 and F18 fimbriae being particularly prevalent (Nataro and Kaper, 1998; Nagy and Fekete, 1999; Koh et al., 2008). Interestingly, the K88 and F18 fimbrial adhesins are often associated with pre- and post-weaning diarrhea, whilst the K99 fimbria has been associated with neonatal diarrhea in pigs (DebRoy and Maddox, 2001). These binding effects are thought to be supplemented by other common adhesins coded by genes such as *fimH* (Bouckaert et al., 2006) and *eaeH* (Sheikh et al., 2014), which are known to provide additional post-attachment binding. Chapman et al. (2006) employed multiplex PCR assays targeting 58 virulence genes to screen 52 clinical *E. coli* isolates from episodes of porcine neonatal and post-weaning diarrhea, and 23 commensal *E. coli* isolates from healthy pigs. Of the 58 genes, 17 were useful in distinguishing commensal from clinical *E. coli*, nine of which (*iha*, *hlyA*, *aidA*, *east1*, *aah*, *fimH*, $iroN_{E.coli}$, *traT*, and *saa*) were

identified for the first time in clinical porcine isolates in Australia (Chapman et al., 2006).

In an attempt to combat *E. coli* expressing these and other virulence genes, antimicrobials have been employed as both curative and prophylactic medicines on farms worldwide. This has placed a selection pressure on both targeted and surrounding microbial populations, and as such resistance to these antimicrobials has become extremely prevalent. For pathogenic *E. coli* in general, the definition and interpretation of virulence has been tempered based on results from large-scale microbial genome sequencing projects (Falkow, 1988; Pallen and Wren, 2007). ExPEC reside in the gut of their host, often in a commensal state, but have the genetic repertoire of virulence genes that enables them to cause life-threatening extraintestinal infections. Due to the degree of redundancy inherent in many ExPEC virulence genes, it remains a challenge to define virulence in ExPEC. These ExPEC-associated virulence genes have also been found in isolates sourced from humans, poultry and swine but rarely in cattle and other ruminants (Johnson et al., 2001; Tan et al., 2012; Manges and Johnson, 2015). Poultry are a well-recognized reservoir of ExPEC that have the potential to cause human disease but the role of swine in this regard has not been investigated sufficiently.

Current Status of Global Antimicrobial Use and Its Influence on the Porcine *E. coli* Resistome

Rationalization of farming practices has had profound effects on the spread of resistance to antimicrobials, and consequently both human and animal health – and the problem continues to grow (FAO, 2009). An estimated 210 million kilograms of antibiotics are produced annually in China, making it the largest producer and consumer of antibiotics globally. Approximately half of the antibiotics produced are used in livestock production (Hvistendahl, 2012; Zhu et al., 2013). China is also the largest producer and consumer of pork; 690 million pigs were produced in 2011 and 38.1 kg of pork are consumed per person per year (Krishnasamy et al., 2015), now reportedly almost double the pork consumption of the USA population. China is said to contain half the world's pig population, with five times the production rate of the USA (the former largest production center) and a higher per capita meat consumption than any developing nation other than Brazil and Latin America (FAO, 2009). Trends are similar for poultry production in China (Krishnasamy et al., 2015). An estimated 34 million kilograms of antibiotics, particularly tetracyclines, sulfonamides, macrolides, and penicillins were used in Chinese pig production during 2012 (Krishnasamy et al., 2015). Combination therapies of chlortetracycline with sulfathiazole and penicillin, and chlortetracycline with sulfamethazine and penicillin, are used widely. Other individual antibiotics that are used less extensively include tylosin, chlortetracycline, oxytetracycline, bacitracin, and bambermycin amongst others (Krishnasamy et al., 2015). Furthermore, pig production in China is responsible for the production of approximately 618 billion kilograms of manure per annum (Wang et al., 2006), much of which is contaminated with antibiotic residues and heavy metals, particularly tetracyclines, sulfonamides and copper; some residues are present at levels in the order of 100s of milligrams per kilogram (Pan et al., 2011; Qiao et al., 2012; Zhu et al., 2013). Elevated levels of zinc and arsenic have also been detected in these studies. Of 149 unique antimicrobial resistance genes 63 were detected significantly more frequently in microbes from manure at different stages of processing on three large pig production farms in China, compared with microbes in manure from control animals that received no antimicrobials; this included genes coding for resistance to antibiotics used to treat humans (Zhu et al., 2013). Samples of pig manure from China were also rich in microbes carrying transposase genes, notably those belonging to the IS*6*-family, predominantly IS*26* transposase (Zhu et al., 2013).

In the USA, estimates suggest that the usage of tetracyclines is as high as in China, both in the combinations described above, and as stand-alone treatments. Additionally, bacitracin, carbadox, lincomycin, neomycin, penicillin, tiamulin, tilmicosin, tylosin, and viginiamycin have all been used in the USA pig production industry (Apley et al., 2012). The USA and China combined produce the majority of the world's pigs through intensive farming practices (FAO, 2009), with China producing up to five times more than the USA (Krishnasamy et al., 2015). However, other countries also have pig production systems that rely heavily on antibiotic usage. In Alberta, Canada, the same suite of antibiotics is used as in the USA, with the addition of dimetridazole (Rajic et al., 2006). A similar pattern of use has also emerged in an Australian survey of pig production, with the use of apramycin and neomycin, penicillins, macrolides, sulfonamides, tetracyclines, lincomycin and spectinomycin, tiamulin, olaquindox, ceftiofur, and dimetridazole reported (Jordan et al., 2009). A number of these antibiotics, including lincomycin, spectinomycin, and dimetridazole are also used in the treatment of infectious human diseases. In an Australia-wide study of 114 porcine-derived *E. coli*, resistance to numerous antibiotics was common: tetracycline (88.6%), ampicillin (71.05%), trimethoprim/sulfamethoxazole (67.5%), streptomycin (69.3%), chloramphenicol (44.74%), neomycin (35.96%), apramycin (34.21%), gentamicin (28.95%), florphenicol (26.32), cefalotin (24.56%), and spectinomycin (21.93%). Resistance to imipenem and amikacin was not detected (Abraham et al., 2015). Of the 114 isolates evaluated 79% were classified as resistant to antibiotics from three or more different classes. Resistance to extended-spectrum cephalosporins (3%) and fluoroquinolones (1%) was detected in isolates belonging to some *E. coli* lineages (ST117, ST744, ST10, and ST1) albeit infrequently (Abraham et al., 2015).

Colistin is a polymyxin antimicrobial used to treat extensively drug-resistant Gram negative infections. It was first used in the 1950s but its use in humans has declined due to concerns about nephrotoxicity and neurotoxicity (Nation and Li, 2009). China, India, and Europe still use colistin extensively for agricultural purposes. In Europe, colistin is used to treat enterobacterial infections in pigs (neonatal diarrhea), poultry (colibacillosis), cattle (neonatal diarrhea in veal calves), sheep, and goats (Timmerman et al., 2006; Pardon et al., 2012); in 2010 it was the fifth most commonly sold antimicrobial after tetracycline,

TABLE 1 | Molecular characterisation of published antimicrobial resistant, porcine-derived *Escherichia coli* isolates.

Country	Resistance genes detected			Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference
	Integron associated	Resistance	Beta-lactamases						
Argentina	*intI2*			Yes	Serogrouping			Yes	Moredo et al., 2007, 2015; Pantozzi et al., 2010
Australia	*intI1, intI2, aadA, dfrA, cmlA, cat1, cat2, ereA, aacC4*	*sul1, sul2, sul3, strA, strB, aphA1, tetA, tetB, tetC*	bla_{TEM}, bla_{CTX-M}, bla_{CMY}	Yes	RAPD Serogrouping Phylotyping	ST100 ST29 ST90 ST746	Replicon	Yes	Chapman et al., 2006; Wu et al., 2007; Smith et al., 2010; Abraham et al., 2012, 2014, 2015
Austria			bla_{CTX-M}, bla_{TEM}, bla_{SHV}	Yes			Replicon		Mayrhofer et al., 2004, 2006; Petternel et al., 2014
Brazil				Yes	ERIC-PCR Serogrouping			Yes	Carvalho et al., 1991; da Costa et al., 2008; Borges et al., 2012; Morales et al., 2012
Canada	*aadA, dfrA, aadB, aacC4, cmlA*	*sul1, sul2, sul3, strA, strB, catA1, aphA, floR, tetA, tetB, tetC*	bla_{TEM}, bla_{OXA}, bla_{CMY}	Yes	ERIC-PCR BOX-PCR Serogrouping				Maynard et al., 2003; Lu et al., 2005; Travis et al., 2006; Duriez and Topp, 2007; Kozak et al., 2009a,b; Rosengren et al., 2009; Deckert et al., 2010; Sheikh et al., 2012
China	*intI1, aadA, dfrA*	*sul1, sul2, oqxAB, aac(6)-Ib-cr, qnrA, qnrB, qnrS, qepA, fosA, floR*	bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{CMY}	Yes	Serogrouping XbaI PFGE ERIC-PCR Phylogrouping	ST10 ST206	Replicon	Yes	Yang et al., 2004, 2014; Yue et al., 2008; Ho et al., 2009, 2013; Ma et al., 2009; Huang et al., 2012; Tian et al., 2012; Liu et al., 2013; Meng et al., 2014; Xu et al., 2014; Gao et al., 2015; Liao et al., 2015
Denmark	*intI1, aacC4*	*sul1, sul2, sul3*	bla_{CTX-M}, bla_{TEM}, bla_{CMY}, bla_{SHV}	Yes	XbaI PFGE Phylogrouping Serogrouping	ST10 ST23	Replicon	Yes	Olesen et al., 2004; Hammerum et al., 2006, 2014; Jensen et al., 2006; Cavaco et al., 2008; Trobos et al., 2009; Jakobsen et al., 2010a,b; Wu et al., 2010; Agerso et al., 2012; Agerso and Aarestrup, 2013; Hansen et al., 2013; Herrero-Fresno et al., 2015
Germany	*intI1, intI2, aadA, dfrA, aadB*	*sul1, sul2, sul3, strA, strB, tetA, tetB, tetM*	bla_{TEM}, bla_{CTX-M}, bla_{OXA}	Yes	XbaI PFGE Phylogrouping Serotyping	ST10 ST58 ST167 ST410	Replicon pMLST	Yes	Kadlec and Schwarz, 2008; Schwaiger et al., 2010; Bednorz et al., 2013; Schierack et al., 2013; Fischer et al., 2014; Dahms et al., 2015; Garcia-Cobos et al., 2015

(Continued)

TABLE 1 | Continued

Country	Resistance genes detected			Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference
	Integron associated	Resistance	Beta-lactamases						
India			bla_{CTX-M}, bla_{SHV}, bla_{TEM}	Yes	Serogrouping			Yes	Murugan et al., 2012; Rajkhowa and Sarma, 2014; Samanta et al., 2015
Italy			bla_{CTX-M}, bla_{TEM}	Yes					Stefani et al., 2014; Luppi et al., 2015
Japan	*intI1, aadA, dfrA, cmlA, aacA4*	*cat1, tetA, tetB, tetC*		Yes	PFGE Phylogrouping			Yes	Kijima-Tanaka et al., 2003, 2005; Asai et al., 2005; Harada et al., 2005, 2006; Kumai et al., 2005; Katsunuma et al., 2008; Kojima et al., 2009; Makita et al., 2016
Korea	*intI1, aadA, dfrA, aadB, cmlA*	*aphA, qnrB, qnrS, aac(6)-Ib-cr, tetA*	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	Yes	PFGE HFERP Phylogrouping Serogrouping			Yes	Choi et al., 2002; Han et al., 2002; Kang et al., 2005; Lim et al., 2007, 2009; Rayamajhi et al., 2008; Unno et al., 2010, 2011; Tamang et al., 2012; Lee et al., 2014
Lithuania	*intI1, intI2, aadA, dfrA, estX, sat2*			Yes					Povilonis et al., 2010; Seputiene et al., 2010
Nigeria		*qnrS*	bla_{TEM}	Yes	ERIC-PCR PFGE Serogrouping		pMLST	Yes	Ojo et al., 2010; Fortini et al., 2011; Adenipekun et al., 2015
Norway	*intI1, intI2, aadA, dfrA, catB, sat1*	*sul1, sul2, strA, strB, tetA, tetB, tetC*	bla_{TEM}, bla_{OXA}	Yes	PFGE	ST10 ST168			Sunde et al., 1998; Sunde and Sorum, 1999; Brun et al., 2002; Sunde, 2005; Sunde and Norstrom, 2006; Trobos et al., 2009
Portugal	*intI1, intI2, dfrA, aadA, estX, sat2, catB*	*strA, strB, qnrB, tetA*	bla_{TEM}, bla_{CTX-M}, bla_{SHV}	Yes	XbaI PFGE	ST10	Replicon pMLST RFLP		Machado et al., 2008; Goncalves et al., 2010; Jones-Dias et al., 2013; Ramos et al., 2013; Rodrigues et al., 2013
Spain	*intI1, intI2, aadA, dfrA, cmlA, sat2, linF*	*sul1, sul3, pmrA, pmrB*	bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA}	Yes	Phylogrouping REP-PCR ERIC-PCR				Brinas et al., 2002; Sabate et al., 2008; Escudero et al., 2010; Marchant and Moreno, 2013; Marchant et al., 2013; Ojer-Usoz et al., 2013; Quesada et al., 2015

(Continued)

TABLE 1 | Continued

Country	Resistance genes detected			Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference
	Integron associated	Resistance	Beta-lactamases						
Switzerland	*intI1, aadA*	*sul1, sul2, sul3, strA, strB, tetA, tetB, tetC*	bla_{CTX-M}	Yes	PFGE Serogrouping	ST3 ST529		Yes	Stephan and Schumacher, 2001; Lanz et al., 2003; Perreten and Boerlin, 2003; Zweifel et al., 2006; Stannarius et al., 2009; Endimiani et al., 2012
Taiwan	*intI1, aadA, dfrA*	*sul1, fosA*	bla_{TEM}, bla_{CTX-M}, bla_{CMY}	Yes	XbaI PFGE	ST744 ST2310			Hsu et al., 2006; Tseng et al., 2015
Thailand	*intI1, aadA, dfrA, linF, sat1*	*sul1*	bla_{CTX-M}, bla_{TEM}	Yes	Phylogrouping			Yes	Hanson et al., 2002; Phongpaichit et al., 2007; Mathew et al., 2009; Lay et al., 2012; Changkaew et al., 2015
UK	*aadA*	*sul2, floR*	bla_{CTX-M}, bla_{SHV}	Yes			pMLST		Jackson, 1981; Enne et al., 2008; Taylor et al., 2008; Freire Martin et al., 2014; Randall et al., 2014; Cheney et al., 2015
USA	*intI1, aadA, dfrA, cmlA, sat1*	*cat2, flo, tetA, tetB, tetC, tetM*		Yes	Serogrouping XbaI PFGE Phylogrouping		Replicon	Yes	Bischoff et al., 2002; Schroeder et al., 2002; Bryan et al., 2004; Singh et al., 2005; Lindsey et al., 2011; Xia et al., 2011; Ju et al., 2012; Tadesse et al., 2012; Roug et al., 2013

penicillins, sulfonamides, and macrolides (European Medicines Agency [EMA], 2013). Colistin is also used in aquaculture (Xu et al., 2012). The recent emergence of colistin resistance in commensal porcine *E. coli* isolates in China via the acquisition of plasmid-mediated *mcr-1* gene has serious implications for the treatment of pan-drug-resistant Gram-negative bacteria, particularly isolates of the extremely drug resistant *Acinetobacter baumannii* and carbapenemase-resistant *Klebsiella pneumoniae* (Liu et al., 2015). The seriousness of this finding is clear due to: (i) high *in vitro* transfer rates among *E. coli* by conjugation and the ability of the *mcr-1* plasmid to transfer and be maintained in globally dispersed pathogenic clones such as *E. coli* ST131, *K. pneumoniae* ST11, and *Pseudomonas aeruginosa*; (ii) the high frequency of carriage of *mcr-1* in *E. coli* from livestock and in retail meats sourced from southern China; (iii) detection of *mcr-1*-like genes in Malaysia (Liu et al., 2016). The apparent high rates of carriage of *mcr-1* in isolates of *E. coli* from animals and the apparent low incidence in human-derived *E. coli* populations has led to speculation that the heavy use of colistin in agriculture in China has been the main driver for the emergence of plasmid-mediated colistin resistance (European Medicines Agency [EMA], 2013; Liu et al., 2016).

Genes conferring antimicrobial resistance have been identified in *E. coli* from diverse pig-related sources (**Table 1**). The genes bla_{TEM}, *sul1/2/3*, *aadA*, *dfrA*, and *tetA/B/C* feature prominently. In order, these genes confer resistance to penicillin, sulfonamides (such as sulphamethoxazole), streptomycin and spectinomycin, trimethoprim, and tetracyclines; all of these are used in the treatment of animals. Additional clinically important cases of resistance are also identified sporadically in the literature, specifically to chloramphenicol (*cmlA, cat*), streptothricin (*estX/sat*), florfenicol (*flo*), quinolones (*qnr*), streptomycin (*strAB*), and fosfomycin (*fosA*). There are also a number of known resistance mutations in the *gyr* and *pmr* genes that confer elevated resistance to quinolones and colistin respectively. Already the presence of these genes together in the same isolate and from disparate countries indicates they are both genetically linked in antimicrobial resistance-encoding loci, and globally disseminated. Detection of the genes bla_{OXA}, bla_{SHV}, and $bla_{\text{CTX-M}}$ coding for the extended-spectrum beta-lactamases, which are relevant to human medicine, is also of concern (Randall et al., 2014). Of note, these data were collected from a variety of publications for each country, and show some selection bias concerning the genes detected. Whole genome sequencing has become a tool to rectify this bias, and will continue to feature in the detection and characterisation of antimicrobial resistance, its evolution and dissemination (Salipante et al., 2015).

Much needs to be done to reduce the reliance on antimicrobials in food production. There is a growing body

TABLE 2 | Published complete porcine *E. coli* genome and plasmid sequences.

