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MECHANISMS OF RESISTANCE

The *tet39* Determinant and the *msrE-mphE* Genes in *Acinetobacter* Plasmids Are Each Part of Discrete Modules Flanked by Inversely Oriented p*dif* (XerC-XerD) Sites

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ABSTRACT The *tet39* tetracycline resistance determinant and the macrolide resistance genes *msrE* and *mphE* were found in an 18.2-kb plasmid, pS30-1, recovered from a global clone 2 (GC2) *Acinetobacter baumannii* isolate from Singapore, that conferred resistance to tetracycline and erythromycin. pS30-1 also contains *mobA* and *mobC* genes encoding MOB_Q family proteins, but attempts to mobilize pS30-1 utilizing a coresident conjugative *repAci6* plasmid were unsuccessful. Eight p*dif* sites, consisting of inversely oriented binding sites for the XerC and XerD recombinases separated by 6 bp, were detected in pS30-1. The *tet39* determinant and the *msrE-mphE* gene pair are each surrounded by two p*dif* sites in inverse orientation. Identical regions in different contexts and many previously unnoticed p*dif* sites were found in a number of different plasmids in GenBank, showing that the *tet39* and *msrE-mphE dif* modules are mobile. A putative toxin/antitoxin system, a gene encoding a serine recombinase, and open reading frames of unknown function were also part of *dif* modules in pS30-1. In general, modules with internal XerC or XerD sites alternate. Two copies of IS*Ajo2-1* (94% identical to IS*Ajo2*) in pS30-1 were inserted 5 bp from a XerC site, and this appears to be the preferred insertion site for this insertion sequence (IS) group. Apparently, *Acinetobacter* plasmids exploit the *Acinetobacter* XerC-XerD recombinases to mobilize DNA units containing resistance and other genes, via an uncharacterized mechanism. The *tet39* and *msrE-mphE dif* modules add to the *oxa24* module and the *oxa58* module redefined here, bringing the total of resistance gene-containing *dif* modules in *Acinetobacter* plasmids to four.

KEYWORDS *tet39* tetracycline resistance, *Acinetobacter baumannii*, antibiotic resistance, *dif* modules, *msrE-mphE* macrolide resistance, plasmids

Acinetobacter baumannii is an important nosocomial pathogen whose treatment is increasingly problematic due to high levels of extensive antibiotic resistance. In addition to resistance genes located in islands in the chromosomes of globally disseminated clones, global clone 1 and 2 (GC1 and GC2) isolates, a number of resistance genes can be carried by plasmids. Some of these genes are part of defined transposons, such as *aphA6* (confers resistance to amikacin, kanamycin, and neomycin) in Tn*aphA6* (1) and *oxa23* (carbapenem resistance) in Tn*2006*, Tn*2008*, or Tn*2009* (2). These transposons have often been found in large conjugative *repAci6* plasmids (1, 3, 4). However, in *Acinetobacter* species two other resistance genes encoding carbapenemases appear to be mobilized by a novel mechanism. The *oxa24* gene and its single base pair variant encoding OXA-72 have been found flanked by inversely oriented XerC-XerD binding sites, or *dif*-like sites (referred to as p*dif* sites from here on), in plasmids from various *Acinetobacter* species (5–8). As this *oxa24* module has several different sequences on

Received 13 April 2017 **Returned for modification** 9 May 2017 **Accepted** 15 May 2017

Accepted manuscript posted online 22 May 2017

Citation Blackwell GA, Hall RM. 2017. The *tet39* determinant and the *msrE-mphE* genes in *Acinetobacter* plasmids are each part of discrete modules flanked by inversely oriented p*dif* (XerC-XerD) sites. Antimicrob Agents Chemother 61:e00780-17. https://doi.org/10.1128/AAC.00780-17.

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either side (5–8), and in one case is found twice in different locations and in the opposite orientation in the pAB120 plasmid (8), it is possible to conclude that a module containing the *oxa24* gene has moved to several different locations. Each p*dif* site is 28 bp long and contains the 11-bp binding sites for the XerC and the XerD recombinases in inverse orientation, separated by a 6-bp spacer of variable sequence (5). Re-27 elements initially identified as direct repeats in the sequence broadly surrounding another carbapenemase-encoding gene, the *oxa58* gene (9), were later recognized as p*dif* sites (5). Several other modules surrounded by inversely oriented p*dif* sites (5), here referred to as *dif* modules, have been identified, and a putative toxin/antitoxin system in pAB120 that is also in a *dif* module may also be mobile (8). These modules have been found in unrelated plasmids (5, 6, 8), indicating movement, and the p*dif* sites are thought to facilitate the mobilization of discrete modules of DNA containing these resistance genes and other genes using the chromosomally encoded XerC-XerD recombination system.

