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LETTER TO THE EDITOR

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# An updated view of plasmid conjugation and mobilization in *Staphylococcus*

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**ABSTRACT**

The horizontal gene transfer facilitated by mobile genetic elements impacts almost all areas of bacterial evolution, including the accretion and dissemination of antimicrobial-resistance genes in the human and animal pathogen *Staphylococcus aureus*. Genome surveys of staphylococcal plasmids have revealed an unexpected paucity of conjugation and mobilization loci, perhaps suggesting that conjugation plays only a minor role in the evolution of this genus. In this letter we present the DNA sequences of historically documented staphylococcal conjugative plasmids and highlight that at least 3 distinct and widely distributed families of conjugative plasmids currently contribute to the dissemination of antimicrobial resistance in *Staphylococcus*. We also review the recently documented "relaxase-*in trans*" mechanism of conjugative mobilization facilitated by conjugative plasmids pWBG749 and pSK41, and discuss how this may facilitate the horizontal transmission of around 90% of plasmids that were previously considered non-mobilizable. Finally, we enumerate unique sequenced *S. aureus* plasmids with a potential mechanism of mobilization and predict that at least 80% of all non-conjugative *S. aureus* plasmids are mobilizable by at least one mechanism. We suggest that a greater research focus on the molecular biology of conjugation is essential if we are to recognize gene-transfer mechanisms from our increasingly *in silico* analyses.

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## Text

### *Conjugation and mobilization*

Conjugation is a highly evolved and efficient mechanism facilitating DNA transfer in bacteria, therefore the characterization of DNA sequences, genes and proteins involved in conjugation is crucial if we are to accurately appraise the potential for gene transfer from bioinformatics analyses. Several bacterial genome surveys have revealed an unexpected paucity of recognized conjugation loci in many bacterial genera.[1,2] In staphylococci, only an estimated 5% of plasmids carry conjugation-gene clusters required for autonomous conjugative transfer. In contrast, the evidence for horizontal transfer of staphylococcal plasmids between distinct lineages is abundant, leading some to propose that bacteriophage transduction may account for much of this transfer.[3-5] However, several new mechanisms of conjugative mobilization have recently been elucidated, which we believe may resolve the paradoxical underrepresentation of conjugative plasmids in staphylococci.[6-8]

Autonomously-transferring conjugative plasmids carry both mating-pore genes and genes for DNA processing, single-stranded DNA (ssDNA) replication and recruitment of ssDNA to the mating pore. DNA is recruited to the mating pore by the relaxase protein, which binds, cleaves and covalently attaches to a recognition sequence called the origin-of-transfer (*oriT*), forming (often with accessory proteins) a nucleoprotein complex referred to as the relaxasome.[9] The relaxasome is recruited to the mating-pore through interactions with a mating-pore component called the VirD4 coupling protein, after which it is transferred to

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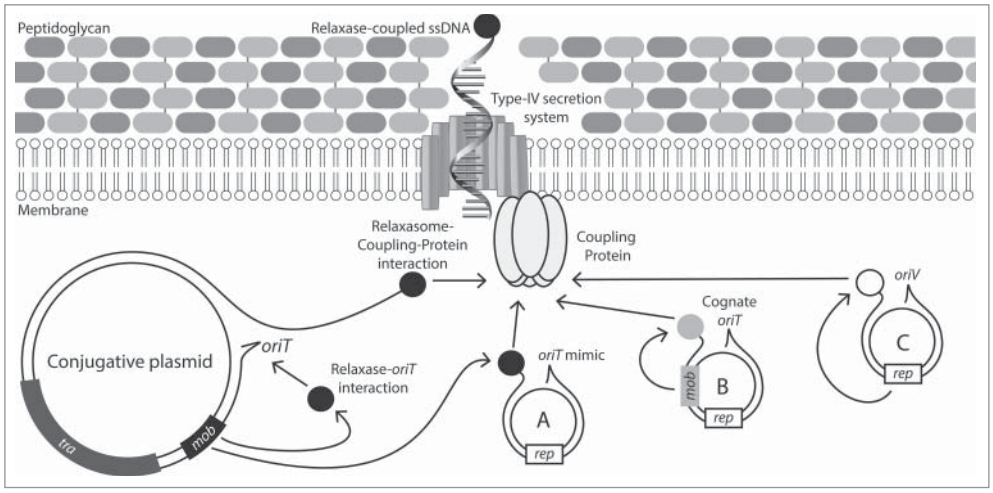


