Evolution of a 72-kb cointegrant, conjugative multiresistance plasmid from early community-associated methicillin-resistant *Staphylococcus aureus* isolates

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Running Head: Conjugative multiresistance plasmid pWBG731

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Abstract. Horizontal transfer of plasmids encoding antimicrobial-resistance and virulence determinants has been instrumental in *Staphylococcus aureus* evolution, including the emergence of community-associated methicillin-resistant *S. aureus* (CA-MRSA). In the early 1990s the first CA-MRSA isolated in Western Australia (WA), WA-5, encoded cadmium, tetracycline and penicillin-resistance genes on plasmid pWBG753 (~30 kb). WA-5 and pWBG753 appeared only briefly in WA, however, fusidic-acid-resistance plasmids related to pWBG753 were also present in the first European CA-MRSA at the time. Here we characterized a 72-kb conjugative plasmid pWBG731 present in multiresistant WA-5-like clones from the same period. pWBG731 was a cointegrant formed from pWBG753 and a pWBG749-family conjugative plasmid. pWBG731 carried mupirocin, trimethoprim, cadmium and penicillin-resistance genes. The stepwise evolution of pWBG731 likely occurred through the combined actions of IS*257*, IS*257*-dependent miniature inverted-repeat transposable elements (MITEs) and the BinL resolution system of the β-lactamase transposon Tn*552*. An evolutionary intermediate ~42-kb non-conjugative plasmid pWBG715, possessed the same resistance genes as pWBG731 but retained an integrated copy of the small tetracycline-resistance plasmid pT181. IS*257* likely facilitated replacement of pT181 with conjugation genes on pWBG731, thus enabling autonomous transfer. Like conjugative plasmid pWBG749, pWBG731 also mobilized non-conjugative plasmids carrying *oriT* mimics. It seems likely that pWBG731 represents the product of multiple recombination events between the WA-5 pWBG753 plasmid and other mobile genetic elements present in indigenous CA-MSSA. The molecular evolution of pWBG731 saliently illustrates how diverse mobile genetic elements can together facilitate rapid accrual and horizontal dissemination of multiresistance in *S. aureus* CA-MRSA.

Introduction

Staphylococcus aureus is an opportunistic human and animal pathogen that causes nosocomial and community-associated infections, ranging from non-invasive soft tissue abscesses to life-threatening sepsis, pneumonia, bacteraemia and endocarditis (1-4). The increasing emergence of antimicrobial resistance in both hospital and community settings is a threat to global health. While methicillin-resistant isolates (MRSA) were once limited to hospital-associated infections (HA-MRSA), in the last three decades distinct community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains have proliferated and overtaken HA-MRSA as a dominant source of infection. Methicillin resistance and often other resistance loci are carried by the chromosomally-integrated SCC*mec* element, though additional antimicrobial-resistance genes in CA-MRSA are generally harboured on circular double-stranded DNA plasmids. In *S. aureus*, it is estimated more than 90% of isolates possess at least one plasmid and of these approximately ~79% carry a plasmid >20 kb, often encoding multiple resistance and virulence determinants (5). Horizontal transfer of these larger plasmids, and associated multiresistance, may occur via conjugation, conjugative mobilization and/or transduction. This rapid and ongoing resistance evolution reduces available treatment options for *S. aureus* infections.

The first MRSA, known as “classic MRSA” appeared in hospitals in the 1960s. By the mid-1970s, large global outbreaks of multiresistant HA-MRSA occurred in hospitals, where these and other HA-MRSA lineages largely remain endemic (6). In contrast, in Western Australia (WA) hospitals were largely unscathed by HA-MRSA, aside from a single outbreak in 1982 by HA-MRSA related to sequence-type (ST) 239, which was also present in eastern parts of Australia during the same period (7). In 1990-1992 the first cases of MRSA found outside hospitals in Australia were documented for patients originating in the far north of WA (8). These earliest CA-MRSA isolates, referred to as WA-MRSA (8) and later as WA-5 (9), were distinct from previously characterized HA-MRSA and the vast majority of WA-5 carried resistance determinants for cadmium, penicillin and tetracycline on a ~30-kb plasmid.

Sequence analysis (9) of a representative WA-5 strain WBG7583, revealed it to be ST8, with an SCC*mec* type IVa (2B) (9). The 30,047-bp plasmid pWBG753 (5) carries a Tn*552*-like β-lactamase region and an integrated copy of the tetracycline-resistance plasmid pT181, flanked by directly-repeated copies of insertion-sequence IS*257*. Interestingly, after the outbreak of WA-5 CA-MRSA, this particular strain and plasmid combination was not documented again. A follow-up study investigating CA-MRSA and CA-MSSA carriage in the same regions of WA in 1995-1996 (9) did not identify any additional ST8 CA-MRSA isolates. Instead, a variety of CA-MRSA

isolates of ST1, ST5, ST45, ST73 and ST78 were isolated, along with the likely CA-MSSA progenitors of the now-dominant Australian CA-MRSA ST93-SCC*mec* IVa (2B) lineage (10). The majority of CA-MRSA isolates in this study (9), like WA-5, carried SCC*mec* Type IVa (2B) (apart from two ST45 isolates carrying SCC*mec* Type V (5C2)) and resistance determinants for penicillin and cadmium, but plasmids in these strains were diverse and did not resemble pWBG753 or carry tetracycline resistance determinants (5). Furthermore, interrogation of a 2003-2004 collection of 4,099 CA-MRSA isolates from WA revealed only 0.66% (thirty-one) were WA-5 and only two of these exhibited tetracycline resistance. This again suggests the original WA-5 CA-MRSA isolates appeared only briefly before being supplanted by SCC*mec* IVa (2B)-CA-MRSA of diverse lineages with a similar spectrum of STs as the indigenous CA-MSSA populations (11).

Amongst the diverse CA-MRSA discovered in the 1995-1996 follow-up study of WA CA-MRSA were strains harbouring a new family of conjugative plasmids named the pWBG749 family. These included pWBG749 and pWBG745 from ST5 CA-MRSA (SCC*mec* IVa(2B)). The pWBG749 family of conjugative plasmids are genetically distinct from the well-characterized pSK41/pGO1 family, which were first isolated in North America in the mid-1970s (12, 13). pWBG749 conjugatively mobilizes a range of large, non-conjugative, multiresistance plasmids also present in CA-MRSA/CA-MSSA isolated in WA in 1990-1995 (14). Unlike the classical model of conjugative mobilization, where plasmids encode their own relaxase gene and a distinct *oriT*, pWBG749 mobilizes plasmids carrying a clone, or 'mimic', of the pWBG749 *oriT* sequence.