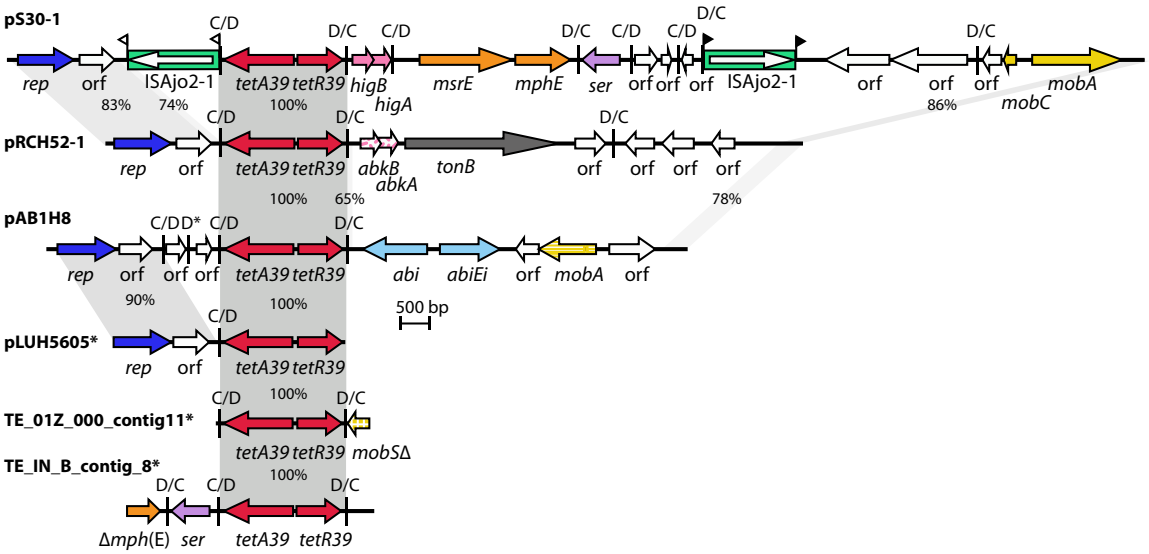
Recently, we found the *tet39* tetracycline resistance determinant in pRCH52-1, a small plasmid recovered from a non-GC1 or -GC2 clinical isolate from Brisbane, Australia, but we were unable to find clear boundaries for a module containing these genes using the two additional sequences available in GenBank at the time (10). Here, we have sequenced a GC2 isolate containing a plasmid carrying the *tet39* determinant and the *msrE* and *mphE* macrolide resistance genes and shown that they are in *dif* modules. Several further *dif* modules were also identified.

RESULTS

***A. baumannii* isolate SGH0823.** The GC2 isolate SGH0823 (also known as S30) was sequence type 2 (ST2) using the Institut Pasteur multilocus sequence typing (MLST) scheme, and in the Oxford Scheme it was ST369 (*cpn60-2*, *gdhB-3*, *gltA-1*, *gpi-106*, *gyrB-3*, *recA-2*, and *rpoD-3*), a single-locus variant of ST208 varying in the *gpi* allele. SGH0823 was found to be extensively antibiotic resistant using the criteria for *A. baumannii* stated in Magiorakos et al. (11). It is resistant to carbapenems (imipenem, meropenem, and doripenem), extended-spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime, and cefepime), penicillins, β-lactamase inhibitors (ticarcillin-clavulanic acid, ampicillin-sulbactam, and piperacillin-tazobactam), quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin and levofloxacin), folate pathway inhibitors (trimethoprim and sulfamethoxazole), tetracyclines (tetracycline, minocycline, and doxycycline), and several aminoglycosides (gentamicin, amikacin, neomycin, streptomycin, and spectinomycin). SGH0823 is susceptible to tobramycin, netilmicin, and colistin.