**Figure 1.** Mechanisms of conjugative mobilization in *Staphylococci*. The conjugative plasmid encodes all genes required for formation of the mating pore, as well as the coupling protein, DNA relaxase and an *oriT*. Mobilizable plasmids can exploit the conjugative-plasmid mating pore by either: (A) encoding a mimic sequence of the conjugative-plasmid *oriT*; (B) encoding a distinct relaxase (Mob) compatible with the conjugative plasmids coupling protein and its own cognate *oriT* or; (C) by carrying a replicative relaxase (Rep) compatible with the conjugative-plasmid coupling protein.

recipient cells through a type-IV secretion system (Fig. 1). Relaxases can additionally be involved in rolling-circle-like plasmid replication in the donor bacterium and recircularization and replication of plasmid DNA in the recipient. Plasmid conjugation systems can therefore be considered an evolutionary amalgamation of type IV protein secretion systems that have evolved to transfer protein-tethered DNA, with rolling-circle replicases (or recombinases)[10,11] that have evolved an association with the type IV secretion system through relaxase-coupling-protein interactions.[12]

Mobilizable plasmids are those which carry DNA-transfer genes required for formation of all or part of the relaxasome, but lack genes required for mating pore formation. Mobilizable plasmids have an ability to exploit conjugative plasmids for horizontal dissemination, but are non-mobile in cells that lack mobile elements carrying compatible mating-pore genes. The vast majority of documented mobilizable plasmids exploit conjugative element mating-pores by encoding their own relaxase (Mob) that acts on the plasmid's cognate *oriT* (Fig. 1).[1]

## *Three distinct families of staphylococcal conjugative plasmids*

Staphylococcal isolates are frequent hosts to diverse antimicrobial-resistance plasmids, but until recently only one family of staphylococcal conjugative plasmids had been characterized at a molecular level. Closely related members of the pSK41/pGO1 plasmid family were first documented by several groups in the early 1980s as the basis of emergent gentamicin resistance.[13-16] In addition to aminoglycoside resistance, these plasmids have been found to variously confer resistance to penicillins, trimethoprim, bleomycin, tetracycline, antiseptics and disinfectants, mupirocin, and macrolides, lincosamides and streptogramin B. The resistance genes responsible for these phenotypes are usually encoded by small plasmids cointegrated between copies of IS*257*/IS*431* within the pSK41/pGO1 plasmid.[17] Notably, plasmids of this type have subsequently been associated with linezolid and high-level vancomycin resistance.[18,19] pSK41/pGO1 family plasmids share a near-identical syntenic backbone that encodes resolution, partitioning and replication functions, and a cluster of approximately 15 transfer genes for proteins implicated in conjugation, including a predicted coupling protein and homologues of type-IV secretion system components[16,20] A conserved relaxase gene, *nes*, is located elsewhere within the plasmid backbone, adjacent to the *oriT* site at which its product nicks the plasmid DNA to initiate conjugative transfer.[21,22]

The pSK41/pGO1 family conjugative plasmids mobilise some smaller plasmids (3.5–14.5 kb) when co-resident in the same cell. Mobilizable staphylococcal plasmids such as pSK639 and pC221 contain a DNA segment encoding the genes *mobCAB*.[23-25] The MobC protein binds to an adjacent cognate *oriT*

sequence that is nicked by the MobA relaxase to initiate transfer.[26] Numerous other small plasmids (3.5–14.5 kb) instead encode a distinct locus implicated as another relaxase-*in cis* mobilization system. The *pre* gene and site, $RS_A$, were originally characterized as a site-specific recombination function carried by staphylococcal plasmids.[27] However, this system has been implicated in mobilization of the *S. aureus* plasmid pUB110 in *Bacillus*[28] and a homologous locus carried by the streptococcal plasmid pMV158 was subsequently shown to comprise a gene, *mobM*, encoding a relaxase that acts on a corresponding *oriT* site to facilitate mobilization of that plasmid.[29,30]

More recently a second distinct family of staphylococcal conjugative plasmids was characterized. pWBG749 was found in a strain from a remote indigenous Australian community in 1995.[3,7,31] pWBG749-family conjugative plasmids carrying penicillin, aminoglycoside and vancomycin-resistance genes have since been identified.[8,32,33] The predicted proteins encoded by the pWBG749 conjugation-gene cluster are distinct from those of pSK41 and include a distinct putative relaxase SmpP and a distinct *oriT* sequence. Interestingly, the majority of pWBG749-like plasmids identified in sequence databases do not appear to carry antimicrobial-resistance loci, perhaps explaining why they have been largely overlooked.[8]