When WA-5 was first isolated, additional ST8 CA-MRSA and CA-MSSA with high-level resistance to mupirocin were isolated (15-17). Again, these strains originated in the northern part of WA where mupirocin was extensively used in the treatment of staphylococcal skin infections. The majority of isolates harboured a ~42-kb non-conjugative plasmid named pWBG715, which encoded resistance to mupirocin, penicillin, trimethoprim, cadmium and tetracycline. One characterized isolate, WBG8101, carried transferrable mupirocin and trimethoprim resistance on what appeared to be a ~75-kb conjugative plasmid (15-17). To gain further insight into resistance-plasmid evolution in some of these earliest CA-MRSA isolates, we present the complete sequence of the conjugative multiresistance plasmid pWBG731. pWBG731 is a cointegrant of two plasmids, the WA-5 pWBG753 plasmid and a conjugative plasmid resembling pWBG745. Copies of IS*257* mediated the cointegration of the two plasmids and likely played a role in the acquisition of mupirocin and trimethoprim-resistance determinants and loss of a cointegrated tetracycline resistance plasmid pT181. We also demonstrate pWBG731 has an *oriT* specificity distinct from pWBG749 and recognizes the same spectrum of *oriT* sequences as the recently characterized conjugative plasmid pC02 (18).

Results

Sequencing of pWBG731

To isolate pWBG731 in a strain devoid of phage, other plasmids and integrative and conjugative elements, pWBG731 was conjugated from its clinical host WBG8101 into the streptomycin and novobiocin-resistant RN4220 derivative WBG4515 (19), producing strain WBG10514. Conjugative transfer of pWBG731 from WBG10514 to the rifampicin and fusidic-acid-resistant RN4220 derivative WBG541 (19) occurred with a mean frequency of 6.7×10^{-5} exconjugants per donor (averaged from six replicate experiments with standard deviation of 5.8×10^{-5}). To obtain a complete sequence of pWBG731, paired-end short-read sequencing was initially carried out using plasmid DNA extracted from WBG10514. However, the pWBG731 sequence could not be unambiguously assembled into less than five contigs due to the presence of several near-identical copies of the 790-bp insertion sequence IS*257*. Attempts to close the pWBG731 sequence using PCR primers specific for sequences flanking the IS*257* insertions were unsuccessful; unexpectedly, all PCR reactions produced ~800-bp products regardless of primer combination and sequencing of each product revealed near identical IS*257* sequences flanked by sequences from each contig. This obvious artefact was likely produced through chimeric PCR product formation during amplification and hybridisation of distinct but near-identical IS*257* sequences (20). To resolve the structure of pWBG731, long-read Pacbio SMRT-cell sequencing was carried out. SMRT-cell subreads (mean length = 2.3 kb) were assembled using the long-read assembler Canu, producing an initial 80-kb circular contig. Following removal of overlapping contig ends and polishing with paired-end short-read sequences, a final circular 72,158 bp contig was produced, with final mapped-read mean coverage depths of 1,329-fold (σ=211) for long-read and 1,220-fold (σ=521) for short-read sequences (Genbank accession MH587574.1).

Structure and evolution of pWBG731

Analysis of the pWBG731 sequence revealed it to be a mosaic comprising parts of at least two staphylococcal plasmids, several additional DNA segments and five copies of IS*257* (IS*431*) (21) (Fig. 1). A 17,865-bp region was derived from a plasmid closely related to pWBG753, which as previously mentioned, carries genes for resistance to cadmium and a remnant of a Tn*552*-like β-lactamase transposon (5, 8). A 34,401-bp region was almost identical to the pWBG749-family conjugative plasmids pWBG745 and pBRZ01 (22) and encompassed a contiguous conjugation-gene cluster *smpA-smpX* (5, 9, 22, 23) (Fig. 1). The pWBG745-like plasmid was flanked by identical, directly-repeated copies of IS*257* (IS*257*#1 and IS*257*#2; Fig. 1) with adjacent 8-bp target-site duplications (TSDs) 5'-TAATCAAA-3' of the pWBG745-like sequence, indicating that cointegration likely resulted from transposition of an IS*257* copy residing in the pWBG753-like plasmid into the pWBG745-like plasmid. Consistent with this notion, pWBG753 contains an identical copy of IS*257* in a corresponding location as IS*257*#1 in pWBG731. However, in pWBG753 this element instead borders a cointegrated copy of a small tetracycline resistance plasmid related to pT181. Another identical IS*257* copy is present at the other end of pT181, along with a flanking 8-bp TSD of pT181 sequence 5'-AAACAAAA-3'. The observed arrangement of these IS*257* copies and TSDs on pWBG753 and pWBG731 are consistent with non-resolved replicative transposition events mediated by IS257#1/IS257#2 present on progenitors of pWBG753 and/or pWBG731. Transposition and cointegration of the pWBG745-like plasmid occurred within the 3' end of a putative gene of unknown function, whereas transposition into pT181 was within the *repC* replication-initiation gene.

The pWBG745- and pWBG753-like regions each contained a *repA_N* type replication initiation gene (24). Either or both could facilitate replication of pWBG731 since they both possess all the sequence features important for replication initiation of this replicon type in staphylococci, including Rep-box repeats within the *rep* coding sequence for initiator binding and candidate promoters for structured antisense RNA and *rep* leader mRNA, which mediate copy-number control (25). Moreover, both replication regions are identical to those of the autonomously-replicating plasmids pWBG753 and pWBG745 (5, 8, 9). Interestingly, the pC02 conjugative plasmid, another large cointegrant plasmid formed from a pWBG749-family conjugative plasmid and one or more multiresistance plasmids (18), also carries two functional *repA_N*-type replication regions and additionally, two functional pWBG749-family *oriT* sites. Remarkably, following conjugative transfer of pC02, relaxase-mediated recombination occasionally splits pC02 at the *oriT* sites to produce two smaller autonomously-replicating plasmids in recipient cells (18). A similar phenomenon is observed for pWBG749-mediated mobilization of the multiple-*oriT* carrying plasmid pWBG762 (23). These observations suggest carriage of multiple functional *repA_N* replication regions by cointegrant plasmids is tolerated in *S. aureus*.

Predicted partitioning (*par*) systems of distinct types are divergently coded from each *repA_N* gene on pWBG731; such systems improve the efficiency of plasmid inheritance during cell division (26). The pWBG753-related *par_2* gene was similar to the unusual pSK1 *par* gene (5). Interestingly, the type Ib *par* system on the pWBG745-like region is more closely related to that found on pWBG749 (97% nucleotide identity) than pWBG745 (90% nucleotide identity). A similar *repA_N-par* gene pairing to pWBG731 was identified on the non-conjugative plasmid pWBG762 (96% nucleotide identity over both *repA_N* and *par* genes), illustrating possible recombination and exchange between *repA_N-par*-gene combinations on these plasmids.