Name	Country	Type	Size (bp)	Achtman MLST/Plasmid Inc.	Disease association/source	GenBank accession	Reference
pND11_107	USA	Plasmid	107,138	I	Neonatal diarrhea	HQ114281	Johnson et al., 2011
pPWD4_103	USA	Plasmid	103,297	I	Post-weaning diarrhea	HQ114284	
pUMNF18_69	USA	Plasmid	69,065	I		CP002891	
pND12_96	USA	Plasmid	92,290	I	Neonatal diarrhea	HQ114282	
UMNK88	USA	Chromosome	5,186,416	ST165 cplx	Post-weaning diarrhea	CP002729	Shepard et al., 2012
pUMNK88_Hly	USA	Plasmid	65,549			CP002733	
pUMNK88_Ent	USA	Plasmid	81,475			CP002732	
pUMNK88_K88	USA	Plasmid	81,883			CP002730	
pUMNK88_91	USA	Plasmid	90,868	I		CP002731	
pUMNK88	USA	Plasmid	160,573	A/C		HQ023862	
pTC1	Hungary	Plasmid	91,019		Weaning diarrhea	CP000913	Fekete et al., 2012
pHK23a	China	Plasmid	73,607	FII	Multi-drug resistant isolate	JQ432559	Ho et al., 2013
pSCEC2	China	Plasmid	135,615	A/C	Maxillary lymph node	KF152885	Zhang et al., 2014
pIFM3804	UK	Plasmid	104,399	I		KF787110	Freire Martin et al., 2014
PCN033	China	Chromosome	4,987,957	ST5147	Extraintestinal infection (brain)	CP006632	Liu et al., 2015
PCN033p1	China	Plasmid	3,319			CP006633	
PCN033p2	China	Plasmid	4,086			CP006634	
PCN033p3	China	Plasmid	161,511			CP006635	
PCN061	China	Chromosome	4,603,777	ST46	Extraintestinal infection (lung)	CP006636	
PCN061p1	China	Plasmid	2,014			CP006637	
PCN061p2	China	Plasmid	5,754			CP006638	
PCN061p3	China	Plasmid	6,222	Q1/FIB/FIC/FII		CP006639	
PCN061p4	China	Plasmid	34,692			CP006640	
PCN061p5	China	Plasmid	103,644	I		CP006641	
PCN061p6	China	Plasmid	145,722	N/FIB/X1		CP006642	
pFSEC-01	China	Plasmid	33,885			KR779901	Zhang et al., 2015
pSD11	China	Plasmid	37,672	X4		KM212169	Sun et al., 2015

of evidence suggesting that food production may be a key driver in the evolution of multiple drug resistance in a number of bacterial species of clinical significance to human health. Livestock represent a reservoir of *E. coli* pathotypes with resistance to more than one antimicrobial. While pigs and poultry are significant sources of ExPEC, cattle are the major reservoirs for Shiga toxin producing *E. coli* (EHEC; Manges and Johnson, 2015) and atypical EPEC (Hornitzky et al., 2005; Dawes et al., 2010). The consequences for the environment of large-scale intensive animal production and a growing human population, estimated to be 9 billion by 2050 (United Nations, 2015) are not fully understood. Much remains to be done to determine how antimicrobial resistance genes are assembled on mobile genetic elements and to identify their unique genetic features. Diagnostics tests that target unique features will be useful in tracking the movement of mobile elements that are carrying complex resistance loci and to identify hotspots where they reside. Metagenomic approaches will have a major role to play in understanding the scale of the antimicrobial resistance problem, detecting and tracking emerging pathogens, and the role of gut flora in human and animal health. Studies on microbial populations from the gastrointestinal tracts of intensively reared livestock are needed to identify and characterize novel pathotypes that will undoubtedly emerge in response to the selection pressures that are commonplace in large-scale animal production facilities. Genomic surveillance studies will have an important role to play in identifying complex resistance loci, particularly how they move through different production systems and natural ecosystems (aquatic and terrestrial), and the impact they have on the carriage of multiple antimicrobial resistance genes in bacteria that colonize and infect humans and animals. Currently, there are just three complete porcine-derived *E. coli* chromosomal sequences published, as well as a variety of complete plasmid sequences, the majority of which have been derived from the study of isolates with closed genome sequences (**Table 2**). This is at odds with the significant role that intensive pig production plays in the release of antibiotic residues into the environment, and we expect more focus to be placed on the sequencing of pig-associated *E. coli* isolates in the near future.

AUTHOR CONTRIBUTIONS

EW drafted an early version of the manuscript and produced the figure and tables. PR and IC contributed to several sections in the manuscript. TC and JH edited drafts of the manuscript. SD wrote most the manuscript.

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Chapter 3: Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS*26*

Author contribution statement: I (Ethan R. Wyrsch) was a primary author of this publication, performing strain handling, DNA extraction, genome assembly and analysis on the F1 strains. I also performed phylogenetic analysis between F1 and F2 and assisted in drafting and editing the final manuscript. Cameron J. Reid performed the DNA extraction and genomic analysis on the F2 collection, performed genomic comparisons between F1 and F2, generated figures and drafted the manuscript. Tiziana Zingali performed antibiotic sensitivity testing, Michael Liu prepared sequencing libraries and Aaron E. Darling generated genome assemblies. Toni A. Chapman provided samples. Piklu Roy Chowdhury assisted in genome analysis. Steven P. Djordjevic, Piklu Roy Chowdhury and Toni A. Chapman conceived the study, with Steven P. Djordjevic also providing funds and assisting in manuscript drafting. Each author contributed to the final editing.

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Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS*26*

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Abstract

Porcine faecal waste is a serious environmental pollutant. Carriage of antimicrobial-resistance genes (ARGs) and virulence-associated genes (VAGs), and the zoonotic potential of commensal *Escherichia coli* from swine are largely unknown. Furthermore, little is known about the role of commensal *E. coli* as contributors to the mobilization of ARGs between food animals and the environment. Here, we report whole-genome sequence analysis of 103 class 1 integron-positive *E. coli* from the faeces of healthy pigs from two commercial production facilities in New South Wales, Australia. Most strains belonged to phylogroups A and B1, and carried VAGs linked with extraintestinal infection in humans. The 103 strains belonged to 37 multilocus sequence types and clonal complex 10 featured prominently. Seventeen ARGs were detected and 97 % (100/103) of strains carried three or more ARGs. Heavy-metal-resistance genes *merA, cusA* and *terA* were also common. IS*26* was observed in 98 % (101/103) of strains and was often physically associated with structurally diverse class 1 integrons that carried unique genetic features, which may be tracked. This study provides, to our knowledge, the first detailed genomic analysis and point of reference for commensal *E. coli* of porcine origin in Australia, facilitating tracking of specific lineages and the mobile resistance genes they carry.

DATA SUMMARY

One hundred and forty-three whole-genome sequences of porcine faecal *Escherichia coli* sequenced in this project have been deposited at the European Molecular Biology Laboratory (EMBL) European Nucleotide Archive under study accession number PRJEB21464 [https://www.ebi.ac.uk/ena/data/view/PRJEB21464]. For individual sample accession numbers, please refer to Table S1 (available in the online version of this article). Further strain data is available in Tables S2–S6.

INTRODUCTION

Escherichia coli is the most frequently isolated Gram-negative pathogen affecting human health [1]. Isolates are frequently resistant to multiple antibiotics and modelling studies forecast that multidrug resistant (MDR; resistant to three or more classes of antimicrobials) *E. coli* infections will account for 30 % of 10 million fatal MDR infections annually by 2050 [2]. In addition to the pathogenic variants, commensal *E. coli* comprise an important component of the gut microbiota. *E. coli* are shed into the environment in high numbers. For example, each gram of faeces from commercially reared pigs contains between 10^4 and 10^8 *E. coli* [3]. It is important to understand the characteristics of these *E. coli* given the huge quantities of faeces generated and disseminated by intensive pig production. China, the world's largest producer of swine, produces an estimated 0.618 billion to 1.29 billion metric tonnes of swine faeces each year [4, 5].

Pathogenic *E. coli* are broadly divided into intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). ExPEC have a faecal origin, having persisted asymptomatically in the gut before opportunistically colonizing extraintestinal sites where they cause a diverse range

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Abbreviations: APEC, avian pathogenic *Escherichia coli*; ARG, antimicrobial-resistance gene; EHEC, enterohaemolytic *Escherichia coli*; EMAI, Elizabeth MacArthur Agricultural Institute; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; ExPEC, extraintesintal pathogenic *Escherichia coli*; IPEC, intestinal pathogenic *Escherichia coli*; IS, insertion sequence; MDR, multidrug resistant; MLST, multilocus sequence typing; VAG, virulence-associated gene.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Six supplementary tables are available with the online version of this article.

of diseases, including urinary-tract infections (UTI), pyelonephritis, wound infections, sepsis and meningitis [6]. ExPEC are thought to have foodborne reservoirs and may enter the food chain via a number of sources [7–11]. The zoonotic potential of commensal porcine *E. coli* as a source of ExPEC that cause disease in humans is unknown. ExPEC cannot be reliably detected in a diagnostic test as they are yet to be shown to possess unique identifying features relative to other pathotypes of *E. coli* [12]. Instead, as we aim to do here, whole-genome sequencing can be used to discriminate strains indistinguishable by other methods, and identify any genetic relationships between *E. coli* strains isolated from pigs and humans.

Horizontal gene transfer, mediated by mobile genetic elements, plays an important role in the evolution of *E. coli*. Commensal or pathogenic bacteria may, in a single horizontal gene transfer event, acquire a mobile genetic element carrying multiple antimicrobial-resistance genes (ARGs), virulence-associated genes (VAGs) and other genetic cargo that encode traits that offer a niche advantage [13–17]. The release of MDR commensal *E. coli* into the environment, such as when pig faeces are used as manure, facilitates horizontal transfer of resistance and virulence genes into other microbial communities in a manner that is poorly understood. ARGs cluster on mobile genetic elements and form complex resistance regions that are often independently mobile. Indirect selection pressure can, in the absence of antibiotic use, lead to the persistence of transferred genes. For example, heavy metals such as copper and zinc in feed formulations for food animals select for ARGs that co-localize with metal-resistance genes [18, 19]. Selection pressure afforded by any one of a number of antibiotics and heavy metals (zinc, cadmium, mercury) that contaminate faecal waste or those used in food-producing and hospital environments is sufficient to select for the retention and spread of complex resistance regions [20, 21]. Understanding of how ARGs assemble on mobile genetic elements, and the extent to which these then traffic through human, food animal and environmental reservoirs, remains limited.

Class 1 integrons are a reliable proxy for the presence of multiple ARGs within bacteria in clinical and veterinary settings [22]. They are gene capture and expression elements that can integrate AMR gene cassettes from the environmental resistome and express them via a promoter residing in the class 1 integrase gene. They are often mobilized by mercury-resistance transposons belonging to the Tn*21* family, which have been disseminated globally on a wide variety of conjugative plasmid backbones [23]. Resistance genes can also be acquired, lost and rearranged in bacteria by genetic events that involve insertion sequences (ISs) such as IS*26*, IS*Ecp1*and IS*CR1* [24–27]. IS*26* is prominent in this regard due to its unique mechanisms of transposition (conservative and replicative), ability to recognize itself, lack of copy number control and ability to mobilize a wide range of ARGs [15, 26, 28–30]. Furthermore, IS*26* is recognized to play a key role in: (i) the evolution of plasmids and genomic islands that carry combinations of VAGs and ARGs [16, 17, 31, 32]; (ii) driving the formation of cointegrate plasmids encoding VAGs and ARGs [33]; and (iii) initiating deletions in large multidrug-resistance plasmids that enhance plasmid stability and expand host range [34].

IMPACT STATEMENT

The data presented in this manuscript describes for the first time, to our knowledge, a genomic analysis of commensal *Escherichia coli* from commercial swine-production facilities in Australia. Pig production routinely involves antibiotic use for disease treatment and prophylaxis, and feed additives containing zinc and heavy metals to control infectious disease. The study is significant because it reports phylogenetically diverse *E. coli* that are multidrug resistant (MDR; resistant to three or more classes of antimicrobials) and carry class 1 integrons altered by IS*26*. This initial descriptive work is important as a basis for the analysis of porcine faecal *E. coli* in all countries that produce swine commercially, due to the scale of global pork production and the vast quantities of faecal waste that are used as manure. The contamination of this waste with MDR bacteria, antimicrobial-resistance genes and unmetabolized antimicrobial residues is a concern. It is necessary to characterize food-chain-associated micro-organisms, such as *E. coli*, with zoonotic potential and multiple resistance genes, as they may pose a threat to public health.

Infectious-disease management relies on the surveillance of antimicrobial resistance and emerging pathogens using a One Health approach. There is currently no published data available that records whole-genome-sequence-based phylogeny, or ARG or VAG carriage in commensal *E. coli* from Australian pigs, and only one comparable study is available from overseas [35]. Here, for the first time, to our knowledge, we present whole-genome sequence analysis of 103 class 1 integron-positive commensal *E. coli* from pigs commercially reared in Australia. We present data characterizing their phylogenetic diversity, carriage of VAGs and ARGs, and an analysis of the class 1 integrons they carry.

METHODS

Management of farms and animals

The study was conducted using *E. coli* sourced separately from two pig-production farm systems located approximately 250 km apart. Farms were designated descriptors F1 and F2. Isolate numbers consist of farm number, a pig number and a letter designating a single isolate from that pig (i.e. F1_404D indicates farm 1, isolate D from pig 404). At both farms, pigs were intensively housed and kept in total confinement. Both farms have used neomycin in the past for the treatment of diarrhoeal disease. No antibiotics were

being used during the first sampling time at F1; however, the pigs sampled at the second sampling time had received a course of neomycin (see below). No antibiotics had been administered to the pigs at F2 prior to sampling.

E. coli strains used in the study

E. coli isolates were collected via rectal-swab sampling of pigs between 19 and 30 days of age. At farm 1, rectal swabs were collected in May 2007 from pigs during an outbreak of diarrhoeal disease, but prior to treatment with neomycin. These pigs were subsequently removed from the shed. The causative agent of the outbreak was unknown. A new batch of healthy sows and their piglets were transferred to this shed and the sows were given neomycin in-feed. Once the piglets were weaned they also received neomycin in-feed for 7–10 days. The second sampling occurred on these piglets in June 2007 after the course of antibiotics. At farm 2, rectal swabs were performed on healthy weaners that were not treated with antibiotics.

E. coli were isolated at the Elizabeth MacArthur Agricultural Institute (EMAI), Australia. Up to ten *E. coli* colonies were selected from individual pigs using MacConkey agar. The total collection from farm 1 was 164 isolates from 33 pigs, whilst from farm 2 was 171 isolates from 23 pigs. All strains were screened by PCR for the class 1 integrase gene *intI1*. This screening indicated that 117/164 (71 %) *E. coli* from farm 1 and 168/171 (98 %) from farm 2 carried *intI1*. Initially, 50 *intI1*-positive isolates from F1 and 100 *intI1*-positive isolates from F2 were selected for whole-genome sequencing. Two enterotoxigenic *E. coli* (ETEC) strains, M10 and ETEC286_3, which were submitted to the EMAI from Australian veterinary services, as clinical, pig-derived strains, were also sequenced and included in the phylogenetic analysis as reference strains.

Storage

All strains were freshly cultured in LB medium and frozen as glycerol stocks made using 500 µl M9 salts solution and 500 µl 50 % (v/v) glycerol and stored at −80 °C. All strains were cultured in LB medium prior to isolation of total cellular DNA used for sequencing.

DNA extraction, whole-genome sequencing and assembly

Total DNA was extracted using the ISOLATE II genomic DNA kit (Bioline) following the manufacturers standard protocol for bacterial cells and stored at −20 °C. Whole-genome sequencing libraries were prepared from separate aliquots of sample DNA using the Illumina Nextera DNA kit with modifications. In brief, the DNA was first quantified using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific). All sample DNA concentrations were standardized to equal concentration to achieve uniform reaction efficiency in the tagmentation step. Standard Illumina Nextera adaptors were used for sample tagmentation. The PCR-mediated adapter addition and library amplification was carried out using customized indexed i5 and i7 adaptor primers (IDT), which were developed based on the standard Nextera XT indexed i5 and i7 adapters (e.g. N701–N729 and S502–S522). Libraries were then pooled and size selected using SPRI-Select magnetic beads (Beckman Coulter). Finally, the pooled library was quality checked and quantified on an Agilent Bioanalyzer 2100 using the DNA HS kit (Agilent). Whole-genome sequencing for the majority of F1 strains and ETEC strains was performed as previously reported [36], using an Illumina MiSeq sequencer and MiSeq V3 chemistry. Whole-genome sequencing of the remaining F1 and F2 strains was performed using an Illumina HiSeq 2500 v4 sequencer in rapid PE150 mode. Sequence read quality was initially assessed using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Illumina raw reads passing quality control were assembled into draft genome sequences using the A5 assembly pipeline, version A5-miseq 20140604 [37]. Genome sequences have been deposited in the European Molecular Biology Laboratory European Nucleotide Archive with study accession number PRJEB21464. Accession numbers for each sample are listed in Table S1.

Strain selection

Sequence data was successfully generated for 141 strains and these were screened by BLAST for *intI1,* ARGs and VAGs, and subjected to Phylosift analysis as described below. These analyses indicated 12 strains were negative for *intI1* and that a number of clones were isolated from individual pigs. We, therefore, excluded *intI1*-negative strains and selected representatives of the clonal isolates, thereby excluding a further 26 strains. The subset of strains that were sequenced were identified as F1+F2 (n=103 from 42 pigs). This subset consisted of 35 strains from 21 pigs sampled at farm 1 and 68 strains from 21 pigs sampled at farm 2; among the F1 strains, 17 were disease-associated strains from 12 pigs (isolate numbers 1–30, designated 'disease' in Tables S1–S5) and 18 were isolated from 11 healthy pigs (isolate numbers 365–409, designated 'healthy' in Tables S1–S5). Only 11 isolates in the collection carried toxin genes (*eltA*, n=2; *eltB*, n=2; *stA*, n=0; *stB*, n=11) associated with porcine ETEC and no ETEC adhesins were detected. Notably, only five of these were from diseased pigs, whilst six were from healthy pigs. This highlights the role that host factors, such as stress and immune health, play in the manifestation of pre- and post-weaning diarrhoea in pigs and we, therefore, argue that this collection should be considered commensal.

Assembly statistics

Comprehensive assembly statistics for 143 sequenced porcine-derived *E. coli,* (141+2 ETEC) are available in Table S1. Isolates not included in this study are highlighted grey. The number of scaffolds per genome ranged from 29 to 1571, with a mean of 235. Each genome sequence had a median sequencing coverage of at least 20 ×, with a maximum of 94× and mean of 54×.

Phenotypic resistance testing

F1 strains were tested at the EMAI using the calibrated dichotomous susceptibility (CDS) test for resistance to 12 antibiotics [38]. The following were tested: ampicillin

(25 µg), cefoxitin (30 µg), nalidixic acid (30 µg), ciprofloxacin (2.5 µg), imipenem (10 µg), sulphafurazole (300 µg), trimethoprim (5 µg), tetracycline (10 µg), neomycin (30 µg), gentamicin (10 µg), azithromycin (15 µg) and chloramphenicol (30 µg). F2 strains were tested for resistance to antibiotics at the i3 Institute, University of Technology Sydney, Australia, using the same method and panel of antibiotics as the F1 collection. F2 strains were also tested with streptomycin (25 µg) and kanamycin (50 µg) (Table S2).

Gene identification and serotyping

Resistance, virulence and plasmid-associated genes were identified using local BLASTN v2.2.30+ [39] searches with an *E* value of 1.0×10^{-3} (Tables S3–S5). The gene databases used were ResFinder, PlasmidFinder, ISFinder, SerotypeFinder and VirulenceFinder [Data references 1–5] [40–44]. Our virulence database was supplemented with additional virulence genes from GenBank, available in Table S6. Genes were considered present if the subject nucleotide sequence was >90 % identical over 100 % of the length of the query sequence. BLAST hits with >90 % identity but covering less than 100 % of the query were considered positive if they were truncated by a scaffold break or insertion. Integrons were characterized in SnapGene (GSL Biotech) using BLASTN output. The collection was then retroactively screened for characterized integrons using BLASTN. Where strains carry two *intI1* genes, *de novo* assembly software is unable to assemble the two complete integrons with Illumina short read data, as it cannot determine which cassette array belongs to which *intI1* copy. The presence of two integrons in strains in this collection was, therefore, initially inferred by BLAST identification of their cassette arrays and downstream regions (e.g. IS*26* deletion signatures), and then confirmed by read-mapping using Bowtie2 and Tablet [45, 46].