In the 1980s at least 3 families of distinct staphylococcal conjugative plasmids were identified in the Grubb laboratory from *S. aureus* isolated from Australia, Africa and Asia, and at least 2 of these plasmid families were clearly distinct from pSK41-family plasmids based on their restriction and incompatibility profiles. Conjugative plasmid pWBG637, was originally isolated in 1985 from a Nigerian hospital *S. aureus* isolate and subsequently identified in several other hospital-associated strains with distinct chromosomal lineages.[34-36] Like pWBG749, pWBG637 does not carry resistance genes.[37,38] pWBG637 is capable of conjugation to other *S. aureus, S. epidermidis* and *Enterococcus faecalis* strains and is able to mobilise the conjugative transfer of several coresident antimicrobial-resistance plasmids.[34,39] In work presented here, we sequenced pWBG637 to further clarify its relatedness to other conjugative plasmids. BLASTN alignments of the pWBG637 sequence (KX086582) revealed it shared 99% nucleotide identity with pWBG749 over 91% of its 38 kb length. Therefore, while members of the pWBG749-family of conjugative plasmids have only recently been sequenced, they have been documented as a distinct family of staphylococcal conjugative plasmids capable of mobilising other antimicrobial-resistance plasmids since the 1980s.

A third distinct family of "Diffusible pigment" conjugative plasmids, which we have designated the pWBG4 family, were first identified as a third unique type of conjugative plasmid in 1985 by Townsend et al. The first of these, pWBG14,[40] confers aminoglycoside, macrolide, lincosamide and spectinomycin resistance and was identified in a strain originally isolated from Royal Perth Hospital in 1968. Conjugative transfer of related plasmids pWBG4 and pWBG25 was subsequently demonstrated.[41,42] A pWBG4-family plasmid conferring trimethoprim resistance, pWBG707, was identified in a Malaysian isolate in 1992.[43] In work presented here, we sequenced pWBG4 (KX149096) and pWBG707 (KX149097). Bioinformatic analyses of these sequences revealed pWBG4 and pWBG707 shared a 19-KB gene cluster of 23 co-oriented open-reading frames encoding predicted products related to VirD2, VirB2 and proteins of various other gram-positive conjugative elements (Fig. 2) (also noted by Shore et al.).[44] Interestingly, pWBG4 was found to be almost identical to pSK73 (GQ915269.1), a recently-sequenced plasmid carried by a strain originally isolated in Melbourne in 1966.

The putative pWBG4 conjugation gene cluster, which we here name *detA-detV*, is clearly distinct from that of pSK41 and pWBG749. Analysis of DetB using CONJscan,[45] placed DetB in the MobC-family of conjugative relaxases. Furthermore, DetA encoded a putative VirD4/MobB coupling protein, thus the *detA-detB* region shared the same Mob-gene arrangement as *mobBC* on the mobilizable plasmids ClodDF13 (of *Escherichia coli* CloDF13) and pUA140 (of *Streptococcus mutans*) and the conjugative *Yersinia enterocolitica* plasmid p29930.[46-48] The *detA-detV* region was additionally identified on several contemporary staphylococcal plasmids associated with the dissemination of *cfr*-gene-encoded linezolid resistance in both human and animal-isolated staphylococci. The pWBG4-family plasmid pSA737, was found in the first American example of *cfr*-mediated linezolid resistance in a human *S. aureus* isolate[49] and was subsequently identified in 19 isolates from 2 Ohio hospitals.[50] pWBG4-family *cfr*-carrying plasmids have now been identified in China (pHK01),[51] Germany (p12-02300),[18] and Ireland (pSAM12-0145).[44] Despite the