The β-lactamase transposon Tn*552* and sequences derived from it, are almost ubiquitous in modern *S. aureus* isolates. In addition to genes for β -lactamase production and its regulation (*blaZ-blaR1-blaI*), Tn*552* carries the *binL* serine-resolvase gene and its target DNA site *resL* (27-29). BinL-mediated recombination between *resL* sites on two copies of Tn*552* resolves cointegrate intermediates produced during replicative transposition (27). *S. aureus* plasmids themselves often carry homologous resolution systems (5), which similarly resolve plasmid dimers formed during plasmid replication or via homologous recombination between plasmid copies. Tn*552* belongs to a group of transposons that target resolution sites, so is often found upstream of *res* genes in staphylococcal plasmids and ICE elements (26) and DNA inversions and deletions can occur between Tn*552 resL* sites and plasmid-resolution sites (29). The Tn*552*-like element in pWBG731 is located upstream of the *res_2* gene, next to sub-site II of that gene's resolution site at a position

equivalent to previously characterized Tn*552* insertion sites (30) and identical to that in pWBG753. While the Tn*552*-like region on pWBG753 shares ~97% identity with Tn*552* (accession X52734), it is incomplete due to truncation by an IS*257* insertion in the transposase gene *orf480* (Fig. 2). Alignment of the pWBG731 Tn*552*-like region revealed further truncation, probably attributable to the action of resolvases. The Tn*552* regions on pWBG753 and pWBG731 are identical up until the *resL* resolution site but diverge beyond the expected resolvase cleavage site within sub-site I, such that a chimeric *res* site is present upstream of a unique resolvase gene *res_3* on pWBG731. Thus, during the evolution of pWBG731 from a pWBG753-like progenitor, a resolvase-mediated recombination event between the *binL* and *res_3* resolution sites appears to have resulted in the deletion of sequences to the right of pWBG753 sub-site I, including *binL*, *orf480* remnant and truncating IS*257* element.

Downstream of the chimeric *res_3 res* site on pWBG731 is a genetically complex 18,304-bp segment extending through to IS*257*#1. The region between IS*257*#4 and IS*257*#5, shares 99% identity to the IS*257*-flanked composite transposon-like structure Tn*4003* containing the *dfrA* trimethoprim-resistance gene, but lacks the third IS*257* copy and intervening segment usually found in Tn*4003*-like elements (31, 32). Additionally, a 192-bp deletion adjacent to IS*257*#4 has removed the 5' end of the *thyE* gene upstream of *dfrA*. Similar IS*257*-flanked deletions have been described previously and can moderate the level of trimethoprim resistance conferred, since they alter the hybrid promoter that drives *dfrA* transcription (33). Tn*4003* was first described in the multiresistance plasmid pSK1, which was also prevalent in Australian *S. aureus* in the 1980s. Such structures were subsequently recognized as cointegrated remnants of a pSK639-like trimethoprim resistance plasmid (34).

IS*257*#5 was itself truncated by a copy of the 148-bp insertion sequence-like element ISLE39. ISLE39 and the related element ISLE49 contain the same terminal-inverted-repeat sequences as IS*257* but lack a transposase gene (Fig. 3). They have been identified on staphylococcal plasmids flanking quaternary ammonium compound-resistance genes *qacB* and *smr* (*qacC*) (35, 36), and similar elements in the pathogenicity island SaPIbov2 flank the biofilm-associated protein gene (*bap*) (37). These sequences represent "Miniature Inverted-Repeat Transposable Elements (MITEs)" and the copies on pWBG731 (which are identical to ISLE39) have now been designated MITE*Sau1* in the ISfinder database (https://www-is.biotoul.fr) to clarify the nature of these elements. MITEs have been largely overlooked because of their small size, but their abundance and significance in both prokaryotic and eukaryotic genomes is increasingly being recognised (38-40). Consistent with this, NCBI BLASTN searches revealed 70 staphylococcal entries containing

one or more complete copies of MITE*Sau1*–like elements in the non-redundant database and 1,293 entries in the refseq_genomes database (NCBI searches conducted July 28, 2019).

MITE*Sau1*#1 (above) and a directly repeated and identical copy MITE*Sau1*#2 flank the 7.8-kb mupirocin-resistance region containing the *ileS2* (*mupA*) gene in pWBG731 (Fig. 1). *ileS2* encodes an alternate isoleucine-tRNA ligase and is present on numerous *S. aureus* plasmids, usually flanked by copies of IS*257*. Plasmid-borne *iles2* regions on diverse plasmids appear to have originated from a common ancestral sequence. Flanking regions often contain genes likely reflecting the context of *ileS2* prior to its capture and repurposing as a staphylococcal mupirocin-resistance locus. Some plasmids, such as pGO400 and pUSA03, carry only the *iles2* sequence, suggesting over time non-essential DNA surrounding *ileS2* has been effectively pruned away during evolution (42). Notably, BLASTN searches of the region from MITE*Sau1*#2 to *ileS2* on pWBG731 revealed it to be the largest region detected so far, containing three additional genes, suggesting it may represent one of the earliest 'unpruned' evolutionary configurations. Intriguingly, the segment downstream of *ileS2* in the *S. aureus* plasmid pV030-8 (accession EU366902) extends further than on pWBG731 but lacks any MITE*Sau1* sequences. Thus, the staphylococcal *ileS2* region appears to have had a complex evolutionary history involving both IS*257* and MITE*Sau1*.