Antibiotic resistance genes. ResFinder identified 12 resistance determinants. The *strA-strB* and *tet*(B) genes (conferring resistance to streptomycin and to tetracycline, minocycline, and doxycycline, respectively) were assigned to AbGRI1, while the bla_{TEM} (ampicillin resistance), *aacC1* (resistance to gentamicin), *aadA1* (spectinomycin resistance), *sul1* (sulfonamide resistance), and *aphA1b* (resistance to kanamycin and neomycin) genes were assigned to AbGRI2. The structure of these islands will be described in detail elsewhere. The *aphA6* gene located in Tn*aphA6* and the *oxa23* gene located in Tn*2008* were localized to a 76.9-kb *repAci6* plasmid, pS30-2. The macrolide resistance genes *msrE* and *mphE* as well as the *tet39* resistance determinant were detected on the same 7.9-kb contig.

pS30-1. The sequence at both ends of the contig containing the *tet39* and *msrE* and *mphE* genes matched the same end of an insertion element IS related to IS*Ajo2*. The opposite end of the IS was found at both ends of another contig. PCR oriented the fragments and amplified single copies of this IS and the products were sequenced to complete assembly of the sequence of the plasmid pS30-1 (Fig. 1, top line). pS30-1 is 18,234 bp and encodes a replication initiation protein, Rep, that belongs to the Rep_3 superfamily (pfam01051) and contains a putative iteron sequence of four identical repeats of 22 bp adjacent to the *rep* gene. The Rep protein of pS30-1 is 99.7% (306/307 amino acids [aa]) identical to the Rep protein of pM131-5 (GenBank accession number



Sequence	*tet39* fragment size (bp)	Left *pdif* site: XerC-XerD	Right *pdif* site: XerD-XerC
pS30-1	2,001	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGCTGTTATGCGAAAC
pRCH52-1	2,001	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGATATTATACGAAGT
pAB1H8	2,001	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATAGAGCTTATGCGAAAT
pLUH5605	1,986	ATTTCGTATAAGGTGTATTATGTTAATT	–
TE_01Z_000_contig11	2,001	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATAGAGCTTATACGAAAT
TE_IN_B_contig_8	2,001	AATTCGTATAACGCGTATTATGTTAATT	ATTTAACATAATAGAGCTTATACGAAAT

FIG 1 Linearized map of pS30-1 compared to other regions containing the *tet39* resistance determinant module. Partial sequences are marked with an asterisk. Arrows indicate the extents and directions of genes and ORFs. The *tetA* gene and the *tetR* genes of *tet39* are shown in red, and *rep* genes are shown in dark blue. Genes encoding putative toxin/antitoxin modules are shown in solid or patterned pink. The *msrE* and *mphE* resistance genes are shown in orange, *tonB* is dark gray, *abi* genes are light blue, and *ser*, encoding a serine recombinase, is shown in purple. Genes encoding proteins showing homology to mobilization proteins are shown in yellow. The *tonB* gene encodes a TonB-dependent transporter homologue. The light green box represents IS*Ajo2-1*, and the arrow inside represents the transposase gene. The extents of regions with significant DNA identities are shown in gray, and the numbers represent DNA identities. Vertical bars indicate p*dif* sites; the orientation of each site is shown above, and flags indicate target site duplications. The picture is drawn to scale according to the GenBank entries listed in Table 1. The sequences of the p*dif* sites that flank the *tet39* module are shown in the table at the bottom of the figure. The bases highlighted in red are different.

JX101644) from an *Acinetobacter soli* (12). Thereafter, the closest Rep protein is RepAci3 (GenBank accession number GU978997), with 91% amino acid identity (278/307 aa). The copy number of pS30-1 relative to the chromosome was estimated to be 10 to 11. The plasmid was transformed into *A. baumannii* ATCC 17978 via electroporation using SGH0823 genomic DNA and conferred resistance to tetracycline (zone size of 6 mm compared to 20 mm for ATCC 17978) and reduced susceptibility to doxycycline (zone size of 18 mm versus 27 mm) but did not affect the zone size for minocycline. The transformants also showed increased resistance to erythromycin (>132 μg/ml) relative to that of ATCC 17978 (16 μg/ml).