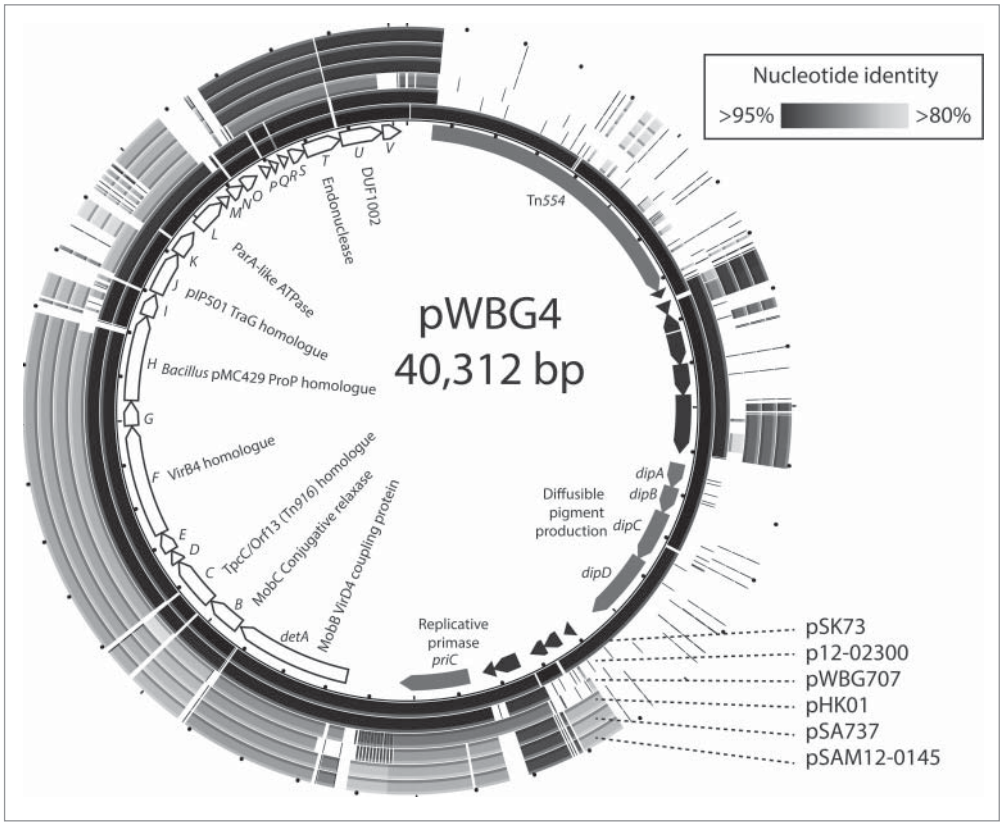


**Figure 2.** pWBG4, a third family of staphylococcal conjugative plasmids. The internal circle represents the gene map of pWBG4, showing the positions and predicted products of the putative pWBG4 conjugation cluster *detA-detV* (white arrows with black outlines) and other open-reading frames. The outer circles represent ungapped circular BLASTN alignments of pWBG4-family plasmids, created using BRIG software.[63]

wide distribution of pWBG4-like plasmids, the clinical importance of linezolid and the conspicuousness of the *detA-detV* conjugation gene cluster, only one of these studies reported laboratory conjugation experiments confirming that the *cfr*-carrying pWBG4-like plasmid was conjugative.[44]

In summary, it is clear that there are at least 3 distinct families of conjugative plasmids (based on their distinct conjugation-gene clusters) in staphylococci. Members of each of the pSK41, pWBG749 and pWBG4 families of conjugative plasmids can be identified in extant and historical staphylococcal lineages and each is currently contributing to the horizontal spread of resistance mechanisms against last-resort antimicrobials such as vancomycin and linezolid.

## *Relaxase*-in trans *mobilization*

There is also a relative paucity of Mob-gene loci on non-conjugative plasmids in *Staphylococcus aureus*.[3,4] Non-conjugative staphylococcal plasmids range from small (<5 kb) rolling-circle plasmids that often only carry genes for replication and a single antimicrobial resistance, to larger (up to ~65 kb) plasmids which carry multiple resistances. A genome survey of staphylococcal plasmids isolated since the 1940s revealed that most staphylococci carry at least one plasmid > 20kb. But, the 3 most common large plasmid families, pMW2, pIB485 and pUSA300HOUMR, which represent 43 % of all plasmids >20 kb identified, lack conjugation or mobilization genes.[3]

A surprising observation from conjugation experiments with pWBG749, is that it is able to mobilise several large multiresistance plasmids that lack Mob-gene loci, including a member of the pIB485 family. In our recent *Nucleic Acids Research* article, we demonstrate that the mechanism by which pWBG749 mobilises non-conjugative plasmids is distinct from that of most previously described systems.[8] Plasmids mobilised by pWBG749 carry sequences that mimic the *oriT* of pWBG749, indicating that the putative relaxase of pWBG749, SmpP, recognizes these *oriT* mimics and recruits the mobilizable plasmid DNA to the pWBG749-produced mating pore. Each pWBG749-

family *oriT* mimic, minimally contains a 126-bp region encompassing three overlapping inverted-repeat sequences (IR1-3), at least one copy of an accessory repeat (AR) and a defined relaxase core region (Fig. 3).[8] The relaxase-*in trans* mechanism is not technically novel, but likely because it has rarely been recognized in nature, like pWBG749, it has been overlooked. What is most astounding from our analyses is the extensive distribution of *oriT* sequences on staphylococcal plasmids. Over 50% of non-identical sequenced plasmids carry 1–3 copies of a pWBG749-like *oriT* mimic (Fig. 3).[8]