Sandwiched between IS*257*#1 and MITE*Sau1*#2 is a region encoding a second set of homologues of the conjugation-cluster genes *smpT*, *smpV* and *smpU*. The *smpT2* gene is 5'-truncated by IS*257*#1 and *smpU2* is 3'-truncated by MITE*Sau1*#2, leaving only *smpV2* intact. The *smp*-cluster gene order for these same genes on pWBG749, pWBG745 and the main *smp* cluster on pWBG731, is *smpT-smpU-smpV*. The distinct *smpT-smpV-smpU* gene order present in this second copy on pWBG731 is also present on pC02 and putative pWBG749-family conjugative plasmids in *S. aureus* MO408 (GI: 477787193) and *S. epidermidis* VCU120 (GI: 418616860) (23). The closest DNA match of this second *smpT-smpV-smpU* cluster, at 70-80% nucleotide identity, was to the pC02 conjugation-gene cluster (43). SmpU shares sequence similarity with DNA topoisomerase III, a protein commonly encoded by diverse conjugative plasmids (44). While SmpT and SmpV genes are conserved on pWBG749-family plasmids, they share no primary sequence similarity with characterized proteins, so it is unclear if the capture of this region on pWBG731 holds any significance for conjugation or if it is merely a remnant of past recombination events. Nevertheless, the presence of a pC02-like region on pWBG731 suggests that more distantly-related members of pWBG749-family conjugative plasmids, or at least parts of them, occasionally cross paths and recombine.

pWBG715 represents an evolutionary intermediate between pWBG753 and pWBG731

At the time of pWBG731 isolation in the 1990s, numerous patients carried CA-MRSA strains containing a ~41.4-kb non-conjugative mupirocin-resistance plasmid (17), typified by pWBG715. pWBG715 conferred the same resistances as pWBG731 but additionally mediated tetracycline resistance. The similarities between resistance profiles of pWBG731 and pWBG715 and the sequence similarity between pWBG731 and the pWBG753, led us to suspect pWBG715 might represent an evolutionary intermediate between pWBG753 and pWBG731 that had retained an integrated copy of pT181. Indeed, the predicted size of a pWBG731-like plasmid with the pWBG745-related region swapped for pT181, was 42,989 bp. Unfortunately, the pWBG715 was lost from WBG7569 during storage, but a related isolate WBG7565 was available that carried plasmid with an identical restriction profile, named here pWBG715b (17). WBG7565 was sequenced using Illumina short-read sequencing (Genbank accession VLNQ00000000). *De novo* assembly and *in silico* MLST profiling confirmed WBG7565, like WA-5, was of ST8 (45, 46). Mapping of the assembled reads of pWBG715b revealed contigs matching with ~99% nucleotide identity to the entire pWBG731 plasmid except for the conjugation-cluster region, consistent with the hypothesis that pWBG715b represented an intermediate plasmid lacking the cointegrated pWBG745-like plasmid (Fig. 4A). The WBG7565 contigs also matched across the entire pWBG753 plasmid except for the region downstream of the Tn*552*-like *resL* site. Importantly this confirmed WBG7565 likely carried an IS*257*-flanked copy of pT181 identical to that on pWBG753. We next constructed a mock reference sequence for pWBG715, where we replaced the pWBG745-related region between IS*257*#1 and IS*257*#2 with the corresponding pT181-like region of pWBG753. The WBG7565 contigs mapped almost entirely over this sequence (Fig 3A). Finally, comparison of electrophoresed ClaI-digested pWBG731 and pWBG715b supported the *in silico*-predicted fragment sizes for both pWBG731 and the mock pWBG715b sequence (Fig. 4B). In summary, it seems likely that pWBG731 evolved in a stepwise fashion from pWBG753 and pWBG715. A pWBG753-like plasmid likely first acquired genes for resistance to mupirocin and trimethoprim from one or more sources in events mediated by the IS*257* transposase and a Tn*552*-like resolution system, to produce a pWBG715-like plasmid. Following this, the pT181-like region of pWBG715 was then replaced with a pWBG745-like conjugation-gene cluster, producing pWBG731.

pWBG731 mobilizes plasmids carrying OT45 and OTUNa *oriT* sequences

There are several lineages of pWBG749-families and their *oriT* sequences have diverged into at least five subtypes (23). Many non-conjugative plasmids carry multiple *oriT*s of different subtypes, suggesting their acquisition might enable mobilization by members of different pWBG749-family lineages. Non-conjugative plasmid pWBG762 for instance, carries *oriT* sequences of subtype OT49, OT45 and OTUNa (14). pWBG749 carries the OT49 *oriT* and only mobilizes recombinant plasmids carrying an OT49 *oriT* mimic (23). Since pWBG731 carried the OT45 *oriT* (Fig. 5), we predicted pWBG731 would mobilize only plasmids carrying the OT45 *oriT* mimic, but not plasmids carrying OT49 or OTUNa *oriT* mimics (23). Individual pLI50 plasmids carrying each of the three pWBG762-derived *oriT* sequences were introduced into RN4220 by electroporation and pWBG731 was subsequently introduced into each strain by conjugation. The resulting exconjugants were used as donors in conjugation experiments to detect mobilization of each pLI50 plasmid to WBG4515. Additionally, donor strains carrying the same pLI50 clones, but with pWBG749e (erythromycin-resistance-marked pWBG749 (14)), were used as donors for comparison. As expected, pWBG731 was not able to mobilize the pLI50 plasmid carrying the pWBG762 OT49 *oriT* mimic. pWBG731 did however mobilize pLI50 carrying the OT45 *oriT* sequence and interestingly, also mobilized pLI50 carrying the OTUNa *oriT* at a similar rate (Table 1). While this result was somewhat unexpected, a similar result has recently been documented for pC02, which harbours an OTUNa *oriT* and mobilizes plasmids carrying both OTUNa and OT45 *oriT* mimics (18).

Discussion

In this work we described the evolution of one of the largest experimentally-confirmed conjugative plasmids in *S. aureus*. In the last few years several of the largest documented (>60 kb) conjugative plasmids have been identified in *S. aureus*, including the 61-kb pC02 and the 85-kb *mecB*-carrying plasmid pSAWWU4229_1 (47) (while conjugative transfer for pSAWWU4229_1 has not been demonstrated it also carries a pWBG749-related conjugation cluster (unpublished)). It is possible these larger conjugative cointegrants may be more prevalent in *S. aureus* than previously documented and are only now being resolved following improvements in high-throughput long-read sequencing technologies (pC02 and pSAWWU4229_1 were also sequenced using PacBio (43, 47)). Given the inherent challenges in assembling larger plasmids carrying repetitive elements from short-read data, it is difficult to speculate if pWBG731-like plasmids are more common in sequence databases than currently appreciated. The evolution of pWBG753, pWBG715 and pWBG731 does however provide remarkable insight into how diverse mobile genetic elements can work together to rapidly repackage multiresistance determinants in a modular fashion and facilitate their subsequent dissemination via conjugation. These three plasmids likely represent only a fraction of the plasmid cointegrants formed, split and recombined during the evolution of pWBG731.