Phylogrouping and multilocus sequence typing (MLST)

E. coli phylogroups were determined using the scheme published by Clermont *et al.* [47]. The genes *chuA* (GenBank accession no. U67920.1), *yjaA* (GenBank accession no. NC_000913.3) and the DNA fragment TspE4.C2 (GenBank accession no. AF222188.1) were sourced from GenBank and identified *in silico* using BLASTN. MLST was performed *in silico* using the PubMLST database (http://pubmlst.org/) and the Achtman *E. coli* MLST scheme (http://mlst.warwick.ac.uk/mlst/).

Phylogenetic analyses

Maximum-likelihood phylogenetic distances between genomes were analysed using the PhyloSift pipeline [48], and a tree was generated using FastTree2 [49]. The tree was visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and iTOL (https://itol.embl.de/). The FastTree2 protocol was modified to resolve short branches, as described previously [50].

RESULTS

Our study collection consisted of 103/335 (31 %) strains of *E. coli* isolated from rectal swabs of pigs from two farms in New South Wales, Australia, that were PCR-positive for the class 1 integron integrase gene, *intI1*. Initial screening indicated that 117/164 (71 %) *E. coli* from farm 1 and 168/171 (98 %) from farm 2 carried *intI1*.

Population structure of *E. coli* isolated from porcine rectal swabs

Strains in our study collection were classified by phylogrouping, *in silico* MLST and *in silico* serotyping. The majority of the strains in our study collection 74/103 (72 %) belonged to phylogroup A, while the remainder belonged to phylogroup B1 (18; 17 %), phylogroup B2 (5; 5 %) and phylogroup D (6; 6 %).

We identified 37 distinct sequence types, 21 of which were previously isolated from swine, as reported by the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli; accessed June 2017). Only seven sequence types were common to both F1 and F2. The most prominent sequence types were ST10, ST361, ST641, ST542, ST48 and ST218. Twenty-five sequence types were represented by a single isolate. Six strains with a single SNP in a reference allele were assigned putative sequence types (Fig. 1, Table S3; denoted by an asterisk). A designation of non-typable was given to the five remaining strains for which one or more alleles could not be determined.

In silico O:H typing using SerotypeFinder predicted 47 serotypes for 85 strains. The remaining 18 strains were O-nontypable with 10 different H types (Fig. 1, Table S3). In general, strains of any given sequence type carried the same O:H alleles, though intra-sequence type variability was observed among eight sequence types (ST10, ST48, ST218, ST542, ST641, ST302, ST4630 and ST1437).

Phylogenetic analysis

To determine genetic relatedness, we used PhyloSift, FastTree2, FigTree v1.4.2, and iTOL to generate and visualize a mid-point rooted, maximum-likelihood phylogenetic tree containing the F1+F2 pig *E. coli* draft whole-genome sequences, two ETEC strains (ETEC286_3 and ETECM_10) and four pig-pathogenic *E. coli* complete genome sequences [*E. coli* UMNK88 (NC_017641.1), UMNF18 (NZ_AGTD01000001.1), PCN033 (NZ_CP006632.1) and PCN061 (NZ_CP006636.1)] (Fig. 1). Tree topology was highly congruent with Achtman MLST and *in silico* serotyping, grouping strains by sequence type, and then further by serotype. Clade structure was generally congruent with phylogroup analyses; however, seven strains belonging to phylogroups B2 and D formed a separate clade. We identified three major clades, with the seven B2/D phylogroup strains forming clade 1. Clade 2 consisted almost exclusively of phylogroup B1 strains, ST641 was the dominant sequence type; however, one phylogroup A strain (F1_4A) was an unexpected member of this clade. Clade 3 was composed of two

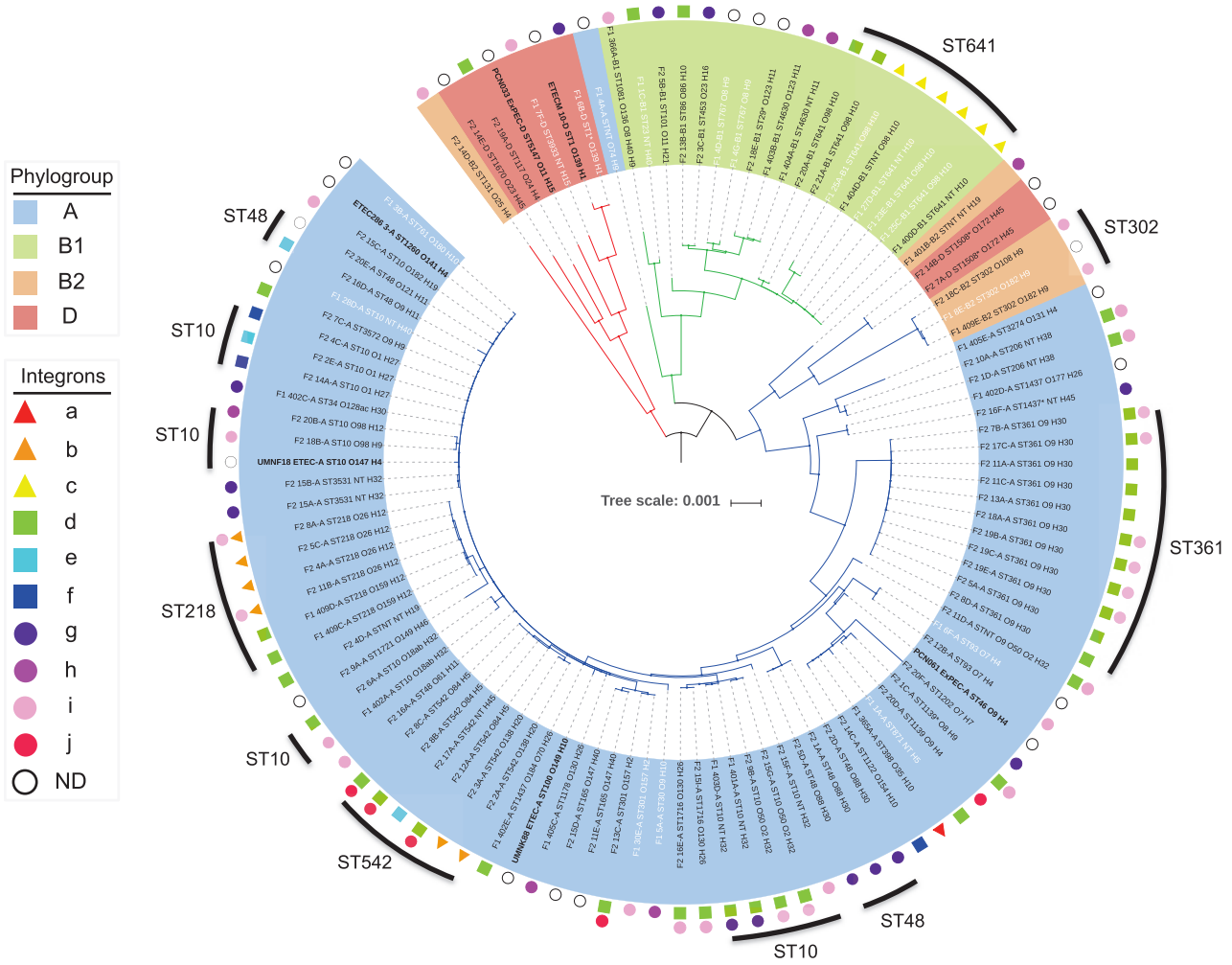

Fig. 1. A mid-point rooted, maximum-likelihood phylogenetic tree inferred using PhyloSift v1.0.1, FastTree2, FigTree v1.4.2 and iTOL. The tree contains all 103 pig *E. coli* isolates sequenced in this study, 2 porcine ETEC strains and 4 reference pig-sourced sequences. The labels of strains isolated from pigs with diarrhoea are in white, and of ETEC and reference strains are in bold. Branches are coloured by clade (clade 1, red; clade 2, green; clade 3, blue). Shading over tip labels indicates phylogroup (A, blue; B1, green; B2, orange; D, red). Tip labels also contain multilocus sequence type and serotype. Asterisks indicate single-locus variants of a given sequence type. The tree scale shows the distance for 1 amino acid substitution per 1000 sites in the analysis. Clusters of the seven most common sequence types have been marked with an outer line. Integrons shown in Fig. 3 are annotated by shapes indicating the presence of *sul1* (triangles), IS*26*-truncated 3′-*CS* (squares) and *sul3* (circles). Integrons (a–j) are coloured red, orange, yellow, green, aqua, blue, purple, magenta, pink and crimson. Strains that were *intI1* positive, but were not characterized are annotated with a white circle. Integrons were not determined for reference genomes used in the analysis.

separate sub-clades, one consisted of six B2 and D strains (three ST302, two ST1508 and a non-typable) and the other exclusively containing phylogroup A strains (ST10 and sequence types within CC10, as well as ST361 and ST542, strains that were common in our study collection).

ARGs and heavy-metal-resistance genes

We identified a total of 17 ARGs in the collection and strains carried between 1 and 15 ARGs each. A total of 100/103 (97 %) strains carried three or more resistance genes. Surprisingly, strains belonging to phylogroup A carried the highest mean number of ARGs (10 per strain). Strains belonging to phylogroup B1, B2 and D each carried a mean of eight ARGs per strain. The most common ARGs among the strains in our collection were: the penicillin-resistance gene $bla_{\text{TEM-1}}$, (84; 82 %); *aphA1*, encoding resistance to kanamycin and neomycin (76; 74 %); the co-linked streptomycin-resistance genes, *strA* and *strB* (73; 71 %); and the tetracycline-resistance gene *tetA* (73; 71 %). Quinolone-resistance genes *oqxAB*, which typically localize on plasmids, were less frequently identified (27; 26 %). Genes encoding extended-spectrum β-lactamases, extended-spectrum carbapenemases and resistance to

macrolides were not detected. Heavy-metal-resistance genes, including the copper-resistance gene *cusA* (103; 100 %), the Tn*21* mercury-resistance gene *merA* (71; 69 %) and the tellurite-resistance gene *terA* (40; 39 %), were identified frequently (Fig. 2).

Five ARGs were identified as gene cassettes carried by class 1 integrons (Fig. 3). Cassettes carried by the majority of strains included those conferring: aminoglycoside resistance, *aadA1* (69; 67 %) and *aadA2* (72; 70 %); chloramphenicol resistance, *cmlA* (60; 58 %); and trimethoprim resistance, *dfrA12* (62; 60 %) and *dfrA5* (51; 50 %). Among sulphonamide-resistance genes, *sul3* was identified in more strains (62; 60 %) than *sul1* (48; 47 %) or *sul2* (46; 45 %) (Fig. 2, Table S3). *sul1* and *sul3* were associated with integrons (Fig. 3).

Structurally diverse class 1 integrons

Among our study collection, we sought to characterize the diversity of class 1 integrons present. It is challenging to assemble complete sequences for such regions using Illumina sequence data, because of the presence of repeated elements. However, we identified numerous structurally diverse class 1 integrons, hereafter referred to as integrons (a–j) (Fig. 1 and 3). Notably IS*26* altered the 3′ region in six of the most common structures (d–i).

Four different class 1 integrons (g–j) carried a *sul3* gene. The first time *sul3* was linked with *E. coli* from a food-animal source in Australia was in 2015 in a highly virulent porcine ST4245 ExPEC strain [50]. Moreover, *sul3* was first reported in a human in Australia in 2017 in a commensal *E. coli* ST95 [51]. In integrons (g) and (h), the *sul3* module, which comprises a putative transposase *tnp440, sul3,* two hypothetical proteins (*orfA* and *orfB*) and 260 bp of the macrolide efflux gene *mefB* truncated by IS*26*, was the same. Integrons (g) and (h) differed from each other in their respective cassette arrays. Integron (i) differed from (g) and (h) both in its *sul3* module, which carried an additional copy of IS*26,* length of the *mefB* gene fragment (111 bp) and an insertion of an IS*1203*-like element in *qacH*. In integron (j), an IS*26* insertion leaves only 197 bp of *intI1* remaining, *mefB* is absent and an IS1-like element is adjacent to *orfB*. Only three of the integrons (a–c) among our strain collection carried a *sul1* gene. Screening indicated that at least 22 strains carry two integron structures. The most common co-carriage pattern was (d, i) (14/22), though (b, i) (2/22), (d, j) (4/22) and (d, g) (2/22) also occurred (Fig. 1, Table S3). Eight sequence types carried more than one integron, including predominant types ST10, ST361 and ST542 (Table S3).

VAGs

To assess the virulence potential of commensal pig *E. coli* strains in our collection, we screened for a total of 94 genes that have been associated with either intestinal disease or extraintestinal disease caused by *E. coli* pathotypes. Twenty-nine of these genes were present in at least one strain (Fig. 2, Table S4). All strains possessed between 3 and 16 VAGs. The mean number of VAGs for each phylogroup was: A, 5; B1, 9; B2, 11; D, 9. The VAGs were present in diverse gene combinations between and within sequence types. Most VAGs were typical of extraintestinal *E. coli* pathotypes (ExPEC), whilst ETEC toxin gene (*eltA, eltB,*

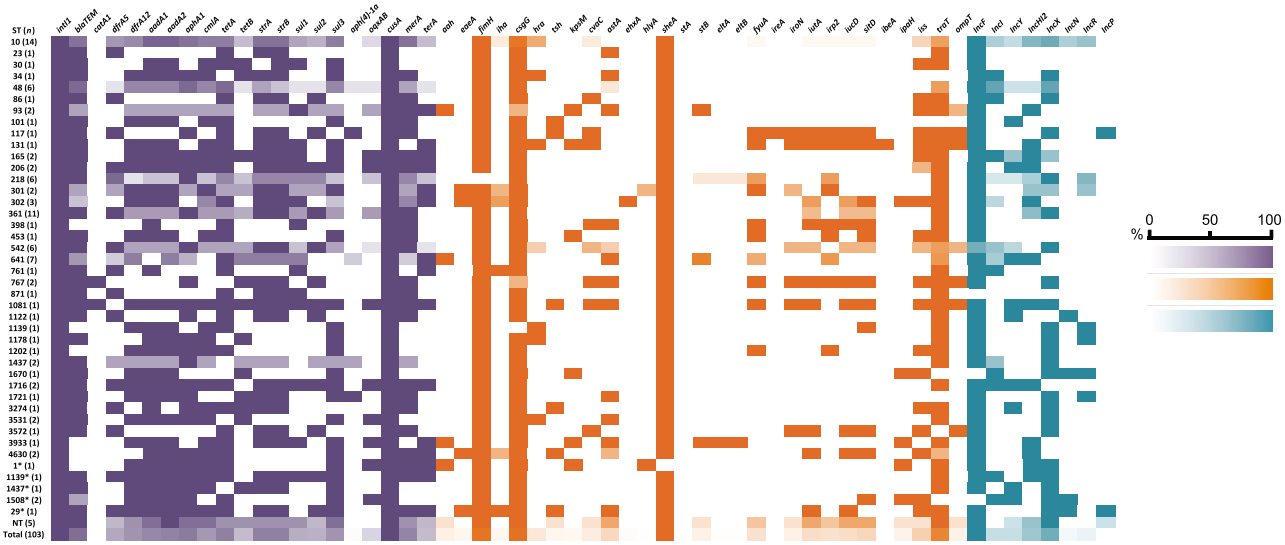

Fig. 2. Heat map depicting carriage of ARGs (aqua), VAGs (orange) and plasmid incompatibility groups (purple) by sequence type. A darker colour indicates high carriage amongst a given sequence type, a lighter colour indicates lower carriage and white indicates no carriage. For full screening data see Tables S3–S5.

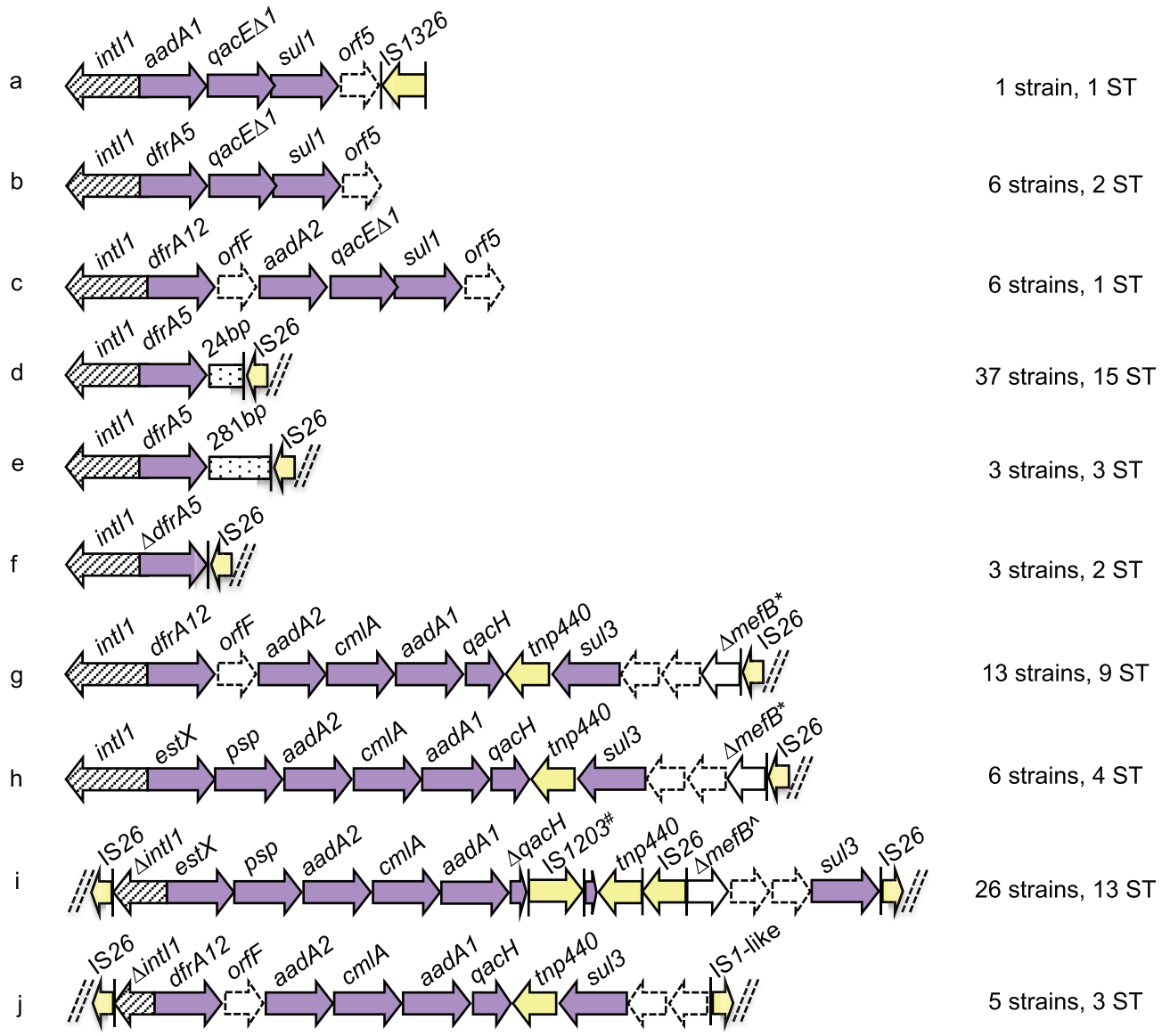

Fig. 3. Schematic diagram (not to scale) of integrons within porcine strains that were sequenced. Arrows represent ORFs. Arrows with broken lines indicate hypothetical proteins. Vertical bars represent inverted repeats. Dashed double diagonal lines represent sequence scaffold breaks. Intergenic sequences are not shown. ARGs (purple) and IS/transposable elements (yellow) are colour coded. *, 260 bp of *mefB* remaining; ^, 111 bp of *mefB* remaining; #, IS*1203*-like.

stA, *stB*) carriage was only observed in 11 strains and no ETEC adhesins were present.