Five variants of the pWBG749-family *oriT* sequence have been identified, named OT49, OT45, OTUNa, OTSep and OT408. Each variant differs in a predicted inverted-repeat sequence 2 (IR2) (Fig. 3). Putative pWBG749-family conjugative plasmids, individually carrying each of the five distinct *oriT* variants, are represented in DNA sequence databases.[8] The divergence of the *oriT* sequences suggests that each pWBG749-family is likely specific for a single *oriT* variant. Consistent with this hypothesis, pWBG749, which carries an OT49 *oriT*, is not able to mobilise pMW2, which carries an OT45 sequence, nor can it mobilise an OT45 *oriT* sequence cloned from the conjugative plasmid pWBG745. A putative ribbon-helix-helix DNA binding protein, SmpO, encoded by all pWBG749-family plasmids, appears to dictate the specificity of mobilization for these *oriT* sequences. Cloning of the pWBG745-encoded SmpO sequence along with the OT45 *oriT*, enables OT45-*oriT* mobilization by pWBG749. This suggests that the pWBG749 relaxase SmpP, is able to interact with both OT49 and OT45 *oriT* variants, but the presence of the

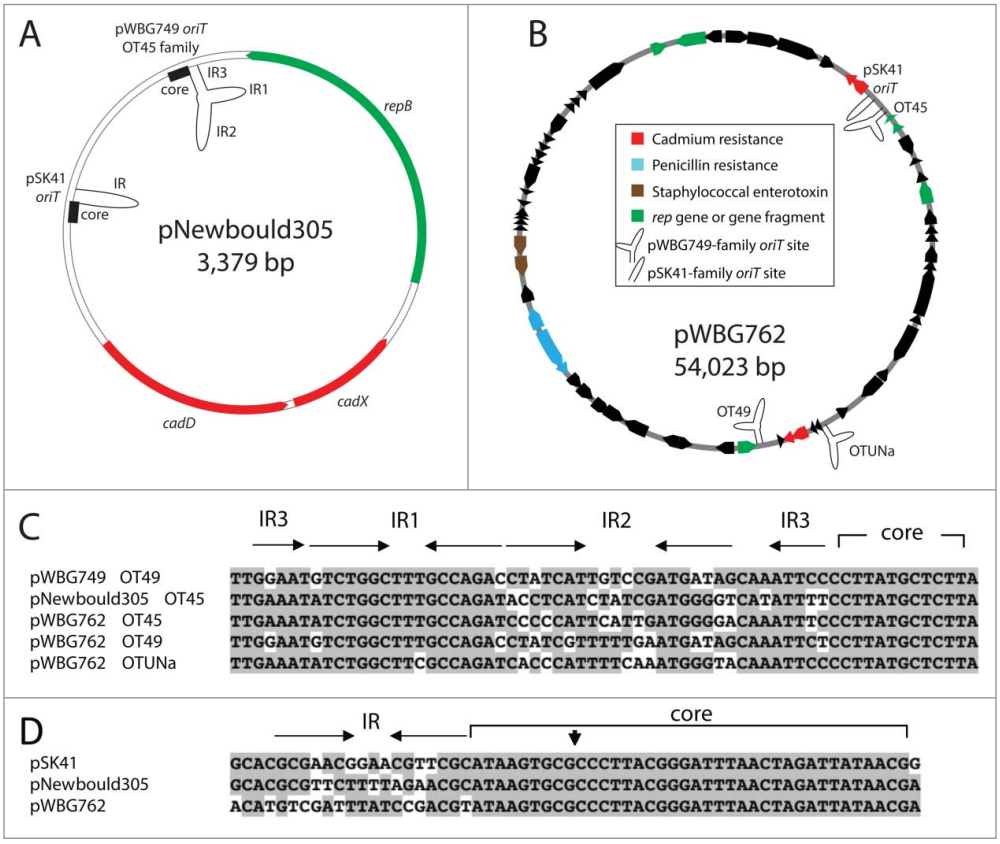


**Figure 3.** The diversity of *oriT* mimics on large and small resistance plasmids in staphylococci. (A) The plasmid map of the rolling-circle plasmid pNewbould305, illustrates the presence of 3 potential mobilization mechanisms. The *repB* gene of pNewbould305 shares 56% amino-acid identity over 96% of its length with the pBS42/pUB110 RepB protein, which enables mobilization by ICE*Bs1*-family elements. Downstream of the *repB* gene is an *oriT* mimic sequence of the pWBG749-family, subfamily OT45 and a pSK41-like *oriT* mimic sequence. (B) The atypically large staphylococcal plasmid pWBG762, carries 4 *oriT* mimics. Three are of the pWBG749 family and one is of the pSK41 family. (C) Alignment of the pWBG749-family *oriT* mimic sequences carried by pNewbould305 and pWBG762 below the pWBG749 *oriT* region, illustrating IR2 sequence divergence. Conserved nucleotides are shaded. The AR1-AR3 repeats of the full *oriT* required for mobilization by pWBG749[8] have been truncated in this figure for clarity (D) Alignment of the pSK41-like *oriT* mimic sequences from pNewbould305 and pWBG762, below the pSK41 *oriT* region, showing divergence of the IR sequence; the Nes relaxase nick site is denoted by a vertical arrowhead.

*oriT*-specific SmpO protein is required for efficient mobilization. The reasons for the divergence of conjugative-plasmid *oriT*s and *oriT* mimic sequences is not clear, but it could signal an underlying frequency-dependent selection against the most common *oriT* variants, perhaps due to a reduction in conjugative-plasmid fitness resulting from mobilizable-plasmid exploitation.

Coincident with the discovery of the relaxase-*in trans* mechanism of mobilization based on the carriage of pWBG749-like *oriT* mimics, sequences resembling the pSK41 *oriT* were similarly detected on a wide range of non-conjugative staphylococcal plasmids, recently reported in our *Journal of Bacteriology* article.