Around 91% of unique non-conjugative *S. aureus* plasmids larger than 20 kb carry at least one *oriT* mimic (48, 49), so the apparent capacity for mobilization via this mechanism is extremely common in *S. aureus*. The pWBG749-family *oriT* sequences have however diverged and pWBG749 cannot mobilize plasmids carrying the OT45 or OTUNa-type *oriT* sequences present on pWBG731 and pC02 plasmids, respectively (Fig. 5) (8). The pWBG731 conjugation-gene cluster, which harbours an OT45 *oriT*, shares only ~75% nucleotide identity to the conjugation-gene cluster of pC02 (18), which harbours an OTUNa *oriT*. This suggests that the OT45 and OTUNa *oriT* sequences have evolved within distinct pWBG749-family lineages. Moreover, trees based on the *oriT* sequences group OT45 and OTUNa *oriT*s into distinct clades (23). Experiments here revealed pWBG731 mobilizes plasmids carrying either an OT45 or OTUNa *oriT*, but not a plasmid carrying an OT49 *oriT*, an identical mobilization profile to that of pC02 (18). This suggests that despite the divergence of pC02 and pWBG731, their *oriT* sequences are functionally equivalent in terms of *oriT* specificity and mobilization. We previously speculated that the more variable inverted-repeat 2 (IR2) region of each *oriT* is involved in *oriT* specificity (23). Re-examination of the OT45 and OTUNa *oriT* sequences (Fig. 5) highlights that the *oriT* regions are more similar in their IR2 repeat regions in comparison to IR2 regions on the other *oriT* sub-types, consistent with this notion. In summary, together with the previous mobilization experiments with

pWBG749e (23) and pC02 (18), there is now direct experimental evidence for the *in trans* mobilization of plasmids carrying each of the OT49, OTUNa and OT45 *oriT* mimic subtypes. These collectively represent 92% of the pWBG749-family *oriT* mimics identified on *S. aureus* plasmids (23, 48).

Plasmid cointegrates of the type observed here in pWBG731, pWBG715b and pWBG753 have been described on numerous occasions, within plasmids and staphylococcal chromosomes (26). Often these IS*257*-mediated cointegrations result in the capture of antimicrobial resistance genes, such as *tet*(K) from pT181 above. The cointegrative capture of a pWBG745-like plasmid into pWBG731 in this case does not appear to have resulted in the acquisition of any identifiable resistance determinants. Rather, it seems that an existing pWBG753-like multiresistance plasmid has acquired the capacity for conjugative self-transmissibility via this single cointegration event. As such, this represents an interesting counterpoint to the process of IS*257*-mediated resistance gene accretion evident in pSK41 family conjugative plasmids. In the course of pWBG731 evolution, the cointegrated pT181-like plasmid in pWBG753 has been deleted, presumably as a consequence of homologous recombination between the flanking IS*257* copies, either before or after cointegration of the pWBG745-like plasmid. In addition to the *tet*(K) resistance gene, this deletion also removed the *pre*/RSa mobilization system present in pT181. Thus, in this multiresistance plasmid lineage, there has been a swap from the capacity for horizontal transmission by mobilization, dependent on a conjugation system co-resident in the same cell, to independent conjugative proficiency. In this regard, it is worth noting that pWBG753 is one of the few *S. aureus* multiresistance plasmids that lacks any recognisable pWBG749- or pSK41-like *oriT* mimic (23, 48, 50), which might explain selective acquisition of *mob* and/or conjugation genes in this plasmid lineage.

Significant sequence diversity is observed between IS*257* copies, which can provide insights into evolutionarily pathways. In this regard it is worth noting that pWBG731 IS*257*#4 is identical to IS*257*L of Tn*4003*, whereas the remnant of IS*257*#5 is identical to Tn*4003* IS*257*R1 (IS*257*L and IS*257*R1 themselves differ by three nucleotide substitutions and a single indel). This is consistent with the similarity between the *dfrA* trimethoprim-resistance region of pWBG731 and Tn*4003* on pSK1. More interestingly, pWBG731 IS*257*#1 and IS*257*#2 are also identical to Tn*4003* IS*257*R1, possibly suggesting that replicative transposition of IS*257*#1 #2, or #5 played a role in the incorporation of sequences between IS*257*#5 and IS*257*#1, resulting in capture of the pT181- and pWBG745-like plasmids in this lineage. Unfortunately, any potentially informative TSD has been removed by the truncation of IS*257*#5 by MITE*Sau1*. The copy of IS*257* truncating the Tn*552* transposase gene in pWBG753 is most similar to IS*257*#4, differing by only a single nucleotide.

Finally, oriented in the opposite orientation to all other IS*257* copies on pWBG731, IS*257*#3 is the most divergent element, differing from the IS*257*#1, IS*257*#2 and IS*257*#5 by 15 substitutions and a single indel.

WA-5 marked the beginning of CA-MRSA in WA, however, it was quickly supplanted by locally abundant *S. aureus* lineages that acquired near-identical SCC*mec* IVa (2B). The appearance of the WA-5 plasmids pWBG753, pWBG715 and pWBG731 was similarly brief. pWBG753 shares very little sequence similarity to extant plasmids in sequence databases aside from its Tn*552*, pT181 and IS*257*-related sequences. It does however share 99.8% nucleotide identity over 83% of its length with the fusidic acid resistance plasmid p11819-97 present in ST80 European CA-MRSA. ST80 became the dominant CA-MRSA lineage in Europe and North Africa throughout the 1990s (51, 52). Phylogenetic analyses of European ST80-MRSA isolates dating back to this period suggest acquisition of p11819-97 and SCC*mec* IV occurred immediately prior to its rapid expansion. So while appearance of WA-5 and pWBG753 was brief in Australia, the related p11819-97 plasmid may have been pivotal in the evolution and dominance of European CA-MRSA during the same period. The cointegrant pWBG731 examined here appears to represent the result of several gene-transfer events that occurred between the pWBG753-harbouring WA-5 strain and incumbent community-associated *S. aureus* lineages carrying pWBG749-family plasmids.

Materials and methods

pWBG731 DNA extraction. A 5-mL tryptic soy broth (TSB) seeder culture (containing 80 µM Cd) was inoculated from a single colony of WBG10514 and grown overnight with shaking at 37°C. From this 500 µL was used to seed 200-ml TSB (with Cd), which was grown in the same conditions for 7h. Cells were harvested by centrifugation, washed in 10 mL TE buffer and then pelleted again by centrifugation. The GenElute HP Plasmid Maxiprep Kit was used (Sigma #NA0310) for plasmid DNA extraction. Prior to Step 2 of the manufacturer's protocol, approximately 2.5 mg of recombinant lysostaphin (Sigma #L9043) was dissolved in 12 mL of 'Resuspension/RNase A' solution. This solution was used to resuspend the cell pellet and the resulting cell suspension was incubated at 37°C for 10-15 minutes (vortexing every few minutes) until it cleared, before proceeding with Step 3 (alkaline lysis). Following elution and subsequent ethanol/sodium acetate precipitation, this yielded 183 µg of DNA (measured using a Qubit v2, ThermoFisher), suspended in 400 µL filtered, deionised H_2O.