Plasmid incompatibility groups

We screened the collection for plasmid replication-associated genes from nine plasmid incompatibility groups that are commonly associated with carriage and mobility of ARGs. IncF was the most common replicon (89; 86 %), followed by IncX (61; 59 %) and IncHI2 (43; 42 %). All replicons were present across multiple sequence types (Fig. 2, Table S5).

DISCUSSION

Globally, there is a poor representation of genomic sequences for commensal *E. coli* isolated from the faeces of pigs, and none in Australia. Here, for the first time, to our knowledge, we have sequenced the genomes of *E. coli* isolated from the faeces of predominantly healthy pigs and determined their Clermont phylogroup, multilocus sequence type (Achtman) and serotype, as well as carriage of ARGs and VAGs. The phylogenetic relationships shared by the 103 strains, the types of resistance genes that reside within the class 1 integrons and the structures of class 1 integrons

were also investigated. Despite sampling only two commercial piggeries, we identified a wide variety of multilocus sequence types. The diversity of isolates differed to previous studies on *E. coli* in pigs [35, 52] and this may be due to our selection of *intI1*-positive strains or simply reflect geographical differences. Our findings suggest that commensal *E. coli* populations residing within the faeces of pigs are often resistant to multiple antimicrobial agents and carry numerous VAGs. Notably, we also identified genetic epidemiological markers for tracking antimicrobial-resistance loci residing on mobile genetic elements in commensal *E. coli*.

Commensal *E. coli* lineages are associated with disease

The dominant lineages in our collection were phylogroup A *E. coli* belonging to sequence types residing within CC10, particularly ST10, ST48 and ST218. ST10 has previously been reported as the dominant sequence type from pigs in Germany, Denmark, Ireland and Spain [3, 35, 52–54]. Our data and the observations of others suggest *E. coli* of CC10 sequence type may be opportunistic, MDR pathogens with a broad animal host range. *E. coli* CC10 can colonize humans, swine, poultry, dogs, migratory birds, rodents, camels and cattle [9, 12, 55–61]. *E. coli* CC10 can also be isolated from raw and treated wastewater, and from urban streams [8]. *E. coli* CC10 is increasingly associated with intestinal disease in humans [62, 63], and extraintestinal infections in pigs [64, 65], dogs [57] and humans, including UTI, pyelonephritis and sepsis [9, 66–68]. *E. coli* CC10 are often MDR, and the resistance genes they carry can encode resistance to extended-spectrum β-lactams [69, 70]. ST10 is a noted ExPEC sequence type in humans and has been identified in food animals, retail meats and the environment [58, 71–74]. The core attributes of ST10 that enable it to colonize diverse niches remain unknown. The phylogenetic diversity we observed within porcine faecal ST10 suggests that such attributes may vary between strains. Whole-genome sequence analysis of *E. coli* ST10 genomes from different regions of the world and from different hosts is needed to understand the full diversity and success of this sequence type.

MDR porcine *E. coli* carry structurally diverse class 1 integrons

Notably, *sul3* was the most frequently identified *sul* gene in our collection and three different *sul3*-containing integron structures were identified. Carriage of class 1 integrons possessing *sul3* has been observed in disease-associated and commensal *E. coli* isolates from animals and humans, as well as in bacterial species other than *E. coli* from different countries [75–77]. In Australia, the carriage of *sul3* by *E. coli* has been reported infrequently, although it has been identified in several uropathogenic *E. coli* isolates [78], in a highly virulent porcine ST4245 ExPEC strain [50], and in a human commensal ST95 *E. coli* on a virulence plasmid that carries multiple ARGs and VAGs [14]. In Europe, class 1 integrons containing *sul3* have been observed in commensal *E. coli* from both humans and animals, indicating they are widely disseminated in a variety of *E. coli* lineages [14, 79–82]. Structures similar to ours have also been reported in different *Salmonella enterica* serovars, suggesting inter-species transfer of class 1 integrons carrying *sul3* may have occurred [75].

The potential role for *sul3* integrons in intraspecies and interspecies exchange of antibiotic resistance makes it desirable both to understand their evolution and to track their movement through bacterial populations. In Fig. 4, we have provided a model that could explain the micro-evolutionary events that created the novel *sul3* integron depicted in structure (i). This integron likely evolved from a progenitor similar to one described by Curiao *et al.* in a human-derived extended-spectrum β-lactamase positive *E. coli* on an IncI1 plasmid from Spain (GenBank accession no. HQ875016.1), as this is the only report to describe IS*26* adjacent to *sul3* [76]. Conceivably, the novel structure (i) emerged from insertion of a second copy of IS*26,* which further truncated *mefB,* followed by an inversion event. To our knowledge, this is the first study to identify a 111 bp *mefB* variant. Integron (i) was observed within the collection in 26 *E. coli* strains of different sequence types, suggesting horizontal transfer of a mobile element(s) carrying the integron, though we were unable to determine which mobile elements were responsible for this. Further work is needed to examine this hypothesis.

IS*26*-mediated deletions of *mefB* can be used to track *sul3*-containing integrons and additional resistance genes they may acquire due to the unique ability of IS*26* to target itself [26]. A number of different truncated variants of the *mefB* gene are carried by *sul3* integrons found in human- and animal-derived *E. coli* [14, 75–77]. Our data suggests the class 1 integrase upstream of the *sul3* module is likely to be functional based on the presence of different antibiotic cassette arrays associated with a 260 bp *mefB* deletion (g, h). BLASTN analysis identified *sul3* integrons carrying Δ*mefB* with an identical 260 bp deletion in porcine isolates P328.10.99.C2 (GenBank accession no. FJ196386.1) and P528.10.99.C4 (GenBank accession no. FJ196388.1) from Great Britain, though the associated cassette arrays were not completely characterized [77]. Furthermore, plasmid pCAZ590 (GenBank accession no. LT669764.1) isolated from poultry in Germany carried an identical integron (*estX-psp-aadA2-cmlA-aadA1-qacI-tnp440-sul3-orf1-orf2-*Δ*mefB*:260bp-IS*26*) to 4(h) with an additional $bla_{SHV\text{-}12}$ gene 73 bp upstream of IS*26* [83]. Although the evolutionary events that lead to this derivative structure are not known, this plasmid illustrates how IS*26* augmented integrons continue to evolve and acquire genes that confer resistance to critically important human antibiotics.

The deletion event in the 3′-*CS* of the integron depicted in (d) (*dfrA5*-IS*26*) may serve as another genetic signature for tracking resistance genes, and bacteria that carry them, through different hosts and environments [15, 84–86]. Previously, we observed the integron structure (d) on plasmids carrying VAGs in atypical EPEC strains isolated from cattle

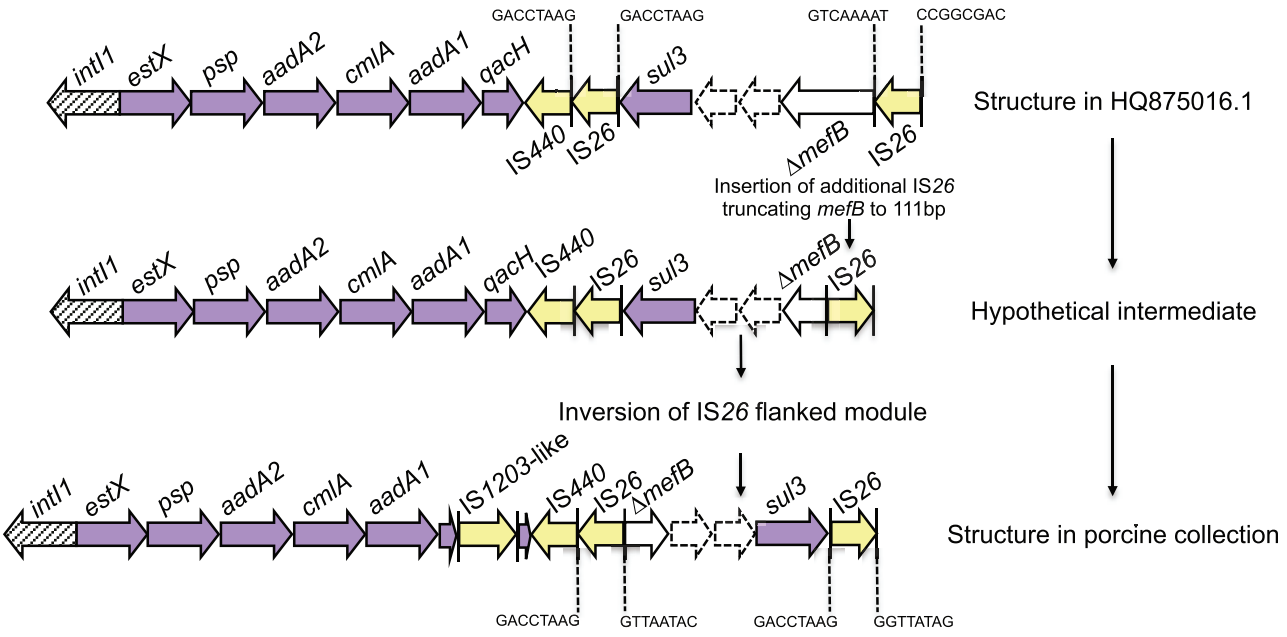

Fig. 4. Schematic diagram (not to scale) of proposed evolutionary pathway to the *sul3*-Δ*mefB* arrangement shown in Fig. 3(i). IS*26* 8 bp direct repeats are annotated.

with gastrointestinal disease and *E. coli* strains linked to EHEC O26:H− isolated from a human patient with haemorrhagic colitis [16, 17]. In each of these earlier cases, the IS*26* that interrupted the 3′-*CS* of the integron formed part of the left boundary of Tn*6026,* an IS*26*-flanked, globally disseminated transposon that harbours multiple ARGs [15–17, 87, 88]. Twenty-seven strains carrying integron (d) possess the resistance genes present in Tn*6026* (bla_{TEM}, *sul2, strAB, aphA1*) suggesting this transposon is also carried in our collection, though further studies are necessary to confirm this. This again highlights that tracking IS*26* deletions is useful for tracking not only the integrons they interrupt, but also additional resistance genes that may be acquired in association with the IS*26*.

The carriage of more than one integron in a number of prominent sequence types in the collection suggests that plasmid or transposon-mediated horizontal transfer of resistance determinants may occur within the microbiota of the porcine gut. This transfer is likely mediated by plasmids present in the collection, though transposons and IS elements may be involved. Long-read sequencing is required to test this hypothesis.

Zoonotic potential of commensal. *E. coli* from swine

In considering the zoonotic potential of pig faecal *E. coli*, we determined the proportion of strains in our collection that carried IPEC and ExPEC VAGs. A limitation of investigating zoonotic potential for extraintestinal disease is the genetic redundancy identified in the virulence attributes from ExPEC. A recent study suggested that the number of virulence factors carried by an ExPEC strain is the only independent factor that can explain extraintestinal virulence in a mouse model of sepsis [89]. Our collection contained two strains possessing large numbers of VAGs, belonging to ST131 and ST117, representative of pandemic ExPEC clones that cause hospital- and community-acquired infections in humans worldwide [58, 90, 91]. They have both been linked with poultry and have only rarely been isolated from porcine sources [9, 58]. The single ST131 strain in our porcine collection carried 10 ARGs and 16 VAGs. The ST117 strain carried 8 ARGs and 16 VAGs, including the full array of iron-acquisition genes *fyuA, irp2, ireA, iroN, iutA, iucD* and *sitD*. Several of these genes are typically encoded on virulence plasmids circulating in APEC [92] and this profile is similar to ST117-O111:H4 strains from poultry reported by Mora *et al.* [93]. The presence of ST117 and ST131 in our collection is intriguing, and warrants further investigation.

Most of the VAGs identified in our collection were those associated with the ability to cause extraintestinal disease in humans, as well as intestinal persistence [6, 94]. Carriage of genes that are under positive selection in uropathogenic *E. coli* [95], such as heat-stable agglutinin gene *hra* [96], murine uroepithelial cell adhesin gene *iha* [97], iron-acquisition genes *fyuA, iutA, iucD* and *sitD*, and the serum survival genes *iss* and *traT*, suggest that some strains may be capable of causing extraintestinal disease in humans. Conversely, it also highlights how many ExPEC VAGs can be considered important intestinal fitness factors. The most intriguing IPEC VAG was intimin gene *eaeA*, found in eight strains, that is characteristic of several intestinal *E. coli* pathotypes, including EHEC, EPEC and atypical EPEC [98].

These strains also carried ExPEC VAGs and may represent hybrid pathotypes.

The frequency of VAGs in phylogroups A and B1, a mean of 5 and 9 VAGs per isolate, respectively, was unexpected because *E. coli* belonging to phylogroups A and B1 are considered to have low virulence potential [99, 100]. The carriage of multiple VAGs in pig *E. coli* is consistent with earlier studies [101, 102]. In China, ExPEC have been isolated from a variety of tissues and bodily fluids of pigs with septicaemia, meningitis and respiratory disease with increasing frequency since 2004 [64, 65]. It is notable that 35 % of 81 isolates in one of these studies belonged to phylogroup A, clonal complex 10 [64]. In European wild boars, which are assumed to be ancestors of domestic pigs in Europe [103], *E. coli* strains carry, on average, 7 or more VAGs, with some strains carrying up to 16 VAGs [104]. Collectively, these observations suggest that *E. coli* phylogroup A and B1, at least those sourced from swine, carry multiple VAGs.

Contribution of food-production animals to the evolution of pathogens and antimicrobial resistance

MDR *E. coli* carrying ARGs associated with mobile genetic elements and VAGs are released into the environment by food-production animals via faecal effluent. In Australia, the capacity for pig production to contribute to the evolution and dissemination of pathogens and ARGs is restricted compared to that of pig-production systems in many other countries, due to a range of factors. Firstly, Australia has a large landmass that is surrounded by ocean, preventing the movement of animals from neighbouring countries. Secondly, importation of food animals into Australia has been restricted since the 1970s [105]. Thirdly, antibiotics such as fluoroquinolones cannot legally be administered to food animals and many others are restricted from use in food-animal production [106, 107]. However, even in the restricted environment in Australia, phenotypic resistance to clinically important antibiotics, including extended-spectrum cephalosporins and fluoroquinolones, has been observed in *E. coli* that belong to globally disseminated *E. coli* lineages ST744, ST100 and ST1 [108]. Globally, genomic surveillance is needed to understand the relative contribution of food-production animals to the complex web of interactions between microbiota and the mobile resistome, to provide baseline carriage rates for antimicrobial genes and VAGs, and to monitor the emergence of novel drug-resistant pathogens [84, 109].

In summary, we report, to our knowledge, the first genomic study of commensal *E. coli* isolated from commercial pigs used for food consumption and provide data to inform assessment of potential risks pig commensal *E. coli* may pose to human health. Our results show that swine are a reservoir: (i) for phylogroup A and B1 *E. coli* that carry VAGs, (ii) the *sul3* gene, (iii) class 1 integrons associated with IS*26*, and (iv) *E. coli* lineages belonging to CC10. Our study has identified several new genetic signatures that may be used in tracking mobile ARGs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Chapter 4: Comparative genomic analysis of a multiple antimicrobial resistant enterotoxigenic *E. coli* O157 lineage from Australian pigs

Author contribution statement: I (Ethan R. Wyrsch) was a primary author of this publication, performing most of the genomic analysis under the guidance of Piklu Roy Chowdhury, generating figures and drafting the initial manuscript. I was also then involved in final editing after co-author circulation. Jerran Santos prepared sequencing libraries. Toni A. Chapman and Sam Abraham performed initial PCR and phenotypic testing for antibiotic resistance. Steven P. Djordjevic, Toni A. Chapman, Aaron E. Darling, Sam Abraham and Ian G. Charles conceived the study and provided supervision, while Steven P. Djordjevic, myself, Piklu Roy Chowdhury, Toni A. Chapman, Aaron E. Darling, Sam Abraham and Ian G. Charles wrote the manuscript.

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Comparative genomic analysis of a multiple antimicrobial resistant enterotoxigenic *E. coli* O157 lineage from Australian pigs

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Abstract

Background: Enterotoxigenic *Escherichia coli* (ETEC) are a major economic threat to pig production globally, with serogroups O8, O9, O45, O101, O138, O139, O141, O149 and O157 implicated as the leading diarrhoeal pathogens affecting pigs below four weeks of age. A multiple antimicrobial resistant ETEC O157 (O157 SvETEC) representative of O157 isolates from a pig farm in New South Wales, Australia that experienced repeated bouts of pre- and post-weaning diarrhoea resulting in multiple fatalities was characterized here. Enterohaemorrhagic *E. coli* (EHEC) O157:H7 cause both sporadic and widespread outbreaks of foodborne disease, predominantly have a ruminant origin and belong to the ST11 clonal complex. Here, for the first time, we conducted comparative genomic analyses of two epidemiologically-unrelated porcine, disease-causing ETEC O157; *E. coli* O157 SvETEC and *E. coli* O157:K88 734/3, and examined their phylogenetic relationship with EHEC O157:H7.

Results: O157 SvETEC and O157:K88 734/3 belong to a novel sequence type (ST4245) that comprises part of the ST23 complex and are genetically distinct from EHEC O157. Comparative phylogenetic analysis using PhyloSift shows that *E. coli* O157 SvETEC and *E. coli* O157:K88 734/3 group into a single clade and are most similar to the extraintestinal avian pathogenic *Escherichia coli* (APEC) isolate O78 that clusters within the ST23 complex. Genome content was highly similar between *E. coli* O157 SvETEC, O157:K88 734/3 and APEC O78, with variability predominantly limited to laterally acquired elements, including prophages, plasmids and antimicrobial resistance gene loci. Putative ETEC virulence factors, including the toxins STb and LT and the K88 (F4) adhesin, were conserved between O157 SvETEC and O157:K88 734/3. The O157 SvETEC isolate also encoded the heat stable enterotoxin STa and a second allele of STb, whilst a prophage within O157:K88 734/3 encoded the serum survival gene *bor*. Both isolates harbor a large repertoire of antibiotic resistance genes but their association with mobile elements remains undetermined.

Conclusions: We present an analysis of the first draft genome sequences of two epidemiologically-unrelated, pathogenic ETEC O157. *E. coli* O157 SvETEC and *E. coli* O157:K88 734/3 belong to the ST23 complex and are phylogenetically distinct to EHEC O157 lineages that reside within the ST11 complex.

Keywords: Enterotoxigenic *Escherichia coli*, Diarrhoeal disease, Swine, Porcine, O157

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Background

Enterotoxigenic *Escherichia coli* (ETEC) are a leading cause of neonatal, pre-weaning and post-weaning diarrhoea (PWD) in pigs. Significant economic losses are incurred as a result of mortalities, reduced growth rates in survivors and medication costs. Antimicrobials have been used to treat infections but the emergence of multiple drug resistant variants poses a serious challenge in controlling ETEC infections in swine production. Dietary zinc supplements, phage therapy, probiotics, antibody dietary formulations and breeding programs to generate swine stock expressing gut epithelial receptors that are not recognised by ETEC fimbriae all represent alternate strategies used by swine producers to control ETEC [1], however these pathogens continue to pose a constant threat to farms.