***tet39* is located between p*dif* sites.** Manual inspection of the region surrounding *tetA39* and *tetR39* in pS30-1 revealed that p*dif* sites flank these genes. The segment containing the *tet39* resistance determinant located between the flanking p*dif* sites is 2,001 bp in size, and, as previously found for the *oxa24* gene, the flanking sites are in inverse orientation (XerC-XerD and XerD-XerC). The same 2,001-bp segment was also found surrounded by p*dif* sites in plasmid pRCH52-1 although this was not noted previously (10). The original sequence for the *tet39* determinant in pLUH5605 (GenBank accession number AY743590) was found to contain a p*dif* site but does not extend far enough to include the p*dif* site to the right. The *tet39* module is also in pAB1H8 (see Table 1 for details of sequences), and close inspection of this sequence revealed three p*dif* sites that were not annotated. Previously unnoticed p*dif* sites

TABLE 1 Sequences used during this study

Sequence name	Sequence length (bp)	No. of p*dif* sites[a]	Source[b]	GenBank accession no.	Reference or source
pS30-1	18,234	8	*A. baumannii*	KY617771	This study
pRCH52-1	11,164	3	*A. baumannii*	KT346360	10
pAB1H8	10,246	3	*A. baumannii*	ANNC01000048	28
pLU5605	3,727	1	*Acinetobacter*	AY743590	29
TE_01Z_000_contig 11	2,446	2	Uncultured bacterium	KU545777	30
TE_IN_B_contig_8	3,953	3	Uncultured bacterium	KU544458	30
EF102240[c]	13,122	4	*A. baumannii*	EF102240	31
pABIR	29,823	13 (2)	*A. baumannii*	EU294228	18
pOXA58_882	36,862	16 (2)	*A. pittii*	CP014479	
pXBB1-9	398,857	12 (9)	*A. johnsonii*	CP010351	16
p255n-1	92,939	9	*A. baumannii*	KT852971	32

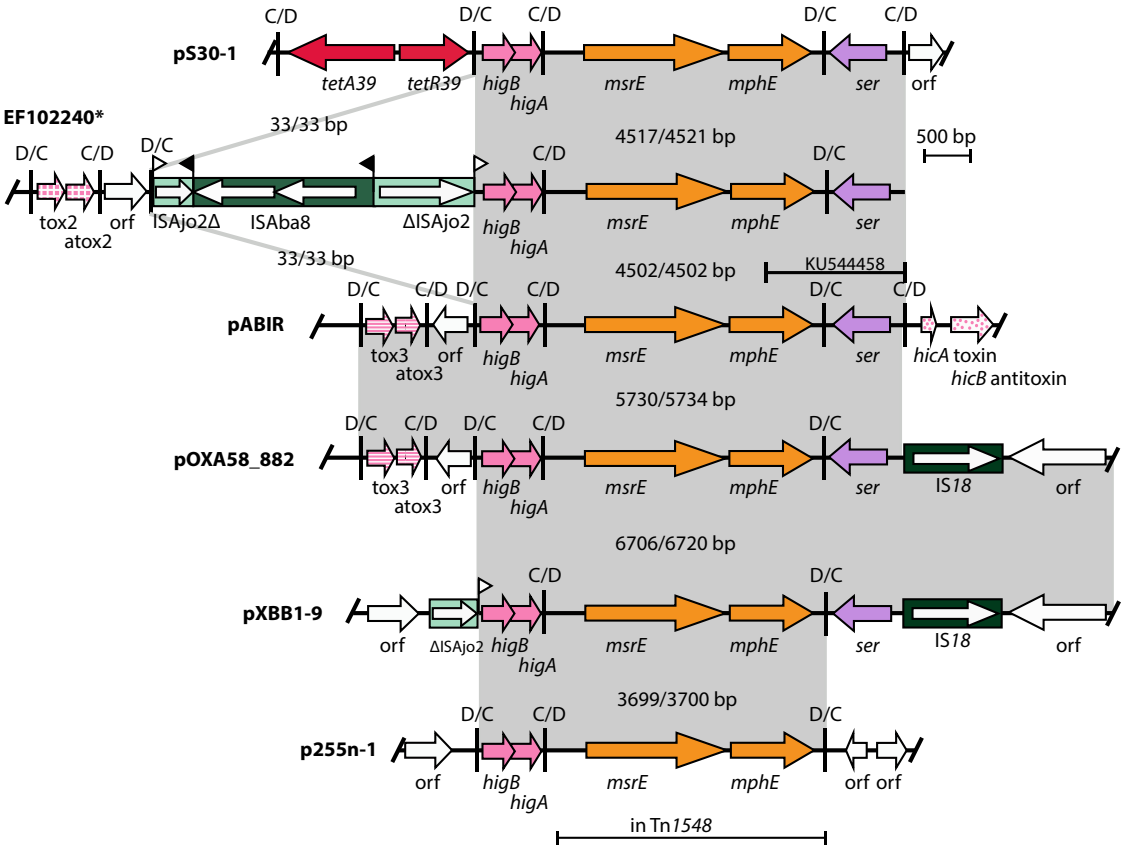
[a]Number of p*dif* sites identified in this study. If any p*dif* or Re-27 site was identified previously, the number found is in parentheses.
[b]*A. pittii*, *Acinetobacter pittii*; *A. johnsonii*, *Acinetobacter johnsonii*.
[c]This sequence is of a cloned insert from a larger plasmid from *A. baumannii* A1.