Sequencing, assembly and sequence analysis. For long-read sequencing approximately 5 µg of purified pWBG731 DNA was used in a single Pacbio RSII SMRT cell (carried out by Macrogen, South Korea), multiplexed with five other *S. aureus* plasmids at similar concentrations. A size-selected ~5.9-kb library was generated with a concentration of 3.6 ng/µL. Pacbio sequencing generated 81,763 sorted, post-filter subreads with a mean length of 2,326 bp, with 43,563 (53%) of reads mapped to the final pWBG731 sequence. Sequence assembly was carried out using the long-read assembler Canu (v1.7) (53). A predicted circular 80,000-bp contig was generated amongst linear contigs assembled from contaminating genomic DNA. Circlator (54) was used to identify overlapping contig ends and reduced the assembly to 72,160 bp. The pWBG731 sequence start position was manually set to the start codon of the predicted *repAN* gene adjacent to the *smpA-smpX* conjugation cluster. Short-read sequencing of pWBG731 was carried out using an Illumina MiSeq. A paired-end library (2 x 300) was prepared using 1.5 ng of pWBG731 DNA and the Nextera XT kit V3 (as described in (55)). Sequencing generated 2.7 million reads, of which 15% mapped (Bowtie 2 (56)) to the final pWBG731 sequence. Assessment of mapped reads with Pilon (v.1.22) (57) identified and corrected two single-bp insertions within polyA/T tracts. The final pWBG731 sequence was 72,158 bp and post-assembly mapping statistics provided in the result section were produced using Bowtie 2 (56) and Qualimap (v.2.2.1) (58)). Whole WBG7565 (pWBG715b) DNA was sequenced using 2 x 151-bp paired-end library and an Illumina NextSeq with a 54-fold mapped depth of coverage. *De novo* assembly was carried out using SPAdes (v3.11.1) (59) which produced 153 contigs > 1 kb and 239 contigs total. BRIG/BLASTN (60) comparisons were carried out with BLASTN and parameter "--word_size 21". MLST profiling

was carried out using mlst software (Seemann T, mlst Github https://github.com/tseemann/mlst) which makes use of the PubMLST database (46). Plasmid maps were created with Benchling (https://benchling.com).

Mobilization experiments. Previously-constructed pLI50 plasmids carrying *oriT* mimics cloned from pWBG762 (23) were introduced into RN4220 by electroporation and pWBG749e or pWBG731 were subsequently introduced by conjugation, using erythromycin or cadmium together with chloramphenicol to select for exconjugants. Resulting strains carrying both plasmids were used as donors in liquid matings with PEG6000 as previously described (23). RN4220 derivative WBG4515 was used as a recipient and streptomycin and novobiocin were used to counterselect against donors.

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595

596 **Table 1. Mobilization of *oriT* mimics by pWBG749e and pWBG731**

Construct name	Conjugative plasmid	Cloned *oriT* mimic subtype	Transfer frequency[a]	
			Conjugative plasmid	pLI50 clone
pLI50	pWBG749e	none	4.2×10^{-5} ($\pm 1.1 \times 10^{-5}$)	Not detected[b]
pLI762-49	pWBG749e	OT49	1.6×10^{-5} ($\pm 4.3 \times 10^{-6}$)	2.1×10^{-6} ($\pm 3.7 \times 10^{-7}$)
pLI762-45	pWBG749e	OT45	1.6×10^{-5} ($\pm 7.1 \times 10^{-6}$)	Not detected
pLI762-UNa	pWBG749e	OTUna	3.3×10^{-5} ($\pm 5.5 \times 10^{-6}$)	Not detected
pLI50	pWBG731	none	5.1×10^{-5} ($\pm 3.5 \times 10^{-5}$)	Not detected
pLI762-49	pWBG731	OT49	6.6×10^{-5} ($\pm 4.4 \times 10^{-5}$)	Not detected
pLI762-45	pWBG731	OT45	1.7×10^{-4} ($\pm 9.0 \times 10^{-5}$)	5.9×10^{-5} ($\pm 1.8 \times 10^{-6}$)
pLI762-UNa	pWBG731	OTUna	2.1×10^{-4} ($\pm 4.7 \times 10^{-5}$)	1.5×10^{-5} ($\pm 1.7 \times 10^{-5}$)

597 [a]Per-donor transfer frequencies are the average of three independent experiments (±standard
598 deviation). [b]Detection limit was approximately 1×10^{-8} exconjugants per donor.