ETEC serogroups O8, O9, O45, O101, O138, O139, O141, O149 and O157 are implicated in diarrhoeal disease during pig development [2-4]. Porcine enterotoxigenic *E. coli* O157 are associated with disease of the small intestine and mediate neonatal, pre- and post-weaning diarrhoea in piglets. This is in contrast to the mode of pathogenesis displayed by EHEC O157, which is associated with food-borne human illness causing diarrhoea and enterohaemorrhagic disease of the large intestine [5-7]. Multiple evolutionary lineages of *E. coli* O157 [8] with different lineages encoding variants of the H-antigen genes, and two distinct *rfbE* alleles within the O157-antigen biosynthesis gene cluster, have been described. In addition, reports of serogroup O157 isolates as ETEC [9] and EPEC [10,11] is indicative of a wider evolutionary range of pathogenic *E. coli* O157, sourced from multiple hosts including humans, pigs and cattle.

Features separating porcine ETEC from other diarrhoeagenic *E. coli* include the presence of the pig-specific fimbrial adhesins F4 (K88), F5, F6, F18 and F41 that facilitate adherence to porcine gut epithelium and toxins including the heat-stable enterotoxins STa and STb, and a heat-labile enterotoxin (LT) that induce diarrhoea [1,12]. Molecular epidemiological studies indicate that other putative virulence genes are also expressed by ETEC causing ND and PWD, including EAST1 toxin, haemolysins, autotransporters, outer membrane proteins, siderophores and additional iron acquisition factors [2,13,14]. Many putative virulence genes are present only in subsets of clinical ETEC isolates suggesting that different virulence gene combinations may contribute to the range of clinical symptoms observed in ETEC infections. Additionally, ETEC readily acquire virulence and antimicrobial resistance genes by lateral gene transfer and strain variants containing new combinations of virulence and antimicrobial resistance genes are continually evolving globally [13,15-18].

In 2008 a piggery in Australia sustained major economic losses from an outbreak of pre- and post-weaning diarrhoea caused by enterotoxigenic *Escherichia coli* (ETEC) with an O157 serogroup. A representative of the ETEC outbreak is isolate *E. coli* O157 SvETEC. *E. coli* O157 SvETEC caused pre- and post-weaning diarrhoea in pigs between 7–15 days of age, affecting farrowing as well as weaner sheds. Severely affected pigs died suddenly with little or no diarrhoea. Gilts were shown to shed the pathogen but remained free of symptoms during the outbreak. Various antimicrobial and management strategies were implemented and all failed to control the ETEC infection in the pig herd. The outbreak resulted in a significant loss of piglets, over a long duration of time and over multiple sheds. Eventually all stock were removed, pens cleaned and restocked. We present here a comparative genomic analyses of O157 SvETEC with a historical ETEC O157 isolate from Australia (O157:K88 734/3) sampled in the 1990s from a clinical neonatal diarrhoea specimen [4,19].

In this study, a combination of bioinformatics and genome sequencing methodologies was used to perform a comparative and phylogenetic analysis of strains O157 SvETEC and O157:K88 734/3. These are the first ETEC O157 genomes deposited in public databases. Our analyses provide insight into the evolution of ETEC O157 isolates in Australian swine populations revealing that they are phylogenetically distinct to other *E. coli* isolates of serogroup O157. We identified a suite of putative virulence factors, antimicrobial resistance genes and mobile genetic elements in the two porcine O157 isolates. In addition, we highlight novel variability between these two related pathogens.

Methods

Strains, isolation, culture conditions and serogrouping

Isolate O157 SvETEC (previously ETEC 95 [19]) was isolated in 2008 from the faeces of an affected piglet from a commercial farm in New South Wales (Australia) that experienced repeated bouts of pre- and post-weaning diarrhoea and high mortality. Isolate O157:K88 734/3 (previously ETEC 24 [19]) was sampled in the 1990s from a clinical submission of common neonatal diarrhoea. Isolates were initially characterised at the NSW Department of Primary Industries Elizabeth Macarthur Agricultural Institute (EMAI) in Menangle [4,13,19]. The strains were sent to the ithree institute at the University of Technology Sydney (UTS), as stab cultures and thereafter regularly cultured in LB broth supplemented with ampicillin (50 μg ml^{-1}) with shaking at 200 rpm at 37°C for approximately 16 hours.

Antimicrobial resistance phenotyping

At EMAI, strains were screened against 18 antimicrobial agents by disc diffusion using the calibrated dichotomous susceptibility (CDS) test Australia, as reported previously [4]. The following antimicrobials were tested: ampicillin (25 μg), amoxicillin/clavulanate (60 μg), ticarcillin/

clavulanic acid (85 μg), cefalexin (100 μg), cefoxitin (30 μg), cefotaxime (5 μg), cefepime (10 μg), nalidixic acid (30 μg), ciprofloxacin (2.5 μg), imipenem (10 μg), sulphafurazole (300 μg), trimethoprim (5 μg), tetracycline (10 μg), apramycin (15 μg), neomycin (30 μg), gentamicin (10 μg), azithromycin (15 μg) and chloramphenicol (30 μg).

Genomic DNA extraction

Sequencing quality gDNA was extracted from 2 mL of each overnight culture using a DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's recommendations.

Whole genome sequencing, assembly, annotation and phylogenetic analysis

Sequencing was performed at the UTS in-house Next Generation Sequencing facility using a bench top Illumina MiSeq® sequencer and MiSeq® V3 chemistry. Sequencing libraries were prepared with 0.5 ng of gDNA following the manufacturer's protocol for the Nextera® XT library preparation kit (Illumina). Sequencing with the MiSeq® sequencer generated 250 nucleotide (nt) long paired end reads of the libraries representing each sample. The quality of the sequence reads was assessed using a locally downloaded version (0.10.1) of FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) software and assembled using the A5-miseq *de novo* assembly pipeline [20] revised to process reads up to 500 nt long [21]. Scaffolds over 1000 nt in length were included in the whole genome sequence analysis. Whole Genome Shotgun sequences were deposited at DDBJ/EMBL/GenBank under the accession numbers JPPP00000000 for *E. coli* O157 SvETEC and JPQX00000000 for *E. coli* O157:K88 734/3. A preliminary annotation of each genome was generated using the automated annotation software RAST [22] and the annotation of antimicrobial resistance genes was performed using the Resistance Gene Identifier (RGI) Version 2 on the Comprehensive Antibiotic Resistance Database website [23]. Individual genes of interest, including those annotated by RAST and the RGI, were manually interrogated using NCBI's BLASTn and BLASTp tools. Insertion sequences (IS) and open reading frames (ORFs) were identified using the online tools IS Finder (https://www-is.biotoul.fr//) and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) respectively. Phage associated gene clusters within the genomic scaffolds were initially identified using the PHAST [24] server. Genomic scaffolds with positive PHAST hits were further verified to be phage-associated in the RAST annotation output and using BLASTn and BLASTp analysis.

An alignment of phylogenetic marker genes was constructed using PhyloSift [25] and a tree was then inferred using FastTree2 [26]. The publicly available FastTree2 software is unable to resolve branches in the phylogeny shorter than 1×10^{-5} substitutions per site. Our dataset appeared to have several short branches; therefore we modified the FastTree2 software to improve short branch resolution and applied it to our dataset. The output was visualised in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The O157 SvETEC and O157:K88 734/3 genomes were analysed alongside 40 complete *E. coli* genomes, 3 complete *Shigella* spp. genomes, 33 draft *E. coli* O157 genomes and 2 draft *E. coli* APEC O78 genomes from the NCBI GenBank database. The *Klebsiella pneumonia* 342 and *Salmonella enterica* subsp. *enterica* serovar Heidelberg str. 41578 genome sequences were included in this analysis as out-groups to confirm the validity of the method; however these sequences were removed from the final phylogenetic tree to facilitate visualizing the fine-scale relationships among *E. coli*.

Comparative genomic and MLST analysis

Comparative genomics used tools available in MAUVE version 2.3.1 [27]. The MAUVE Move Contigs tool was used to tile scaffolds generated by the *de novo* A5-miseq assembler against the reference *E. coli* APEC O78 finished genome. From this, the best alignment was chosen based on the highest weight score, an indicator of whether the predicted rearrangement exists, and lowest number of Locally Collinear Blocks (LCBs). Scaffolds that tiled against the finished APEC O78 genome were sorted and identified in this study as the subset representing the 'core' genome. The subset of scaffolds that did not align against the finished APEC O78 genome were designated the 'accessory' genome. The progressiveMauve module was used for comparative analysis of the genomes and to generate the figure. Regions of interest identified from whole genome comparisons were further analysed using iterative BLASTn and BLASTp searches.

The PubMLST (http://pubmlst.org/) database was used to identify the sequence type of the isolates using the Achtman *E. coli* MLST scheme [28] (http://mlst.warwick.ac.uk/mlst/).

Results

Whole genome sequence statistics and phylogenetics

De-novo assembly of the O157 SvETEC genome generated 236 scaffolds with 60-fold coverage and a predicted genome size of 5547789 nt. The N50 value for the assembly was 96352 nt. For O157:K88 734/3, de-novo assembly generated 226 scaffolds with 78-fold coverage, a predicted genome size of 5449663 nt and an N50 value of 91578 nt. In both cases, 50% of the respective genomes were assembled into the largest 18 scaffolds. Scaffolds were sorted into cohorts putatively representing the core and accessory genomes of O157 SvETEC and O157:K88 734/3 using the criteria described above. The O157 SvETEC core genome consisted of 96 scaffolds totalling 4789945 nt while the O157:K88 734/3 core genome comprised 94 scaffolds

totalling 4809185 nt. The accessory genome of O157 SvETEC was 738983 nt in length and was represented in 112 scaffolds while the O157:K88 734/3 accessory sequence totalled 616231 nt in 97 scaffolds.

Phylogenetic analysis using the assembled genome sequences was performed to gain insight into the evolution of ETEC O157 isolates in Australian swine populations. *In-silico* identification of the genes used in the updated Clermont *et al.* phylotyping method [29] determined that O157 SvETEC and O157:K88 734/3 belonged to phylogroup C. This concurred with previously reported data [4].

The PhyloSift phylogenetic analysis was performed to examine the swine isolates in the context of *E. coli* population structure. Figure 1 shows that neither *E. coli* O157 SvETEC nor *E. coli* O157:K88 734/3 clustered with the other *E. coli* serogroup O157 isolates and grouped together into a single clade with the most closely related completely closed reference genome being an avian pathogenic *E. coli* (APEC) of serogroup O78 [GenBank: CP004009]. Notably, the subclade included a variety of other isolates including *S. sonnei* 53G [GenBank:HE616528], known pathogenic *E. coli* serogroups such as O111 [GenBank:AP010960], O104 [GenBank:CP003301] and O26 [GenBank:AP010953] and a draft *E. coli* O157:H43 genome sequence [GenBank:AHZD02000001]. *E. coli* O157 draft and complete genomes as well as *E. coli* O55 genomes grouped together as a large clade in the tree, clustering with a confidence value of 1. The findings of our phylogenetic analysis were supported by eMLST analysis in which the O157 SvETEC and O157:K88 734/3 isolates represented a novel sequence type, ST4245 [4]. For comparative purposes, we also sequence typed APEC O78, the closest neighbour of the porcine O157 isolates and found it to be ST23, a member of the same clonal complex.

Comparative genomics

The scaffolds representing the core O157 SvETEC genome had 4,741 predicted ORFs while the scaffolds representing the core O157:K88 734/3 genome consisted of 4,732 predicted ORFs. Both genomes displayed 94 RNA-encoding ORFs. A BLASTn comparison, facilitated by Mauve, of the O157 SvETEC and O157:K88 734/3 genomes was performed on the scaffolds representing the core genomes of both ETEC O157 against the finished reference genome of *E. coli* APEC O78 (Figure 1). Common features between the two ETEC O157 core genomes included an enterotoxin-encoding scaffold and the transposon Tn*7*, which were not present within the reference genome. Scaffolds carrying genes encoding enterotoxins LT and STb aligned to core genome sequences of both strains, likely due to an IS*911* gene aligning each scaffold to the same IS element in the APEC O78 genome sequence. BLASTn analysis of the LT/STb enterotoxin encoding scaffolds indicated similarity with pUMNK88_Ent [GenBank:CP002732] with both scaffolds exhibiting 99% sequence identity over 77% of the query sequence for O157 SvETEC and 79% for O157:K88 734/3. These data suggest that the enterotoxin genes reside on a plasmid. Variability between the core O157 genomes was mostly limited to phage associated regions scattered throughout each genome alignment. Six phage-associated regions, designated Phage Regions S1-S6, were identified within the O157 SvETEC core genome and seven, designated Phage Regions K1-K7 were identified in the O157:K88 734/3 core genome (Table 1 and Figure 2). Two of the six phage regions in the O157 SvETEC genome were identified to be intact prophages by PHAST whereas five of the seven O157:K88 734/3 phage regions were identified as intact prophages. The majority of these phage associated scaffolds were putatively composed of >85% phage and hypothetical gene content. Phage Region 1 of O157:K88 734/3 was most similar to a lambda phage and encoded the increased serum survival gene *bor*. Two identical phage-associated regions were shared by both O157 genomes. Phage Region 6 of O157 SvETEC and Phage Region 7 of O157:K88 734/3 were identical over 19.3 kb. Phage Region 4 of O157 SvETEC was 99% identical to Phage Region 5 of O157:K88 734/3. Two smaller regions in O157 SvETEC and one in O157:K88 734/3 were manually identified as phage associated regions and are represented by unlabelled boxes in Figure 2. In addition, the scaffold encoding the K88 adhesin operon in the O157:K88 734/3 genome also encoded several phage-associated genes that were lacking in the homologous O157 SvETEC scaffold. As such, this scaffold was aligned into the O157:K88 734/3 core genome due to these phage-associated genes. Variation between all three genome sequences was also observed around the APEC O78 O-antigen biosynthesis genes.

The accessory genome scaffolds of O157 SvETEC and O157:K88 734/3 were examined for ORFs. O157 SvETEC comprises 1008 predicted ORFs over 112 scaffolds including 8 RNA predictions, whereas O157:K88 734/3's comprises 851 predicted ORFs over 97 scaffolds including 8 RNA predictions. To further assess variability between the two genomes a comparative BLASTp analysis of the predicted amino acid sequence of the accessory genome ORFs of O157 SvETEC and O157:K88 734/3 was performed (Additional file 1: Table S1). Several scaffolds and consequently their predicted peptide sequences were determined to be unique to O157 SvETEC. The majority of genes common between the accessory genomes were plasmid related and included *tra* operon genes associated with IncF and IncI plasmids, and hypothetical proteins. Fragments of complex antimicrobial resistance loci (CRL) were also shared between the two accessory genomes including a Tn*21*-like mercury

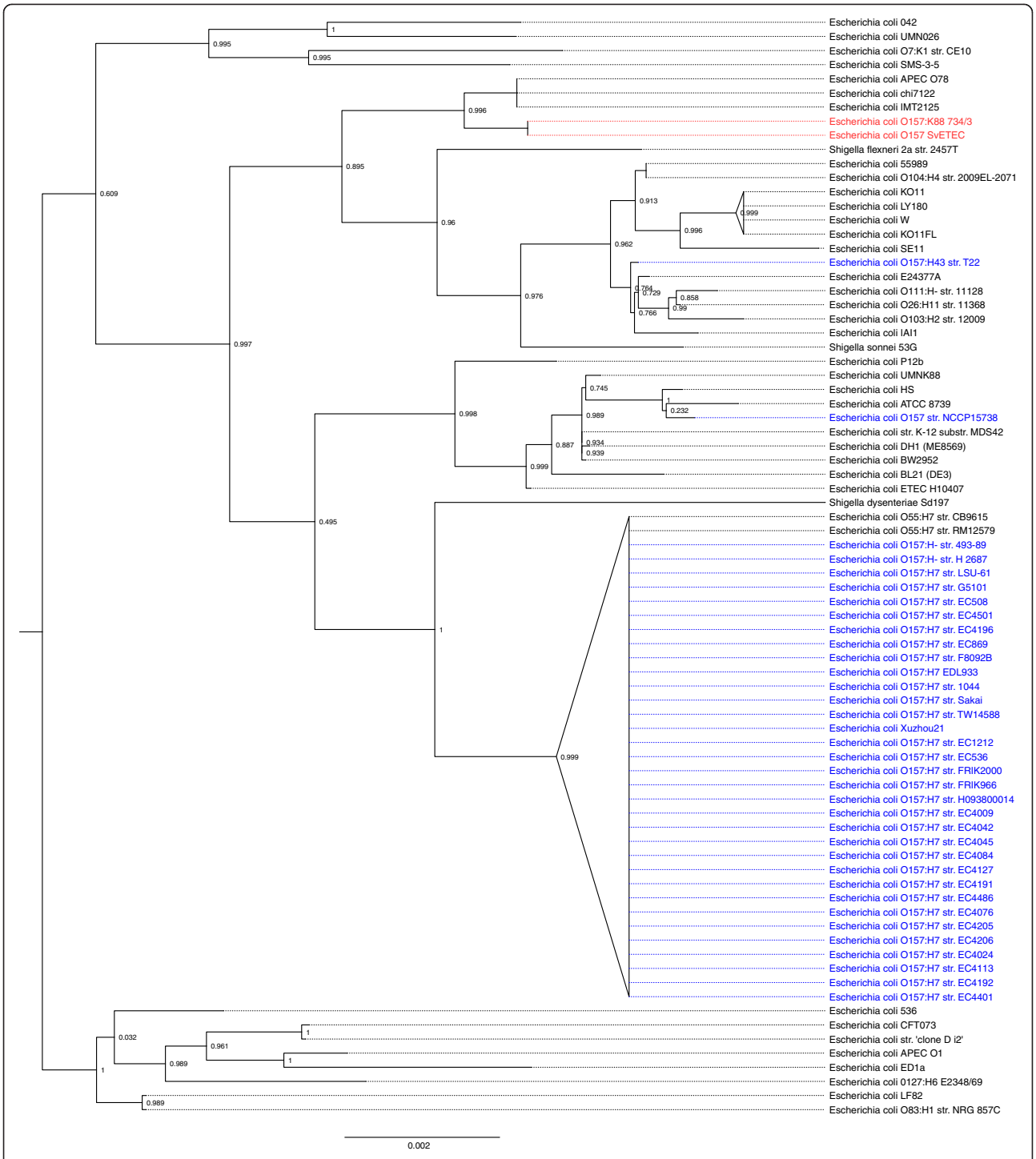

Figure 1 A phylogenetic tree produced using PhyloSift. The numbers located on each node is a confidence value between 0 and 1, with values near 1 indicating that the branch at that node exists with high probability. The scale is number of substitutions per site. *E. coli* O157 SvETEC and *E. coli* O157:K88 734/3 were placed into a single clade (red). The most closely related isolate was an avian pathogenic *E. coli* O78. Various completed and draft *E. coli* O157 genomes taken from the GenBank database clustered together (blue), with a confidence value of 1.

resistance operon and scaffolds associated with clinical class 1 and class 2 integrons. Several phage-containing or phage gene-associated scaffolds were also identified within the accessory genomes. Two approximately identical scaffolds of 29133 nt and 29044 nt respectively for O157 SvETEC and O157:K88 734/3 were identified as most similar to Enterobacteria phage P1 (NC_005856) but were predicted to be incomplete by PHAST. Both accessory