[52] These pSK41-like *oriT* mimics possess a core sequence identical to the conjugative *oriT* sequence, which includes the Nes nick site. However, they differ in an adjacent inverted repeat that forms a DNA hairpin important for interactions with the Nes relaxase (Fig. 3D). Nonetheless, purified pSK41 Nes binds and processes 2 different *oriT* mimics *in vitro*, albeit at lower efficiency than its cognate *oriT* sequence. Furthermore, pSK41 mobilises a recombinant plasmid carrying a copy of the pSK41 *oriT*, suggesting that pSK41-like conjugative plasmids are also capable of performing the relaxase-*in trans* mechanism of mobilization. However, pSK41 did not mobilise recombinant plasmids containing either of the 2 *oriT* mimics that had been shown to be substrates *in vitro*. It would seem likely that nucleotide differences in the inverted repeat flanking the pSK41-like *oriT* mimics confer specificity on the mobilization process, paralleling the variant specificity described above for pWBG749 relaxase-*in trans* mobilization. The involvement of accessory proteins, analogous to pWBG749 SmpO, in pSK41 relaxasome formation is currently under investigation. Four pSK41 mimic sequence types were identified on 83 non-conjugative staphylococcal plasmids, but notably none were identical to the *oriT* sequence found on all sequenced pSK41/pGO1 family conjugative plasmids.[52] Taken together, these observations raise the provocative possibility that many characterized plasmids carry *oriT* mimics specific for divergent pSK41-like conjugative plasmids that are yet to be detected.

### What proportion of non-conjugative staphylococcal plasmids are potentially mobilizable?

The discovery of the relaxase-*in trans* mechanism of mobilization greatly increases the number of non-conjugative plasmids in *S. aureus* that can be considered to be potentially mobilizable, as illustrated by our previous analysis of a database of 280 non-identical *S. aureus* plasmids collected from NCBI.[8,52] Only 18 (6 %) of these plasmids carry a relaxase gene resembling that of either pWBG749 (SmpP; n = 4) or pSK41 (Nes; n = 14). Examination here of the remaining 262 non-conjugative plasmids (Table S1) for the mobilization relaxase genes *mobA* and *pre*, and the recently identified *oriT* mimic sequences (pWBG749-family and pSK41-family) revealed a remarkable stratification in their distribution with respect to plasmid size. *mobA* or *pre* genes were found almost exclusively on plasmids in the 3.5–14.5 kb size range. 74% (n = 49) of plasmids in this size-range encode one of these genes (but never both). No smaller plasmids carried these genes, and for plasmids over 14.5 kb (n = 135), only 2% (n = 3) contained *mobA* or *pre* (likely pseudogenes were not included); these likely represent small plasmid cointegrates present in larger chimeric plasmids. Notably, 89% (n = 120) of plasmids over 14.5 kb, which includes many multiresistance and pathogenicity plasmids, contained at least one pWBG749-family (87%) or pSK41-family (38 %) *oriT* mimic sequence. Equally strikingly, only a single plasmid in the 3.5–14.5 kb size range contained an *oriT* mimic, and that plasmid didn't have *mobA* or *pre*. Thus, mimics appear to be remarkably under-represented in 3.5–14.5 kb plasmids, which are typically mobilizable by virtue of either a *mobA* or *pre* system with a cognate *oriT*. In contrast, mimics were carried by 39 % of plasmids less than 3.5 kb (n = 65), but none were found on plasmids less than 2.5 kb (n = 33).