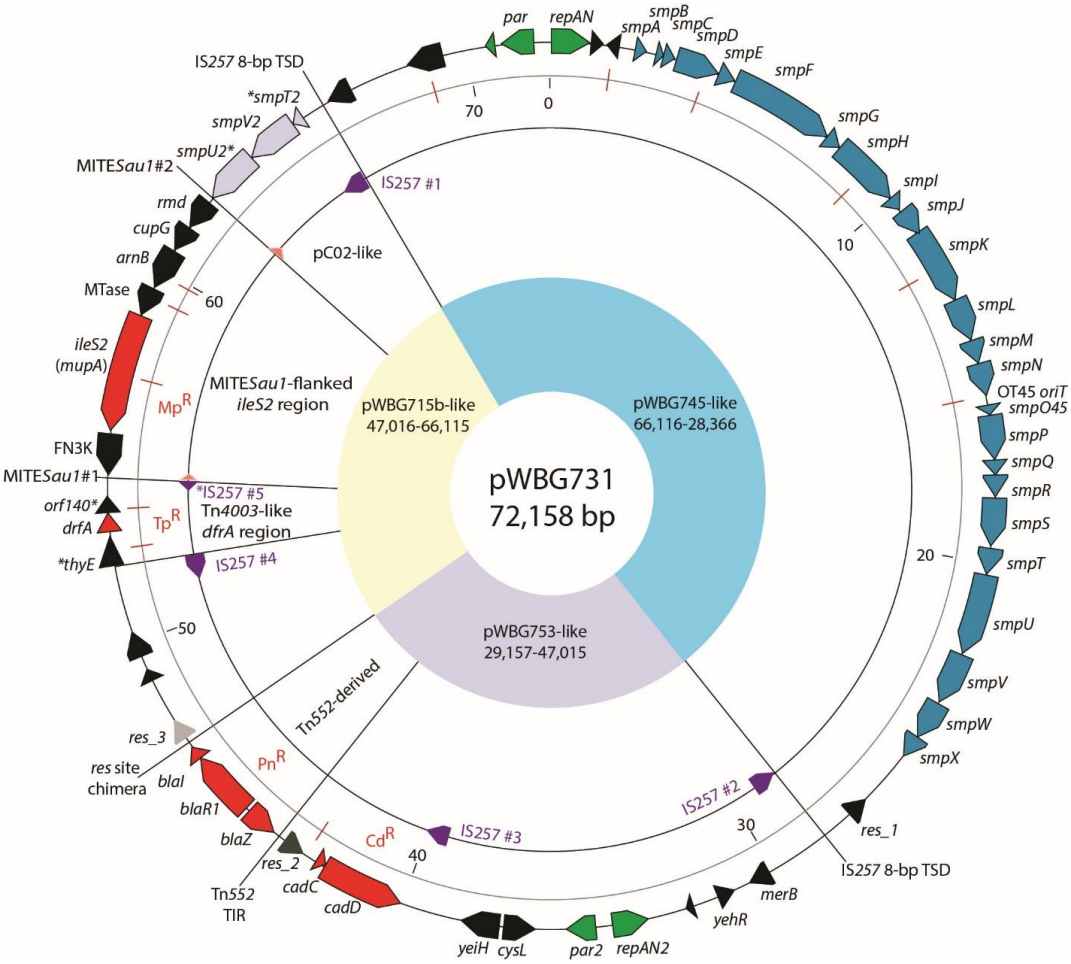


Figure 1. The 72-kb conjugative multiresistance plasmid pWBG731. (*A*) Map of the pWBG731 sequence highlighting sequence features. The outer ring shows positions of predicted genes and key *cis*-acting sequences likely involved in its evolutionary construction. Asterisks before or after a gene name indicates a 5' or 3' gene truncation, respectively. The grey ring shows the sequence ruler (in kilobasepairs) and positions of ClaI sites (corresponding to ClaI-digested DNA in Fig. 4B) shown as red lines. The next inner ring highlights positions of IS*257* and MITE*Sau1* elements. All IS*257* copies except IS*257*#5 carry an intact transposase gene. Yellow, blue and grey sectors indicate regions nearly identical to those present on plasmids pWBG745, pWBG753 and pWBG715.

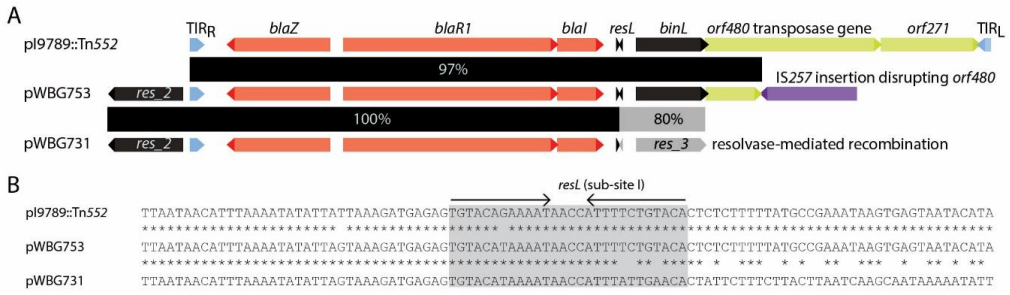


Figure 2. Tn*552*-derived regions on pWBG753 and pWBG731. (A) Gene maps of the Tn*552* and Tn*552*-like sequences on pI9789, pWBG753 and pWBG731, showing the positions of terminal inverted repeats (TIR), *resL* sites and other genes or insertion sequences. (B) Sequence alignment of the *resL* sites on the same three plasmids. The region of the *resL* site is the main recombination site of the *resL* region "sub-site I". Accessory sub-sites II and III are identifiable upstream of the *binL* and *res_3* coding sequences.

Figure 3. Alignment of MITE*Sau1*–like elements. Alignment (M-Coffee (41)) of distinct MITE*Sau1*–like sequences from pWBG731, pSW49 (Genbank accession AM040730.1), pWBG759 (GQ900401.1), SaPIbov2 (AY220730.1) and pAvX (MH785253.1). Non-identical nucleotides are shown in the alignment with MITE*Sau1*. The terminal inverted repeats of the elements are indicated by horizontal arrows, and the ends of pWBG731 IS*257*#1 are presented at the top for comparison, with non-matching nucleotides shown in lowercase. MITE*Sau1* of pWBG731 is identical to ISLE39 (accession AF535086.1).

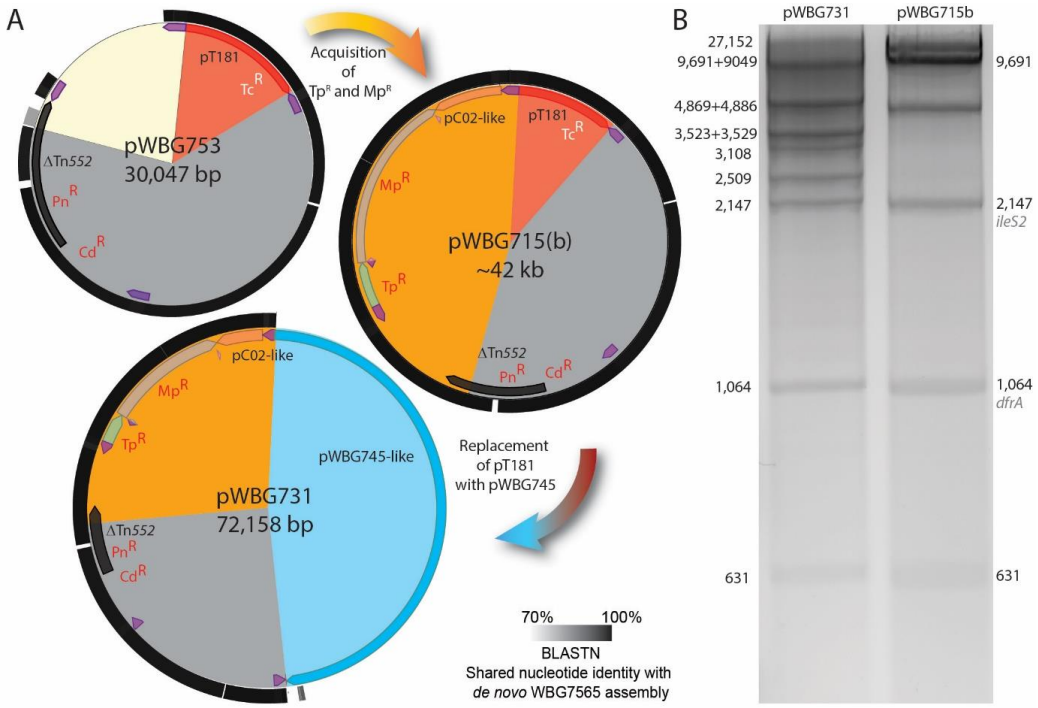
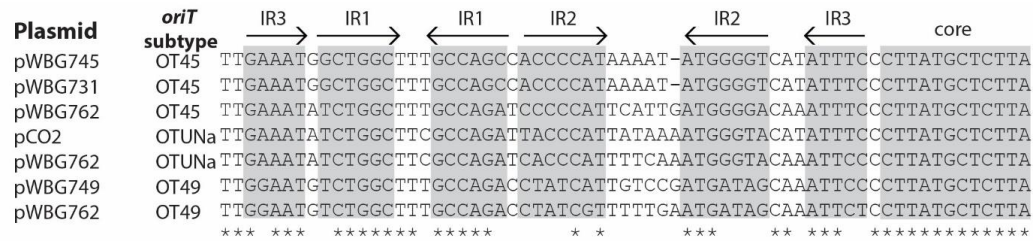