were also found in the partial sequences TE_01Z_00_contig11 and TE_IN_B_contig8 (Fig. 1). As the sequences on each side of the region surrounded by the p*dif* sites are different in each of the structures shown in Fig. 1, it is possible to conclude that the *tet39 dif* module is mobile.

The *msrE-mphE* gene pair is surrounded by p*dif* sites. Two inversely oriented p*dif* sites were also found flanking a 2,950-bp segment including the *msrE* and *mphE* macrolide resistance genes in pS30-1 (Fig. 2), and the presence of the *pdif* sites is consistent with this being a separate mobile unit. A search of the nucleotide database with this segment revealed a number of other plasmids and partial sequences that contain the same segment (Table 1), and these sequences were inspected for the presence of p*dif* sites (Fig. 2). A total of 41 additional p*dif* sites were found. However, the *msrE-mphE dif* module is always adjacent to another *dif* module carrying a toxin-antitoxin gene pair (see below) on one side and, in all but one case, adjacent to a gene encoding a serine recombinase on the other. These macrolide resistance genes are also found in Tn*6180* located in AbGRI3 in some GC2 isolates (13) and in the closely related transposon Tn*1548* (14) but the *dif* module is incomplete (Fig. 2, bar at bottom). In these transposons, the p*dif* site on the right is present and followed by a third sequence on this side. However, the p*dif* site on the left is missing, and a 120-bp segment next to it has been replaced by an IS*Ec29*.

Additional *dif* modules in pS30-1. In addition to the two modules containing antibiotic resistance genes described above, four other potential *dif* modules surrounded by inversely oriented p*dif* sites were identified in pS30-1. One, located between the *tet39* and the *msrE-mphE* modules, contains two overlapping open reading frames (ORFs) that encode a putative toxin/antitoxin system. The first ORF produces a 107-aa protein of the HigB toxin family, while the second translates to a 91-aa protein with a helix-turn-helix DNA binding domain, likely performing the function of the counterpart antitoxin HigA (15). This module was found in the same position in all of the plasmids containing a complete *msrE-mphE* module (Fig. 2).

Downstream of the *msrE-mphE* module in pS30-1 is a region encoding a small serine recombinase (203 aa), here named *ser*, surrounded by inversely oriented p*dif* sites. The *ser* gene is also found following the *msrE-mphE* module in all available sequences except that of p255-1 (Fig. 2). However, in sequences that contain *ser*, there are two variants based on whether a p*dif* site in inverse orientation to the left-hand site of this module is present on the right. pS30-1, TE_IN_B_contig_8 (KU544458) and pABIR form one group where there is a p*dif* site on the right. EF102240 is also a member of this group although available sequence extends only 4 bp into the XerC binding site of the right p*dif*. The right p*dif* sequence and 29 bp of the *ser* module directly prior to it have been replaced by 33 bp of unrelated sequence and then an IS*18* in pOXA58_882 and pXBB1-9. No site resembling a XerC-XerD binding site is found in the sequence directly



Sequence	*msrE-mphE* fragment size (bp)	Left p*dif* site: XerC-XerD	Right p*dif* site: XerD-XerC
pS30-1	2,950	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAAAATTTCTTATGTGAAGT
p255n-1	2,950	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAAAATTTCATATACGAAAT

FIG 2 Comparison of sequences containing the *msrE* and *mphE* resistance genes. Partial sequences are marked with an asterisk. Arrows indicate the extents and directions of genes and ORFs and are colored according to the scheme used in Fig. 1. The light green box indicates IS*Ajo2-1*, while the dark green box indicates IS*18*; the internal arrows represent the transposase gene. Vertical bars indicate p*dif* sites; the direction of each site is shown above, and flags indicate target site duplications. Regions with significant DNA identity are shown in gray along with the respective base pair ratios. The picture is drawn to scale from the GenBank entries listed in Table 1. The table below the figure shows the two sequences of p*dif* sites that flank the *msrE* and *mphE* genes. The p*dif* sites of pS30-1 are identical to those of all of the plasmids shown except for those of p255n-1, for which variant bases are highlighted in red.

following the IS*18*. It is possible that a recombination event has occurred in the *res* site associated with *ser*.