Table 1 Phage associated regions identified by PHAST

Strain	Region	Concatenated[1] Boundaries (bp)	Size (kb)	Ordered scaffold numbers	PHAST Annotation	Annotation Identity[2] (Phage Identity[3])	PHAST Prediction[4]
E. coli O157 SvETEC	S1	1316571-1334538	17.9	83, 114, 2	Stx2 converting I NC_003525	24% (92%)	Incomplete
	S2	2204749-2280987	76.2	59, 58, 107, 53, 39	mEp460 NC_019716	22% (90%)	Intact
	S3	2966791-2982739	15.9	32, 81, 1	P1 NC_005856	50% (85.7%)	Incomplete
	S4	3287635-3331585	43.9	1, 11	TL 2011b NC_019445	78.57% (98.2%)	Intact
	S5	3449037-3477233	28.1	44, 70, 14	P1 NC_005856	39.39% (84.8%)	Incomplete
	S6	4542510-4561835	19.3	31	APSE 2 NC_011551	15.78% (47.3%)	Incomplete
E. coli O157:K88 734/3	K1	1322304-1357135	34.8	67, 55, 141, 6	lambda NC_001416	54.76% (97.6%)	Intact
	K2	1555282-1584506	29.2	34, 83, 58	Fels 2 NC_010463	57.14% (100%)	Incomplete
	K3	1810316-1849764	39.4	19, 41	mEp460 NC_019716	17.94% (92.3%)	Intact
	K4	2254746-2314331	59.5	54, 65, 94, 98, 132, 136, 38	mEp460 NC_019716	29.11% (92.4%)	Intact
	K5	3315282-3359241	43.9	2, 23	TL 2011b NC_019445	78.18% (98.1%)	Intact
	K6	3476711-3497698	20.9	30	Fels 2 NC_010463	86.2% (100%)	Intact
	K7	4558357-4577682	19.3	7	APSE 2 NC_011551	15% (45%)	Incomplete

[1]Identified regions were generally over multiple scaffolds but were identified as concatenated sequence. [2]Percent identity of the region compared to the PHAST annotation. [3]Percentage of identified ORFs that encode phage or hypothetical genes. [4]PHASTs prediction of whether each prophage encodes the genes necessary for lysogeny.

genomes encoded plasmid transfer genes relating to the plasmid incompatibility groups F and I, however the O157 SvETEC accessory scaffolds also encoded IncH plasmid-associated genes. BLASTn analysis of the 66 kb (Scaffold 26) encoding IncH1 plasmid-associated genes identified seven GenBank entries each with 96-99% query coverage and 98-99% identity with the scaffold. Each entry was of a plasmid and included *Serratia marcescens* plasmid R478 [GenBank:BX664015], *Salmonella enterica* subsp. *enterica* Serovar Cubana str. CFSAN002050 plasmid [GenBank:CP006056] and *Escherichia coli* APEC O1 plasmid pAPEC-O1-R [GenBank:DQ517526]. Several other scaffolds within the O157 SvETEC accessory genome also aligned with high identity to these plasmids. These scaffold generally encoded hypothetical proteins with the exception of a copper/heavy metal (*cus/czc*) resistance operon (Scaffold 43) and a tellurium/tellurite (*ter*) resistance operon (Scaffold 35).

The O157 SvETEC accessory genome was also characterised by the presence of a Tn*7*-like transposon operon, hygromycin resistance, the enterotoxin STa and a second allele of STb, all of which were lacking in the O157:K88 734/3 accessory genome. A 17.8 kb region of another accessory O157:K88 734/3 scaffold was identified to contain an incomplete phage most similar to Enterobacteria phage P1 (NC_005856). Further BLASTn analysis

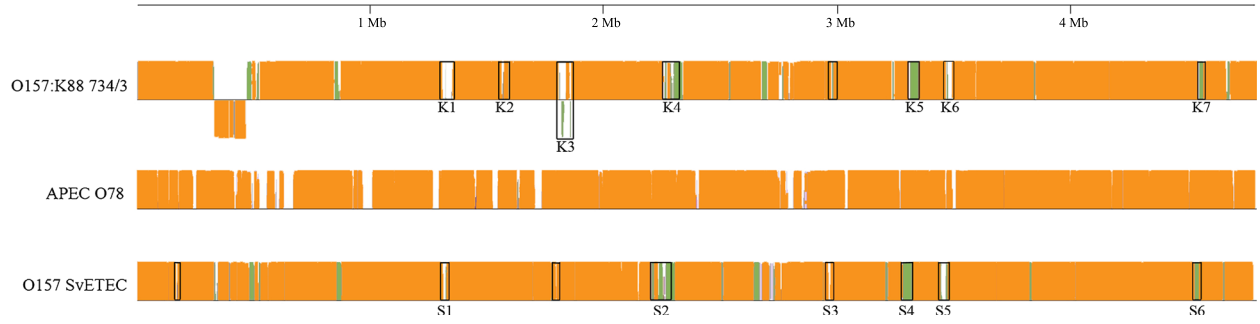

Figure 2 Comparative progressiveMauve analysis between the core scaffolds of each whole genome sequence and a reference genome, the closely related *E. coli* APEC O78. Predicted sequence homology among all three genome sequences is represented by orange regions. Green regions indicate sequence shared between the O157 core scaffolds only. Regions within black boxes are identified phage sequences and phage-associated regions. Sequence below the line of O157:K88 734/3 represents a predicted inversion, based on scaffold tiling to the APEC O78 reference.

suggested both ETEC O157 genomes contained only a single copy of the P1-like phage, which has been split amongst various core and accessory scaffolds due to the reference tiling. Aside from these specifics, much of the variation between the accessory genomes of the two strains lies in plasmid associated hypothetical proteins, mobile elements and CRL-associated scaffolds.

The O-antigen biosynthesis genes within both ETEC O157 draft genome sequences were split across several scaffolds making detailed analysis difficult. Despite this, high synteny was observed between the O157-antigen encoding scaffolds with both isolates encoding the Sakai-type *rfbE* gene (gene truncated at 1032 bp by a scaffold break in O157:K88 734/3) and also containing the genes *yoeB* and *yefM* identical to the *E. coli* strain PV00-24 O-antigen biosynthesis gene cluster [GenBank: AB602253.1][8].

Virulence factors

As both the ETEC O157 pathogens belong to the novel ST4245 sequence type we were particularly interested to identify potential virulence determinants within their genomes. Those we identified through sequence analysis are detailed in Table 2, including a variety of adhesin and toxin genes and their associated operons. Both O157 SvETEC and O157:K88 734/3 were positive for the enterotoxins LTp (*eltAB)* and STb (*estB*) whereas the heat stable enterotoxin STa (*estA*) was only identified in O157 SvETEC. A further distinction highlighted by sequence analysis was that a second allele of *estB* was present in a separate scaffold within the O157 SvETEC genome. This allele differed by a single nucleotide polymorphism and was identical to the STb gene of *Escherichia coli* F18+ strain EC2173 plasmid pTC1 [GenBank:CP000913]. The *fliC* gene was, in both isolates, the H19 allele.

Antimicrobial resistances

Multiple antibiotic resistances were observed in the phenotypic analysis of O157 SvETEC and O157:K88 734/3. Both isolates were resistant to ampicillin, nalidixic acid, sulphafurazole, trimethoprim, tetracycline and neomycin. Each isolate displayed distinct additional resistance phenotypes against apramycin and chloramphenicol for O157 SvETEC and against cephalexin and cefoxitin for the O157:K88 734/3 isolate [4]. Various genes known to provide these antimicrobial resistances were identified within the genome sequences (Table 3). The *gyrA* sequence of both isolates was determined to contain a single S83L amino acid change compared with the *E. coli* K12 substrain MG1655 *gyrA* gene.

Discussion

In this study, we were able to characterise the evolutionary background of two non-EHEC *E. coli* O157 strains, and identify genetic differences between these two ETEC

Table 2 Virulence genes associated with pathogenicity present in ETEC O157

Type	Gene	O157 SvETEC	O157:K88 734/3	Details
Adhesins	*faeG*	+	+	K88 (F4) Fimbrial adhesin
	fimH	+	+	Type 1 fimbriae, D-Mannose-specific adhesin
	csgG	+	+	Facilitator of fibronectin-binding curli assembly
	eaeH	+	+	Highly conserved adhesin
	sfmA	+	+	Fimbrial-like adhesin
	ecpA	+	+	*E. coli* common pilus
	mat	+	+	*mat* operon
Toxins	*east*1	+	+	Enteroaggregative *E. coli* heat-stable enterotoxin
	hlyA	+	+	α-Hemolysin
	hlyE	+	+	Hemolysin E
	eltB	+	+	Heat-labile enterotoxin B subunit
	eltA	+	+	Heat-labile enterotoxin A subunit
	estA	+		Heat-stable enterotoxin a
	estB	+	+	Heat-stable Enterotoxin b
	estB	+		Heat-stable Enterotoxin b (second allele)
Siderophores	*fyuA*	+	+	Iron uptake receptor
	irp2	+	+	Iron repressible protein
Other	*bor*		+	Serum survival
	traT	+	+	Surface exclusion and serum survival
	fliC	+	+	Flagellin structural protein, H antigen determinant (H19 allele)

Table 3 Antibiotic and heavy metal resistance genes identified within ETEC O157

Resistance gene/Operon	O157 SvETEC	O157:K88 734/3	Phenotypic function/Resistance	Resistance observed
intI1	+	+	Class 1 Integron Integrase	
intI2	+	+	Class 2 Integron Integrase	
bla_{TEM}	+	+	Ampicillin	+
ampC	+	+	Cephalosporins	O157:K88 734/3 only
gyrA	+	+	Quinolones	+
aphA1	+	+	Aminoglycosides	+
APH(4)-IA	+		Hygromycin B	O157 SvETEC only
strAB	+	+	Streptomycin	+
aadA	+	+	Streptomycin and Spectinomycin	+
dfrA	+	+	Trimethoprim	+
aacC	+		Apramycin	O157 SvETEC only
sul1	+	+	Sulphonamides	+
sul2	+	+	Sulphonamides	+
sul3	+		Sulphonamides	+
macAB	+	+	Macrolides	
tet operon	+	+	Tetracycline	+
cmlA	+		Chloramphenicol	O157 SvETEC only
emr operon	+	+	RND Efflux Pump	
gadX	+	+	Acid Resistance Regulator	
mdt operon	+	+	RND Efflux Pump	
cus operon	+	+	Heavy Metals	
mer operon	+	+	Mercury	
ter operon	+		Tellurium	

strains known to adopt different pathogenic strategies; *E. coli* O157:K88 734/3 associated with neonatal diarrhoea and *E. coli* O157 SvETEC associated with severe pre- and post-weaning disease. Our analysis demonstrated an evolutionary contrast between ETEC O157 and EHEC O157 and identified various small differences in toxin and antimicrobial resistance gene content between the two ETEC O157 isolates.

Molecular evolution of ETEC O157 strains

The PhyloSift analysis demonstrated that the O157 SvETEC and O157:K88 734/3 ETEC isolates are most closely related to an APEC O78 isolate. This is interesting because previous work on APEC O78 isolates has reported such isolates to be closely related to human ETEC isolates [30]. PhlyoSift analysis also demonstrated a degree of relatedness between the ETEC O157 isolates, *E. coli* W and enterohaemorrhagic *E. coli* O111:H$^-$. These results highlight the similarity of various *E. coli* pathotypes and serogroups when observed from a genomic perspective. In doing so these results further stress that clonal ancestry can play a minor role in predicting pathogenesis compared with the lateral acquisition of virulence factors. The PhyloSift analysis, which utilised the DNA sequences of 37 conserved marker genes, also demonstrated a contrast between the genetic relatedness of most *E. coli* O157:H7 genomes from the GenBank database and our ETEC O157:H19 genomes. Two O157 genomes proved exceptions to this observation; *E. coli* O157:H43 str. T22 from Hungary [31] and O157:H42 str. NCCP15738 from Korea (GenBank:ASHB01000001.1). Clustering of *E. coli* O157:H7 isolates and the divergence of non-H7 strains in the phylogenetic analysis supports the idea that H-antigen typing may be a useful indicator of *E. coli* O157 lineage. In addition, the atypical (*stx*- and *eae*-negative) O157:H43 str. T22 encode CDT and long polar fimbriae [29], which underscores how O157 isolates carrying different H-antigens may carry different repertoires of virulence genes.

Observations of *E. coli* relatedness similar to these have been made previously in relation to the parallel evolution of EHEC pathogenesis within the distantly related serogroups such as O157, O26 and O111 [32], where pathogenesis was mediated by similar laterally acquired elements such as plasmids and prophages. This phylogenetic divergence also suggests genetic recombination played a role in ETEC O157 development, with the ETEC O157 isolates encoding the "Sakai-type" O157 O-antigen biosynthesis

gene cluster [8]. The acquisition of this biosynthesis gene cluster is an event known to have also shaped the evolution of enterohaemorrhagic *E. coli* O157 [33].

Multi-Locus Sequence Typing analysis of the O157 SvETEC and O157:K88 734/3 genomes supported their close evolutionary relationship to each other and to the ST23 complex which includes APEC O78. In the MLST database *E. coli* ST23 isolates have been sourced from avian, porcine, human and bovine hosts. Enterotoxigenic *E. coli* O157 strains of porcine origin from different geographic locations have been submitted to the MLST database as ST90 (ST23-complex) although we could find little mention or analysis of these strains in the literature.

Comparison of ETEC O157 core and accessory genomes

Analysis of scaffolds representing the core genome from O157 SvETEC and O157:K88 734/3 highlighted the close relationship of these strains. Genetic differences were largely associated with the acquisition of prophage-associated sequences. Some prophages were common between the two isolates suggesting they may have been acquired prior to an evolutionary divergence. One phage in O157:K88 734/3 contained the *bor* gene which is known to be associated with increased serum survival. The *bor* gene has a lambda phage origin and is generally associated with avian extraintestinal pathogenic *E. coli* [34].

The variability of the accessory genomes for each isolate is mediated mostly by plasmid sequences, particularly IncH1 plasmid genes and heavy metal resistance operons, indicating that the differences in pathogenesis between these two isolates is essentially associated with plasmid content. Attempts to purify plasmids from our isolates using various plasmid extraction kits and standard alkaline lysis methods were unsuccessful. It is possible that the plasmids are extremely large, beyond the capability of the extraction methods used, or they are embedded in the chromosomes of these isolates. We were unable to confirm the presence of genomic islands in our analysis because of the reference genome based tiling exercise employed to separate the subset of scaffolds potentially encoding the core genome and accessory genomes. Large mass plasmids encoding resistance to multiple antibiotics in *E. coli* isolated from the faeces of food animals in Australia have been described [35,36] and as such may play a role in the pathogenesis of these isolates. Heavy metals are used frequently in pig production as antimicrobials and the identification of copper and mercury resistance loci in both strains and tellurium resistance in O157 SvETEC are likely provide significant survival advantages to these isolates. Antimicrobial resistance genes, discussed below, provide further advantages to these isolates. Such genes are often found on plasmids carrying mercury resistance transposons that serve as conduits for the lateral transfer of such genes [37,38].

Virulence factors

E. coli virulence factors that are routinely screened for globally [4,39,40] were identified in the O157 SvETEC and O157:K88 734/3 genomes. These included the porcine ETEC specific K88 fimbrial adhesin, and several adhesins commonly seen in other pathotypes of *E. coli*. The *fimH* gene, which encodes a mannose-specific adhesin in extraintestinal pathogenic *E. coli* [41] has also been observed in commensal *E. coli* isolates or porcine origin [42]. BLAST analysis identified various adhesins, however most of these are common to *E. coli* isolates and have no definitive role in pathogenicity. The primary difference between the virulence profiles of the O157 SvETEC strain and the O157:K88 734/3 strain is that the latter lacks the heat-stable enterotoxin a (STa) and a second copy of STb. Links between the enterotoxins present in an isolate and the form of pathogenesis caused by ETEC have been described previously [43,44] and these differences may impact pathogenesis. Previous work has also shown that the pTC plasmid-like variant of STb, which is homologous to the extra STb allele encoded in O157 SvETEC, is not enterotoxigenic *in vivo* [45,46].

Other virulence associated genes identified in the O157 SvETEC and the O157:K88 734/3 genomes included the flagellin structural protein *fliC*. The presence of the *fliC* gene, which encodes the H19 flagellar antigen [47], is important. Previous studies have demonstrated a correlation between the expression of flagellum and the K88ac fimbrial antigen [48] where the expression of both *fliC* and *faeG,* the major subunit of the K88 adhesin, correlates with adhesion to porcine epithelial cells *in vitro*. The characterisation of the flagellum also allows us to identify the serotype of both isolates as *E. coli* O157:H19:K88. Pathogenic *E. coli* of the serotype O157:H19 have been very sparingly reported [9] and studies involving isolates of this serotype have focussed on triclosan tolerance [49,50]. These studies do indicate that O157 strains have a capacity to adapt to multiple pathogenic lifestyles, having been identified as verocytotoxigenic, enterotoxigenic and enteropathogenic *E. coli*.

Another factor that appears to influence ETEC pathogenicity is the host's intestinal development and environment. Environmental conditions have an impact on enteroheamorrhagic *E. coli* pathogenesis and the regulation of virulence factor expression [51]. A similar situation has been observed *in vitro* for ETEC, with LT expression modified by pH [52]. Also, it has been demonstrated that the porcine intestinal epithelium can develop a resistance to F18-mediated adherence [53] and the stress of early weaning reduces the ability of piglets to cope with ETEC infection [54]. This data indicates that whilst it is important to characterise pathogenic *E. coli*, host characteristics and the intestinal environment may play a role in altering pathogenicity and account

for the variation in disease severity between these isolates given their highly similar virulence factor content.

Antibiotic resistance

The greatest disparity between the O157 SvETEC and O157:K88 734/3 isolates is their antibiotic resistance. Both have quite comprehensive resistance profiles being resistant to beta-lactams, quinolones, aminoglycosides and cephalosporins. In context, one of the most important antimicrobial resistance genes in either strain is *aphA1* which confers resistance to neomycin and kanamycin, with neomycin being a drug of choice for the treatment of infection during pig development within Australian pig farms. The majority of the observed resistance phenotypes have been accounted for including a known nalidixic acid resistance mutation in the DNA gyrase gene *gyrA*. Large complex antimicrobial gene resistance loci centred around both class 1 and class 2 integrons were identified within these genome sequences, however the prevalence of the insertion sequence IS*26* in these regions precluded the automated assembly of the loci.

Conclusion

O157 SvETEC and O157:K88 734/3 appear to be part of a poorly characterised lineage of *E. coli* O157, which differs significantly from EHEC O157. Differences in pathogenicity between the strains may stem from differences in acquired virulence factors, antimicrobial resistance, and phage related genes (the majority of which are uncharacterised hypotheticals) with *E. coli* O157 SvETEC encoding a larger toxin repertoire compared to O157:K88 734/3. Further studies with these strains will focus on the assembly of antibiotic resistance gene loci and their association with mobile genetic elements.