Figure 4. Stepwise evolution of pWBG753, pWBG715 and pWBG731.

(A) Plasmid maps of sequenced plasmids pWBG753 and pWBG731 and a map for the predicted sequence of pWBG715 (not to scale relative to each other). Outermost rings on each plasmid map indicate percentage nucleotide identity to contigs from a BLASTN query of the *de novo* sequence assembly WBG7565, which carries pWBG715b, against each plasmid sequence. The grey sectors indicate the pWBG753 backbone conserved on all three plasmids; the yellow region on pWBG753 represents the region replaced with the orange region on pWBG715; the red sector indicates the pT181 region on pWBG715 that was replaced by the blue pWBG745-like conjugative plasmid on pWBG731. (B) Agarose electrophoresis of ClaI-digested pWBG731 (left) and pWBG715b (right) DNA. Fragment sizes for pWBG731 are indicated on the left and recognizable corresponding fragments in the pWBG715 digest are indicated on the right.



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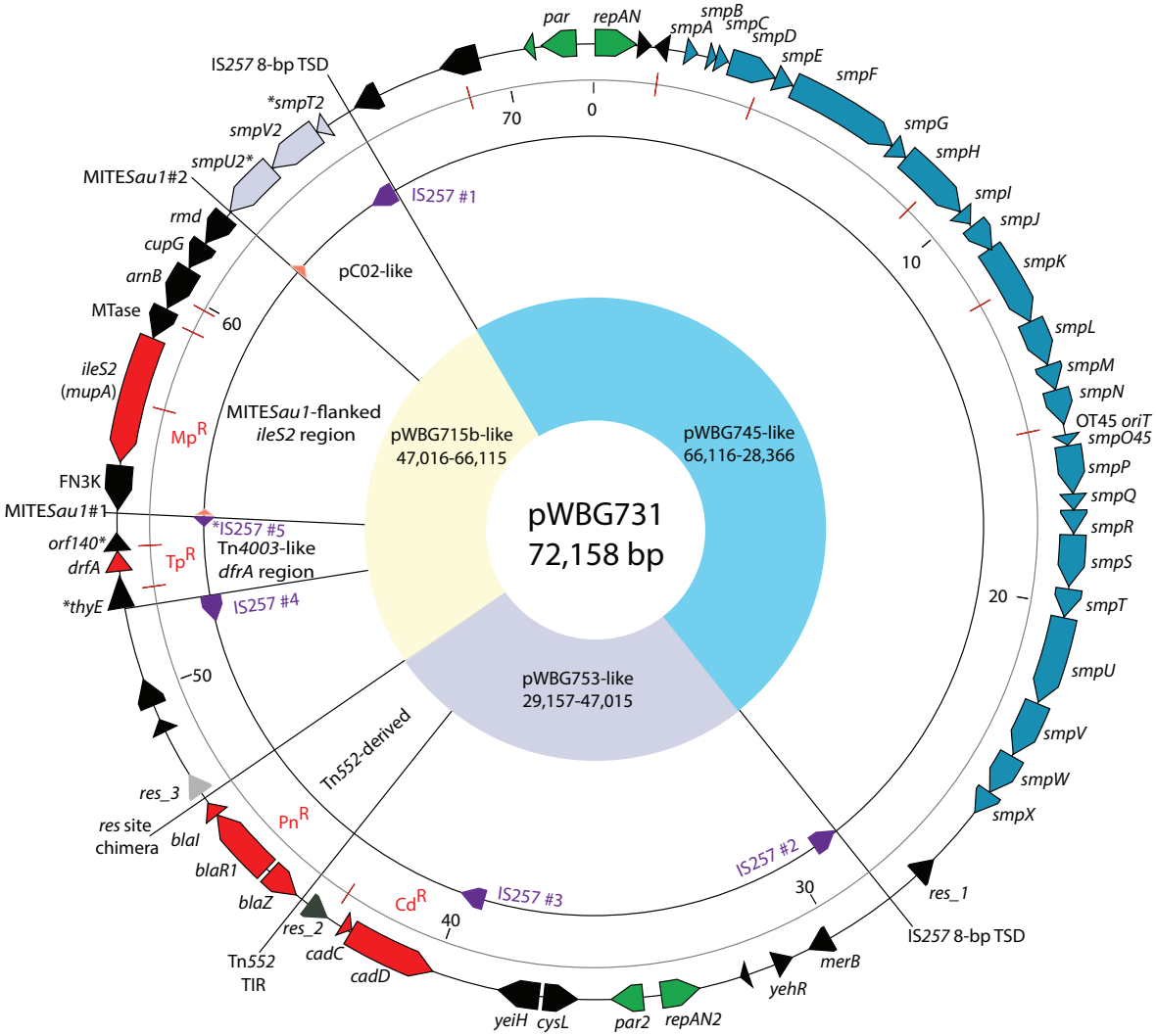
641 **Figure 5. Comparison of *oriT* sequences on pWBG749-family plasmids**. An alignment of the
642 *oriT* sequences (excluding upstream AR1-AR3 regions) on conjugative plasmids pWBG745,
643 pWBG731, pC02 and pWBG749, along with *oriT* mimic sequences of subtypes OT49, OT45 and
644 OTUNa present on pWBG762. Comparison of the IR2 regions reveals the similarity between the
645 IR2 regions of the OT45 and OTUNa *oriT* sequences in contrast to the IR2 region of OT49
646 sequences.

647



Antimicrobial Agents and Chemotherapy