Of 262 non-conjugative *S. aureus* plasmids (Table S1), 56 % (n = 146) were found to carry at least one *oriT* mimic, with pWBG749-family mimics present on 53 % (n = 138) and pSK41 mimics evident in 23 % (n = 61); many plasmids possessed both mimic types (20 %; n = 53). Some plasmids, such as pWBG762, carried 3 copies of pWBG749-family mimics as well as a single pSK41-family mimic. Perhaps most significantly, whereas very few large (>14.5 kb) multiresistance/pathogenicity plasmids were previously thought to be potentially mobilizable (i.e., encode *mobA* or *pre*), the detection of *oriT* mimics on 89% of these plasmids suggest nearly all these plasmids are potentially mobilizable by pWBG749 or pSK41-family plasmids through a relaxase-*in trans* mechanism. The

prevalence and conservation of *oriT* mimics not only implies far greater potential for mobilization than previously anticipated, but also carries important implications for our understanding of the evolution of staphylococcal plasmids and their hosts. Specifically, plasmids carrying these mimics effectively bear the evolutionary fingerprints of conjugative plasmids and implicate conjugative mobilization as major evolutionary driver in staphylococci.

Overall, 74% of non-conjugative *S. aureus* plasmids may have the capacity to be mobilised by either a relaxase-*in trans* (56 %) or the classical relaxase-*in cis* (19%) mechanism. The estimate for potential mobilization may be higher still if we consider the recently discovered "replicative relaxase" mechanisms of mobilization. The Grossman laboratory recently described the mobilization of rolling-circle-replication plasmids pC194, pBS42 and pHP13, which lack genes encoding classical Mob proteins, by the ICE*Bs1* family of integrative and conjugative elements. This mobilization mechanism is dependent on the plasmid-encoded rolling-circle replication protein, suggesting that these Rep proteins interact directly with the VirD4 coupling protein of ICE*Bs1*-family conjugation systems, recruiting ssDNA to the mating pore in a Mob-independent manner (Fig. 1).[6] ICE*Bs1* carries a conjugation gene cluster related to that of Tn*916*, a family of integrative and conjugative elements that are frequently carried by staphylococci.[53] BLASTP searches of non-conjugative plasmids revealed that 39% (n = 103) carried a Rep gene closely resembling that of pC194/pBS42/pHP13 ($E = 10^{-30}$) (Table S1). If these plasmids are indeed mobilizable through a replicative relaxase mechanism, this would raise the proportion of non-conjugative *S. aureus* plasmids with potential for mobilization to 80% (n = 210). We predict that this proportion may approach 100% as further types of conjugative plasmids and new mechanisms of plasmid mobilization are discovered in coming years.

## Conclusions

While only around 5–6% of *S. aureus* plasmids are conjugative, it appears that the majority of non-conjugative plasmids, including most large multiresistance plasmids, are potentially mobilizable. While the prevalence of conjugative plasmids in isolated *S. aureus* is low, the acquisition of Mob and *oriT* sequences by most non-conjugative plasmids evidences that conjugative mobilization is a frequent enough event that most *S. aureus* plasmids have evolved to take advantage of it. The presence of multiple *oriT* sites on numerous plasmids, suggests that previous and frequent exposure to variants of both pWBG749 and pSK41-family plasmids has facilitated the transfer of the most widely distributed large multiresistance plasmids such as pMW2, pIB485 and pUSA300HOUMR. The most disturbing consequence of this mobilization, from the perspective of antimicrobial-resistance, is that while horizontal transfer of pWBG749 or pSK41 is apparently rare, when it occurs, it may facilitate the transfer of any mobilizable plasmid present in the same cell. If documented vancomycin-resistance gene carrying derivatives of pSK41[22] and pWBG749[32] were to become endemic in *S. aureus* populations for example, we might expect gene transfer rates for all compatible mobilizable plasmids to increase as well, along with their resistance and virulence-gene cargo. Given the high prevalence of *oriT* and Mob-carrying plasmids in *S. aureus*, it is perhaps fortuitous for our healthcare systems that the prevalence of conjugative plasmids in *S. aureus* is only 5–6%. However, this low apparent incidence may reflect a sequence data sample heavily biased toward disease-causing organisms. Clinical strains are isolated from specialized niches so might be quite distinct from the broader population, or yet to be defined reservoirs, from which they originate, where mechanisms of horizontal gene transfer might be more significant.