Additional file

Additional file 1: A spreadsheet containing a BLASTp comparison of all identified open reading frames in the accessory genomes of both O157 SvETEC and O157:K88 734/3 draft genome sequences. Data includes scaffold numbers, ORF annotations and BLASTp homology as a percentage.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SPD, TC, AD, SA, IC conceived the study. PRC and EW conducted the comparative genomic analyses. JS prepared the libraries for genome sequencing. SA and TC conducted initial PCR and phenotypic testing for antibiotic resistance. SPD, TC, EW, PRC, AD, SA and IC wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 5: Complete Sequences of Multiple-Drug Resistant IncHI2 ST3 Plasmids in *Escherichia coli* of Porcine Origin in Australia

Author contribution statement: I (Ethan R. Wyrsch) was primary author of this manuscript, performing the genomic comparisons, generating figures and writing the initial manuscript. I also performed DNA extractions for long-read sequencing. Toni A. Chapman provided samples and guidance. Piklu Roy Chowdhury assisted with data interpretation. Michael Y. Liu and Cameron J. Reid performed additional DNA extractions and sample preparation. Matthew Z. DeMaere performed genome assemblies. Steven P. Djordjevic conceived the study, provided funds and together with me and Piklu Roy Chowdhury, wrote the final manuscript. Final editing contributions came from all authors.

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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**).

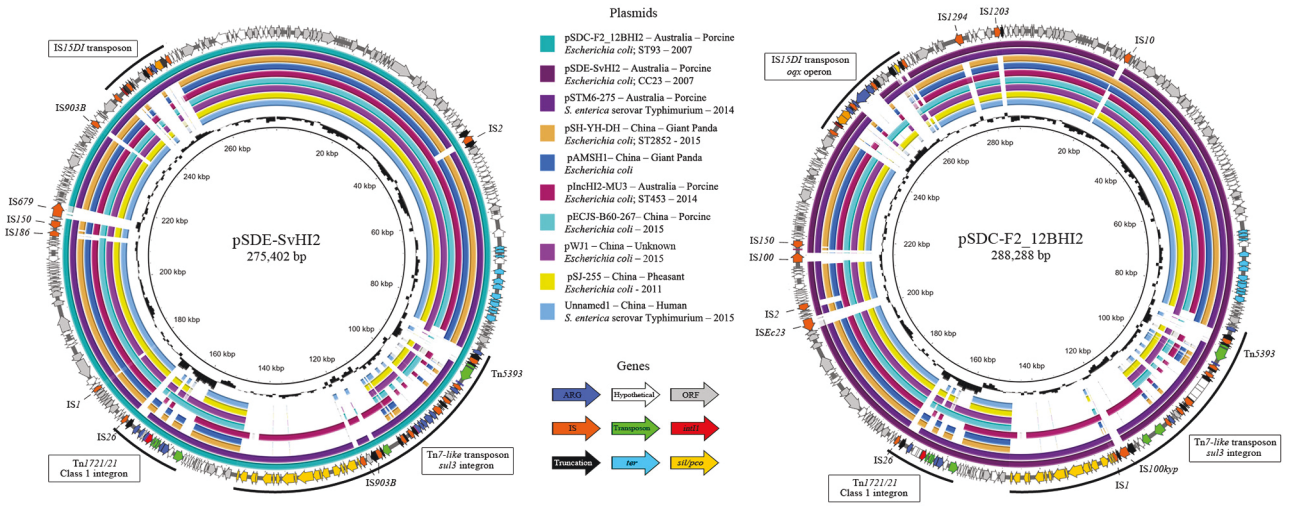
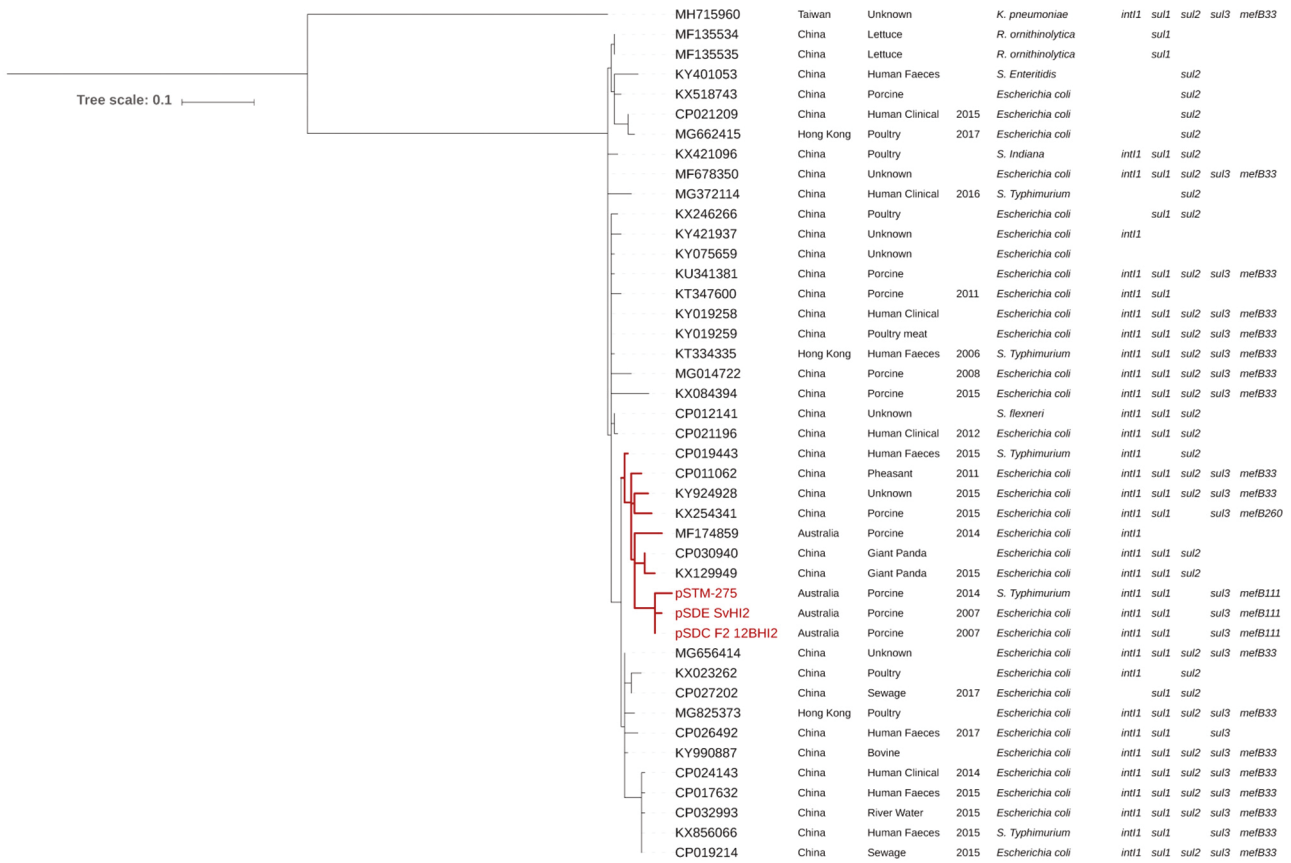


FIGURE 1 | Plasmid maps and alignments of pSDE-SvHI2 **(Left)** and pSDC-F2_12BHI2 **(Right)**. Maps display plasmid annotations, with genes color coded by function. Annotations highlight important complex resistance structures and insertions on each plasmid. Inner rings show BLASTn alignments against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



Plasmid	Country	Source	Year	Host	Integron Profile
MH715960	Taiwan	Unknown		*K. pneumoniae*	*intI1 sul1 sul2 sul3 mefB33*
MF135534	China	Lettuce		*R. ornithinolytica*	*sul1*
MF135535	China	Lettuce		*R. ornithinolytica*	*sul1*
KY401053	China	Human Faeces		*S. Enteritidis*	*sul2*
KX518743	China	Porcine		*Escherichia coli*	*sul2*
CP021209	China	Human Clinical	2015	*Escherichia coli*	*sul2*
MG662415	Hong Kong	Poultry	2017	*Escherichia coli*	*sul2*
KX421096	China	Poultry		*S. Indiana*	*intI1 sul1 sul2*
MF678350	China	Unknown		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
MG372114	China	Human Clinical	2016	*S. Typhimurium*	*sul2*
KX246266	China	Poultry		*Escherichia coli*	*sul1 sul2*
KY421937	China	Unknown		*Escherichia coli*	*intI1*
KY075659	China	Unknown		*Escherichia coli*	
KU341381	China	Porcine		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KT347600	China	Porcine	2011	*Escherichia coli*	*intI1 sul1*
KY019258	China	Human Clinical		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KY019259	China	Poultry meat		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KT334335	Hong Kong	Human Faeces	2006	*S. Typhimurium*	*intI1 sul1 sul2 sul3 mefB33*
MG014722	China	Porcine	2008	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KX084394	China	Porcine	2015	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
CP012141	China	Unknown		*S. flexneri*	*intI1 sul1 sul2*
CP021196	China	Human Clinical	2012	*Escherichia coli*	*intI1 sul1 sul2*
CP019443	China	Human Faeces	2015	*S. Typhimurium*	*intI1 sul2*
CP011062	China	Pheasant	2011	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KY924928	China	Unknown	2015	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KX254341	China	Porcine	2015	*Escherichia coli*	*intI1 sul1 sul3 mefB260*
MF174859	Australia	Porcine	2014	*Escherichia coli*	*intI1*
CP030940	China	Giant Panda		*Escherichia coli*	*intI1 sul1 sul2*
KX129949	China	Giant Panda	2015	*Escherichia coli*	*intI1 sul1 sul2*
pSTM-275	Australia	Porcine	2014	*S. Typhimurium*	*intI1 sul1 sul3 mefB111*
pSDE SvHI2	Australia	Porcine	2007	*Escherichia coli*	*intI1 sul1 sul3 mefB111*
pSDC F2 12BHI2	Australia	Porcine	2007	*Escherichia coli*	*intI1 sul1 sul3 mefB111*
MG656414	China	Unknown		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KX023262	China	Poultry		*Escherichia coli*	*intI1 sul2*
CP027202	China	Sewage	2017	*Escherichia coli*	*sul1 sul2*
MG825373	Hong Kong	Poultry		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
CP026492	China	Human Faeces	2017	*Escherichia coli*	*intI1 sul1 sul3*
KY990887	China	Bovine		*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
CP024143	China	Human Clinical	2014	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
CP017632	China	Human Faeces	2015	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
CP032993	China	River Water	2015	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*
KX856066	China	Human Faeces	2015	*S. Typhimurium*	*intI1 sul1 sul3 mefB33*
CP019214	China	Sewage	2015	*Escherichia coli*	*intI1 sul1 sul2 sul3 mefB33*

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.

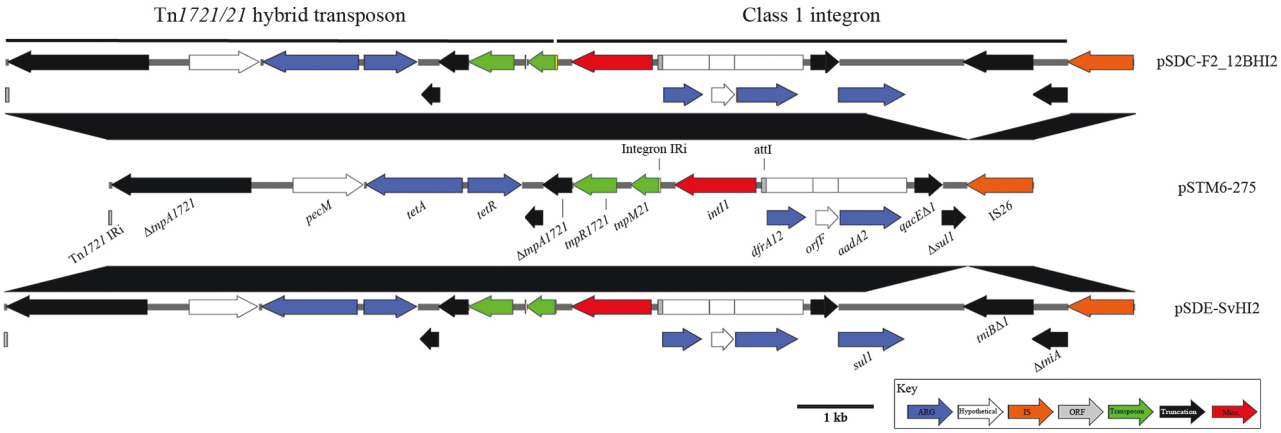


FIGURE 3 | Diagrams and comparisons of Tn*1721*/Tn*21* hybrid transposons carrying a class 1 integron. Clinical class 1 integron-based locus, with comparisons to the highly similar structure found in pSTM6-275 (center). Genes are colored by function, with black connecting regions indicating homology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

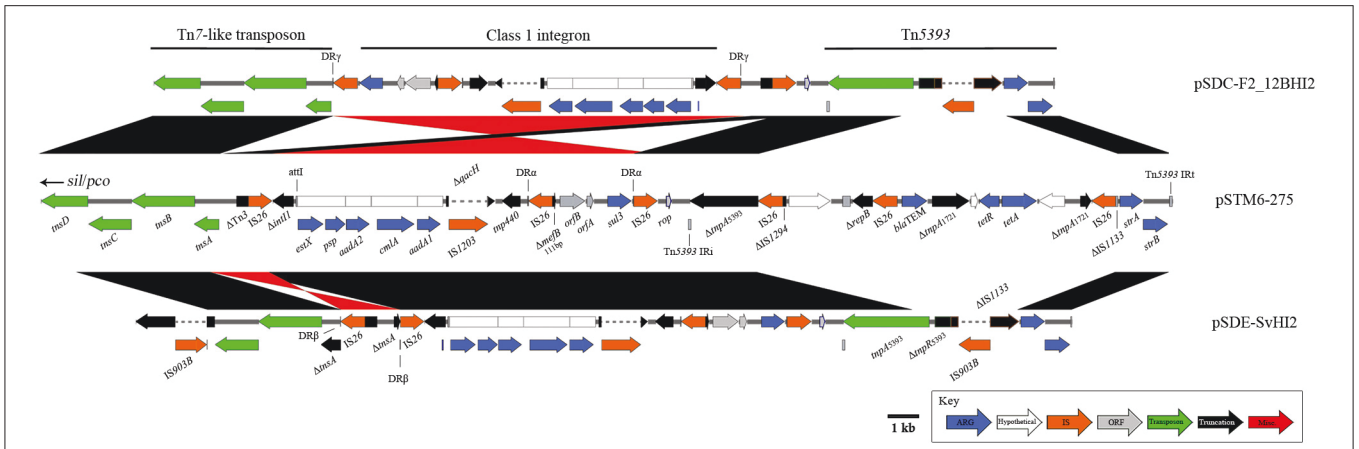


FIGURE 4 | Diagrams and comparisons of *sul3*-associated resistance structures carrying an IS*26* truncated *intI1* Structural comparisons between related *sul3* encoding regions, comprising a putative transposon, *sul3*-CS integron and Tn*5393*. Alignments in black show homology, with inversions indicated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

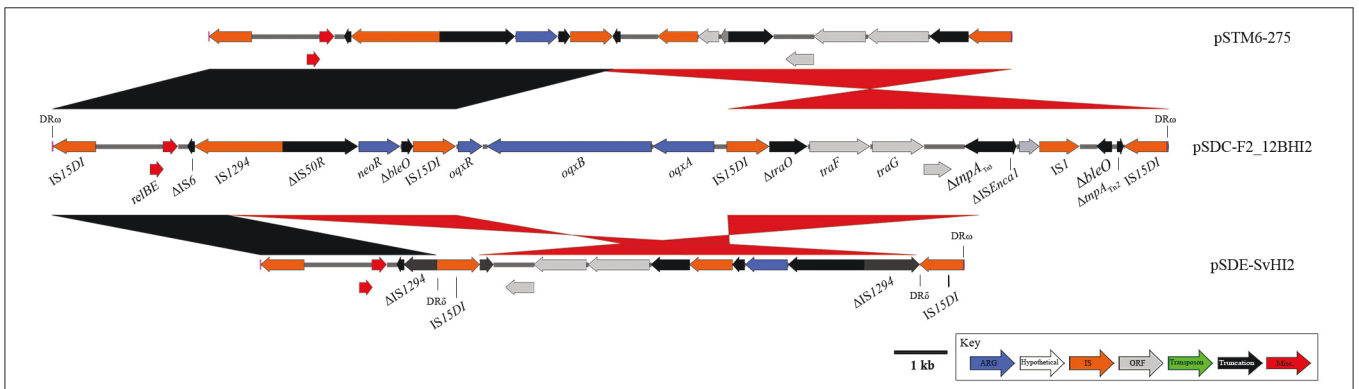


FIGURE 5 | Diagrams and comparisons of an IS*15DI*-mobilized composite transposon. Composite transposon captured by IS*26* variant *IS15DI*, mobilizing the neomycin resistance gene *neoR*. pSDC-F2_12BHI2 (center) also hosts a Tn*6010*-like element, inserted at the central IS*15DI*.

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 Δ$tnpA_{Tn3}$.

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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Chapter 6: General Discussion

6.1 The epidemiology of antimicrobial resistant *Escherichia coli* and the mobile elements circulating in porcine production animals

Data summarised in Chapter 2 was drawn from published literature exploring *Escherichia coli* of porcine agricultural origin around the globe and identified 22 countries with AMR present. Available genotyping data showed that many were the expected reservoirs for antimicrobial resistance mechanisms protecting against non-restricted antibiotics, but the literature is skewed by limitations in gene target selection. Data from most countries show the presence of integrons, with known gene cassettes for aminoglycoside (*aadA*), trimethoprim (*dfrA*) and chloramphenicol (*cmlA*) resistance featuring prominently (Chapter 2, Table 1). Also seen were the sulphonamide resistance genes *sul1*, *sul2* and *sul3*, potentially linked to class 1 integron structures. Tetracycline and penicillin resistance (*tet* and *bla*$_{\text{TEM}}$) gene variants were common. In some countries medically relevant resistances, including the extended-spectrum beta-lactamases (ESBLs) *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CMY}}$ and *bla*$_{\text{OXA,}}$ and potentially fluoroquinolone resistance (*qnr*), have also been detected. Various studies have also generated some sequence typing data on these strains and have identified ST10 as a dominant sub-clade within many porcine samples. Six separate studies in Australia identified *intI1* and *intI2*, and a range of gene cassettes (*aadA, dfrA, cmlA, cat1, cat2, ereA, aacC4*), the three *sul* genes, *strAB*, *aphA*, *bla*$_{\text{TEM}}$ and *tet*. This is a profile matching known surveyed antimicrobial use (1). ESBL genes were also detected in sporadic isolates linked to known off-label cephalosporin usage (2). As far as broad epidemiological analysis, limited sequence data is available from *E. coli* of porcine origin.

6.1.1 Whole genome sequencing of porcine isolates

Generation of whole genome sequences allows us to correlate sequence types, serotypes and phylogroups with more detailed relationships from phylogenetic analyses and gene data characterising AMR, virulence, and the presence of mobile elements. As part of the commensal *E. coli* study (Chapter 2), WGS data from this project (designated F1 and ETEC) was combined with, and compared to, sequence data from a second Australian site (F2) (Figure 6.1). Like F1, samples in F2 were generally selected for sequencing because of the presence of *intI1*. F2 samples were not associated with antibiotic usage or disease. A total of 141 sequences were uploaded to GenBank from F1 and F2, assembled using A5-miseq, but

only 103 were included in much of the final analysis due to either a lack of *intI1* or clonal exclusion to reduce the effect of over-sampling of individual pigs.

Epidemiological distribution of drug-resistant commensal *Escherichia coli*

The distribution of phylogenetic groups from these farms correlates well with known *E. coli* population structures, with a proportionally largest group A, then B1, D and B2. This gives us an indication we sampled a potentially normal *E. coli* population, despite the integron-positive skew. There were also few instances of over-sampling a clone from any single animal. This is an indicator of the infiltration of multiple-drug resistance into commensal strains. Between F1 and F2, 37 STs were identified, and 7 were shared between the two sites. ST10 and its relatives ST48 and ST218 represented a considerable proportion of the phylogroup A population, matching data from international animal samples. There was a clear intermixing of sequence types from both farms overall, giving an indication of potential commonalities between them. Within F1 too, there was some transfer of closely related strains between sample groups.

E. coli ETECM/10, an O139:H1-ST1 strain, matched a close homologue from the F1 samples, 6B from the outbreak cohort. F1_368E, a phylogroup A strain from the treatment cohort, also has pathogen characteristics (O149:H10-STNT) and is related to the international reference porcine pathogen UMNK88, an O149:H10-ST100 strain. Interestingly, there is another small clade of phylogroup A-ST1721 strains of serogroup O149:H45, related to ST218-O26:H12 strains. Another notable clade was the ST301s, a set of O157:H2 strains from F1 and F2, distinct from the ETEC O157 strains characterised separately.

Virulence gene carriage amongst the commensal population was limited, with only 11 hosting ETEC-associated virulence toxins (*stb, eltAB*), but none hosted the adhesins vital pathogenicity. Additionally, a range of genes were detected in most strains that are known to contribute to extraintestinal pathogenesis. Iron acquisition genes, serum resistance (*iss*) and general adhesins like *fimH* and *csgG* were common. Two strains stood out, and both belonged to known ExPEC lineages; ST131 and ST117. Both sequence types, as well as the genes we identified within them, are known to circulate in poultry and humans (3).

Plasmids replicons (incompatibility groups) were detected in high numbers across the collections with IncF, IncI, IncY, IncHI2 and IncX the most prominent. Using WGS data, however, it is difficult to place specific mobile elements in context, and this heavily

obfuscates assigning specific resistance profiles to plasmids, particularly in cases where there are multiple CRL profiles. Phage profiles weren't generated for this reason.

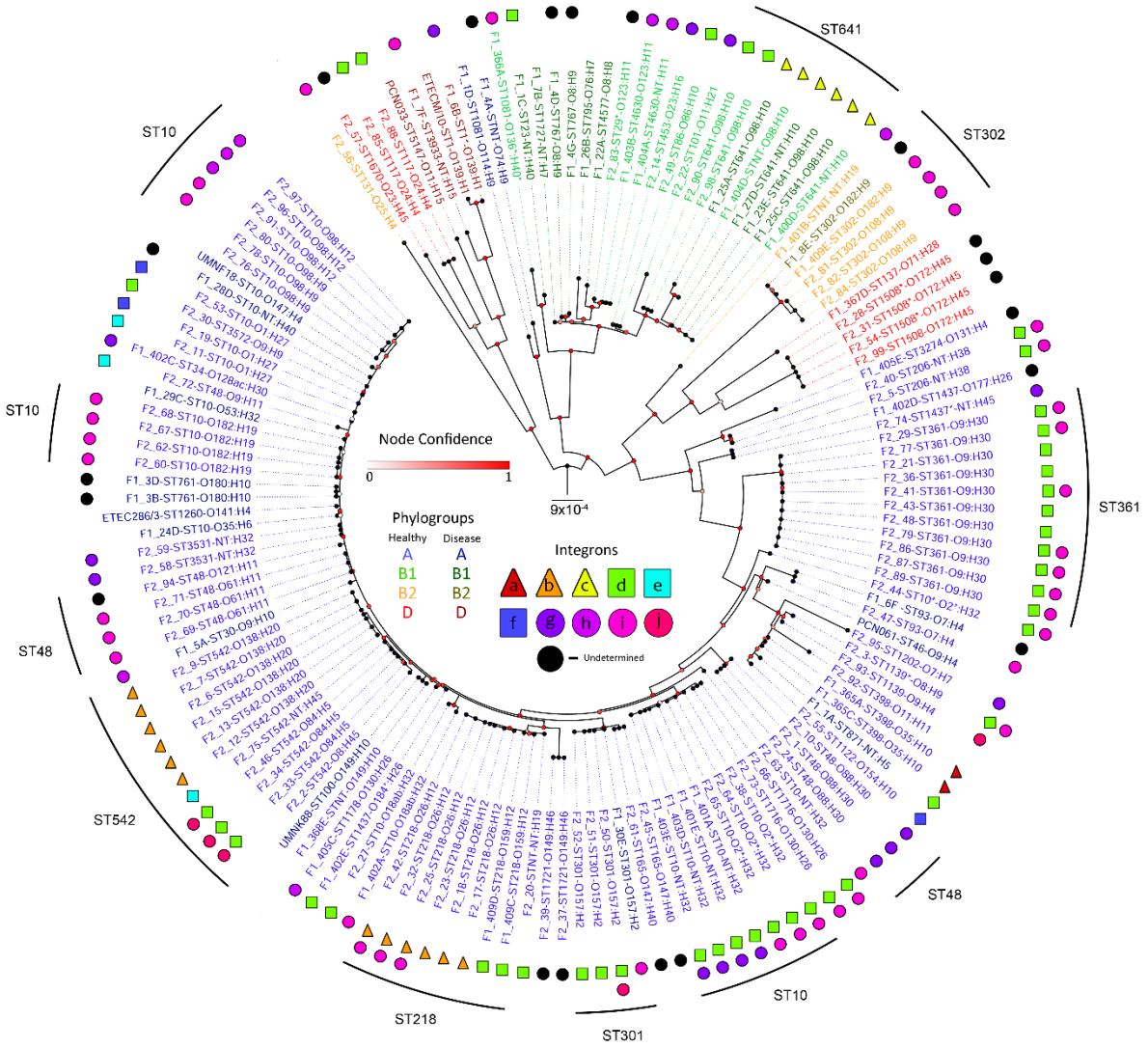

Figure 6.1: A mid-point rooted, maximum-likelihood phylogenetic tree generated from PhyloSift v1.0.1 and FigTree v1.4.2. The tree contains all draft whole genome sequences presented from both Australian facilities, plus two of the enterotoxigenic strains and four reference sequences, for a total of 149 isolates. Tip names contain ST and serotype data, and colouring shows the phylogroup data (A-blue, B1-green, B2-orange or D-red, darker for disease associated strains), with major MLSTs labelled on the outer ring. Node confidence values have been coloured on a 0 to 1, white to red scale. The tree scale (centre) shows the distance for 0.0009 substitutions per site. Integron subtypes are shown as shapes at the tip edges. Note this tree differs slightly from that in Ch. 3 Figure 1 due to the full sample size inclusion, and an altered naming structure for F2.

Antimicrobial resistance genes and signatures

Across the entire collection, no extended-spectrum beta-lactamases or medically relevant resistances were identified. This is a sign antimicrobial stewardship is a success in this case.

In the 103 *intI1* positive sequences, ten separate structures were identified hosting the integrase (See Chapter 3 – Figure 3). As these data come from short-read assemblies, scaffolding limits the context we can draw from this analysis. Interestingly, the *sul2* and *sul3* derivatives of the clinical class 1 integron were more common than the original *sul1*-hosting structure (Figure 5). Most prevalent was a *dfrA* hosting structure with a known IS*26* insertion associated with Tn*6026* (Ch. 3, Figure 3d). Of the 37 strains hosting this structure, 27 also carried *sul2, strAB,* bla_{TEM} and *aphA1* (aminoglycoside resistance), the genes mobilised in Tn*6026*. The remaining ten may have lost the genes through IS*26* mobilisation, but, again, Illumina short read sequencing is not often suitable for determining context. This signature was identified across 15 sequence types. Second most prevalent was the *sul3* integron associated with a $mefB_{111\text{bp}}$ remnant (Ch. 3, Figure 3i). This structure also had an IS*26* insertion into *intI1*, an *estX-psp-aadA2-cmlA-aadA1* cassette array and an IS*1203*-like insertion into *qacH*. It was seen in 26 strains across 13 sequence types. The *intI1* may have been functional and integrated the cassettes pre-interruption, or a separate *intI* gene functioned *in-trans* to facilitate integration. Two $mefB_{260\text{bp}}$ structures were identified, one with a cassette array matching the $mefB_{111\text{bp}}$ structure and a second, more prevalent structure hosting *dfrA12* and *orfF*, rather than *estX* and *psp* (Ch. 3, Figure 3h and 3g). Neither of these structures had the IS*1203*-like insertion into *qacH*. Integrons still hosting *sul1* had either *aadA1, dfrA5,* or *dfrA12-orfF-aadA2* as a cassette array.

Other resistance genes were identified in the collections outside of these scaffolds, including a large amount of tetracycline resistance and beta-lactam resistance, with lesser instances of quinolone (*oqxAB*) and hygromycin B resistance. Heavy metal resistance, to mercury, copper and tellurite, was also common. The transposons Tn*21* (*mer* mercury resistance associated) and Tn*1721* (*tet* associated) were commonly detected, particularly Tn*1721*, likely accounting for the large amounts of tetracycline resistance in the isolates. The insertion sequence often implicated in the formation of these resistance structures, IS*26* of the IS*6* family, was almost universally present in the samples. A low level of *intI2* carriage was also detected, likely contributing to some of the matches to AMR gene cassettes like *dfrA* and *aadA*. The class 2 integron is generally associated with Tn*7*, which targets a specific chromosomal insertion site within *glmS*.

Enterotoxigenic O157 pathogens

The EMAI provided two pathogens of a serogroup associated with enterohaemorrhagic *E. coli*, and with a known source in the ruminant gut, O157 (4). These ETEC strains, *E. coli*

O157 SvETEC and O157:K88 734/3, were isolated in 2008 and the mid 1990's respectively, both as the cause of disease in young piglets. O157 SvETEC caused severe disease and resisted treatments.

A phylogenetic analysis of these strains and a range of reference sequences (Chapter 4 – Figure 1) demonstrated that they were a clonally related compared to a broad range of reference sequences, and one of several clades of *E. coli* hosting the O157 biosynthesis genes. Interestingly, the LPS biosynthesis gene *rfbE* of both ETEC matched exactly with the O157 pathogen *E. coli* O157:H7 str. Sakai, part of the highly studied ST11 clade, whilst the ETEC are both O157:H19, ST4245 of clonal complex 23. The draft genome sequences of these strains were compared to a close reference from CC23, *E. coli* APEC O78, helping sort acquired sequence from chromosomal and allowing some characterisation of phage scaffolds (although reference associations aren't reliable for identification of specific phages in this way), plasmid-associated scaffolds and identification of genes encoding AMR, virulence, and mobility.

The ETEC O157 strains encoded a full suite of virulence genes (Ch. 4, Table 2). Starting with toxins, both carried the LT and STb genes, with O157 SvETEC also hosting an STa and a second STb gene allele. These were complemented by the alpha-haemolysin *hlyA*. On top of a suite of chromosomal adhesins, both encoded the K88 (F4, *fae*) adhesin prevalent in enterotoxigenic *E. coli*. Their resistance profiles were also both broad and indicative of their agricultural sources (Ch. 4, Table 3). The class 1 and 2 integrase genes were present, plus *sul1* and *sul2* in both, with *sul3* also in the more recent strain, with copper and mercury resistance present in both and tellurium also only in the most recent strain. A fosmid library was successfully generated from O157 SvETEC, and PCR characterisation of the resultant clones suggested the presence of multiple integrons, as we suspected given the range of *sul* genes present, however sequencing of the products demonstrated the presence of CRLs that lacked reference at the time, so the context of most of these genes was left unresolved using draft genome sequence data.

These strains represent an interesting and potentially dangerous lineage. Clearly it has persisted for some time and hosts a range of virulence factors. Their combination with the antimicrobial resistance genes leaves farmers few antimicrobial treatment options, making it a potentially important target for epidemiological studies.

6.1.2 IncHI2 plasmids hosting multiple-antimicrobial resistance in *Enterobacteriaceae*

Long read sequencing was performed on two strains from this collection hosting *sul3* with a *mefB*$_{111bp}$ signature remnant (Chapter 5). The pathogen *E. coli* O157 SvETEC, and commensal ST93 strain F2_12B (O7:H4) were selected, and a Unicycler assembly resolved two large IncHI2 plasmids hosting multiple CRLs. Plasmids pSDE-SvHI2 and pSDC-F2_12BHI2 were 275,402 bp and 288,288 bp, respectively, and subtyped as IncHI2:ST3.

IncHI2 plasmids are known disseminators of drug resistance globally, and uniquely carry a *ter* tellurite resistance operon. The prototypical IncHI2 plasmids are ST1, and several other types have been sequenced. A SNP analysis of the closest reference sequences, including each sequence type, revealed a clade of ST3 plasmids sourced from mainland Asia and Australia (Ch. 5, Figure 2). These plasmids were sourced mostly from human and animal hosts in China, Hong Kong, and Laos, from multiple pathogens, and each encoded drug resistance (Ch. 5, Table 1).

Importantly, one of the sequences identified was a recent, highly homologous Australian plasmid sourced from porcine *Salmonella* Typhimurium 1,4,[5],12:i:- in 2013, pSTM6-275 (5). Comparative analyses between these plasmids showed that the Australian plasmids carried similar CRLs, and these were unique compared to the remaining ST3 plasmids (Ch. 5, Figure 1). Beyond the resistance loci, the only variation between the Australian plasmids was in IS element insertions and a few SNPs, giving it a reasonably conserved backbone.

Of the resistance loci, two host a class 1 integron and one is mobilised by an IS*26* homologue, IS*15DI*. The smaller of the integron structures is hosted in a Tn*1721/21* transposon (Ch. 5, Figure 3), a structure seen rarely that has combined the *tetAR* tetracycline resistance operon with the modulator protein of Tn*21*, *tnpM.* The cassette in each case was *dfrA12-orfF-aadA2*. The 2007 structures were identical in this case, both with an IS*26* insertion in the Tn*402* remnant *tniA* providing the border of the structure. In pSTM6-275, the IS*26* border is now within a truncated *sul1* gene. As *sul3* is also present on the plasmid, this loss of a resistance gene may have had no effect on the fitness of host.

The largest of the structures is a transposon, currently designated *Tn7*-like, mobilising a copper and silver resistance operon (Ch. 5, Figure 4). Matches to database sequences show this transposon has apparent roots in Asia. The *sul3-mefB*$_{111bp}$ integron structure has then been mobilised into the *tnp* region of this transposon, potentially by IS*26*. The integron

hosted the *estX-psp-aadA2-cmlA-aadA1* cassette, matching WGS data. This is then adjacent to Tn*5393,* which is a known distributer of *strAB*, but the variants from 2007 both have an IS*903B* element inserted into the IS*1133* usually present. There are numerous insertions and inversions that alter these structures individually, but the most influential is possibly the insertion of tetracycline (Tn*1721* remnant) and penicillin resistance genes into the 2013 structure, further consolidating agriculturally relevant resistances into the structure. Insertions individually present in the 2007 structures inform us that they were variants of the progenitor of the structure in pSTM6-275, but can't be direct progenitors. The total contribution to the resistance profile by the structure is resistant to streptomycin/spectinomycin, chloramphenicol (amongst others for *cmlA*) and sulphonamides, with the addition of tetracycline and penicillin in the more recent variant.

The last structure is mobilised by *IS15DI* and is composed of a combination of IS elements and plasmid fragments, also hosting a neomycin resistance gene (Ch.5, Figure 5). In pSDC-F2_12BHI2, *IS15DI* had also introduced an *oqx* operon, encoding quinolone resistance. Inversions and some gene loss in pSDE-SvHI2 again alter the individual CRLs.

Across the IncHI2:ST3 plasmids available, loci encoding similar genes to these CRLs are present, including *sul3* and the *oqx* operon, but the context of these genes seems uniquely Australian for now, and with the high levels of AMR encoded by these plasmids, tracking any current distribution will be an important first step in tackling AMR within pigs.

6.2 Summary and future challenges

Genomic epidemiology has become a truly powerful tool only very recently, with the rise in WGS data, computing capacities and software to perform sequence analysis. As part of this project, a collection of *E. coli* from pigs was successfully sequenced, providing baseline data for the presence of serogroup-sequence type combination amongst drug-resistant strains, and characterising the virulence and ARG content, integron types and the dominant plasmid replicons. Several enterotoxigenic pathogens were also sequenced, including a novel O157 lineage belonging to CC23, and a dominant plasmid lineage encoding AMR with a lot of redundancy, plus three heavy metal resistance operons. This plasmid has been identified on two agricultural sites, and in the severe pathogen O157 SvETEC and a separate porcine pathogen, isolated six years later, at which point it had acquired penicillin resistance, and added a second tetracycline resistance operon to the largest structure. The likelihood that these plasmids will remain prevalent is high, and the detailed annotation and comparisons

performed here may aid in future epidemiological surveillance by serving as a reference point for the development of multiple-drug resistance. While a range of IS elements are present in the collection, IS*26* clearly plays an active role in the rearrangement, consolidation, and mobilisation of these CRLs. The gene profiles in the collections supports a high prevalence of Tn*1721*, Tn*21*, Tn*5393* and Tn*6026*, with IncF, IncI, IncY, IncHI2 and IncX plasmids likely contributing to their mobilisation.

6.2.1 Completing reference sequences

The study of multiple-drug resistance and mobile elements its greatly hindered by the limitations of short read sequencing and assembly. With known reference sequences, however, WGS is a widely applicable tool to identify shorter key signature sequences and sequence profiles. As such, completing reference sequences of, at least at first, the dominant and unknown mobile elements is important. Long-read sequencing is becoming more accessible as it develops, and its ability to construct reference sequences will improve detailed epidemiological analysis.

Regarding this work specifically, a two more virulence and resistance plasmids (IncFII, IncI1), an enterotoxigenic P1 phage, and numerous chromosomal islands were identified in the completed *E. coli* O157 SvETEC assembly. After detailed annotation, the distribution of these structures within the general sample population can be determined.

6.2.2 Expanded sampling

The additional data from the F2 site greatly improved the inferences we can make regarding integron dominance and ST prevalence amongst the porcine samples, and as the pool of available data increases, our ability to track AMR and pathogen development through genetic signatures will improve.

For future sampling to have epidemiological significance, we must consider how multiple-antimicrobial resistance develops and persists after exposure to antimicrobials. A ‘One Health’ approach targets human, animal, water and terrestrial environments and will allow the characterisation of disparate sites under the influence of the same selective pressures. Identifying and managing the level of interconnectivity between environments and the impact they have can lead to healthier and more controlled microbial populations. Globally, animal production has been on the rise since the 1970s (6), and Australian pork production represents a small fraction of the global total- just 0.4%. China and the USA account for most of the production, with both consuming vast amounts of antimicrobials in the process. With

similar feed and growth strategies, and antimicrobial stewardship in place, the list of antibiotics used between countries overlaps significantly, generating the same selective pressures in animal production facilities and any downstream contaminated sites, across international borders. What effect this will have on the global microbiome is already apparent, and with the approaching universal distribution of specific resistance mechanisms, and no chance of reducing antibiotic use, we will require detailed genomic analysis to truly follow and predict bacterial evolution in the future.

6.3 References

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Appendix

Supplementary files for published works are available through the respective journals.