There are very few examples of the relaxase-*in trans* mechanism documented for other mobile genetic elements. [54-57] Considering its simplicity, it is intriguing why the relaxase-*in trans* mechanism of plasmid mobilization has not been widely detected in other species. We suspect that in general, mobilization has been favored in *S. aureus* due to selection against large plasmid size and/or carriage of conjugation genes. *S. aureus* hosts relatively small plasmids compared to other genera, suggesting constraints on plasmid size may be particularly strict.[3,4] Conjugation-gene clusters are much larger than mobilization loci. The carriage of mobilization loci by most *S. aureus* plasmids may represent an evolutionary trade-off that enables smaller plasmid size while maintaining a potential for transfer in the presence of a conjugative plasmid.[58] Carriage of an *oriT* mimic likely has even a smaller impact on plasmid size than Mob-gene

carriage and the accumulation of multiple *oriT* mimics likely increases the opportunity for transfer. An interesting observation from the analysis of plasmid sizes and putative mobilization mechanisms, is that only 3% of plasmids over 14.5 kb carried a Mob or Pre gene, but 89% of the same plasmids carry an *oriT*. This could suggest that for large plasmids, there may be some deleterious effect of Mob-gene carriage. Indeed, plasmids such as pWBG762 and pMW2, carry *pre* pseudogenes, suggesting that they have been captured incidentally through plasmid cointegration events and subsequently mutated. Further molecular investigations are required to fully understand the nature of the selective forces shaping conjugative mobilization and reveal if the relaxase-*in trans* mechanism is indeed a phenomenon largely unique to staphylococci, or if it has simply been overlooked in other species.

Bacterial whole-genome sequencing has exploded in recent years and is even replacing our routine typing methods in pathogen and antimicrobial-resistance surveillance. With these increased data come the promise of an enlightened view of bacterial genome evolution and an increased understanding of the events that lead to the emergence of highly pathogenic or resistant strains. An understanding of horizontal-gene-transfer events and the mobile elements that facilitate them, is central to our understanding of bacterial evolution. We and others envisage that in the near future we will have the capacity to track horizontal spread of resistance and virulence elements throughout bacterial populations, which in turn will reveal opportunities in which we can control or prevent gene transfer from occuring.[2] However, how well we extract meaningful information from the multitude of sequenced genomes depends entirely on how well we understand the genes, proteins and molecular mechanisms underlying these processes. It is clear from our analyses that even for a clinically important and routinely sequenced pathogen like *S. aureus*, until recently, we have been oblivious to a DNA transfer mechanism that has impacted the evolution of the majority of its characterized plasmids. Several novel and diverse conjugation and mobilization systems have recently been recognized in bacteria[6,8,11,52] so we would be foolish to assume that there are not additional gene-transfer mechanisms operating that are yet to be recognized.[6,58]

## Materials and methods

Plasmids pWBG637 and pWBG707 were conjugated into *S. aureus* WBG4515[59] and then the whole genomes of the resulting strains were sequenced using Illumina MiSeq with the MiSeq Reagent Kit v2 (250 × 2). Reads were assembled using SPAdes v. 3.6.0 and contigs were extended using SSPACE Standard v. 3.0.[60,61] Plasmid pWBG4 DNA was extracted and digested with combinations of BamHI, EcoRI, ClaI and HindIII and cloned into pBluescript SK+. Each clone was sequenced using primer walking and Sanger sequencing, producing 413 sequences covering pWBG4 in both directions. A clone carrying a 5.7 kb ClaI fragment containing *dipA-dipC* conferred diffusible pigment production in *E. coli* (Fig. 2). Plasmid annotations were initially carried out using Prokka (1.12-$\beta$), prior to manual annotation.[62] Detection of Mob-gene loci was carried out as previously described[8] and the non-conjugative *S. aureus* plasmid list is provided in Table S1.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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