Another module in pS30-1, encoding a protein of no known function, is surrounded by inversely oriented p*dif* sites (Fig. 1, top line), and the p*dif* site upstream of the *mobC* gene and the first p*dif* site following the *repA* gene are also in inverse orientation. Two other segments that include open reading frames of unknown function are bounded by p*dif* sites in direct orientation, and whether these have resulted from the fusion of adjacent modules causing loss of an intervening p*dif* site or whether they are functional remains to be established.

IS*Ajo2-1*. The two copies of IS*Ajo2-1*, the 1,482-bp insertion sequence identified in pS30-1, share 94% nucleotide identity with IS*Ajo2* (16), and the encoded transposase is 97% identical to that of IS*Ajo2*. Like IS*Ajo2*, IS*Ajo2-1* has 24-bp inverted repeats with 20 bp identical and produces a 5-bp target site duplication. Interestingly, both copies of this IS in pS30-1 (Fig. 1) and the IS*Ajo2* in EF102240 (Fig. 2) are located 5 bp from the XerC side of a p*dif* site, with the IS facing in the same direction with respect to the

orientation of the p*dif* site. This indicates that these ISs may target a specific location in an orientation-specific manner. If so, their presence will point to p*dif* sites.

Mobilization genes in pS30-1. pS30-1 encodes a 479-aa protein that shares 31% amino acid identity with parts of the 710-aa MobA of RSF1010 (GenBank accession number NC_001740), the prototype for the MOB_Q family of *mob* genes (17). The reading frame upstream of *mobA* in pS30-1 translates to an 88-aa protein that shares 42% identity with the 95-aa MobC protein of RSF1010. The presence of *mobA* and *mobC* genes indicates that pS30-1 may be able to be mobilized if a suitable conjugative plasmid is also present in the cell. SGH0823 contains a conjugative *repAci6* plasmid (pS30-2) that carries Tn*2008* and was found to transfer ticarcillin resistance at a frequency of 2.3×10^{-5} transconjugants/donor. The ability of pS30-2 to mobilize pS30-1 was tested by selecting for the presence of transconjugants exhibiting tetracycline resistance in addition to rifampin and ticarcillin resistance. Only rifampin- and ticarcillin-resistant transconjugants were recovered. Though pS30-1 was not mobilized by the *repAci6* plasmid, it may be mobilized by a different type of conjugative plasmid.

DISCUSSION

The fact that the *tet39* module is flanked by various different sequences allowed it to be defined. However, it is likely to include only one p*dif* site, and which p*dif* site should be included is not clear as there is variation in the spacer sequence in both the p*dif* site on the left and in the p*dif* site on the right. The finding that the *tet39* determinant and the *msrE-mphE* gene pair are each part of mobile *dif* modules adds to those carrying the *oxa24* and *oxa58* genes (5–9, 16, 18), bringing the number of antibiotic resistance determinants found in *dif* modules in *Acinetobacter* plasmids to four. In addition to the antibiotic resistance genes, several toxin-antitoxin gene combinations were found in *dif* modules (Fig. 1 and 2), and these likely contribute to the stability of any plasmid carrying them. Additional modules identified in this study mostly encode hypothetical proteins of unknown function.

IS*Ajo2-1* identified here and IS*Ajo2* (16) are currently placed in the IS*1202* group in the IS*NCY* (not characterized yet) category. Our searches revealed that a relative of IS*Ajo2* with 90% nucleotide identity or higher is present in 10 additional sequences in GenBank. In each case, a putative XerC binding site of a p*dif* site was found 5 bp or, in a few cases, 6 bp away from the same end of the IS. Another IS in this group, IS*Aba32*, has also been found in an *A. baumannii* plasmid, pD36-4, that is part of the D36 genome (19). IS*Aba32* is also 1,482 bp in size and creates a 5-bp direct duplication upon insertion; however, its inverted repeats are slightly longer at 26 bp with 20 bp identical. The sequence of IS*Aba32* is 73.3% identical to that of IS*Ajo2*, and the encoded transposase is 72% identical to that of IS*Ajo2*. Interestingly, examination of the sequence adjacent to the copy of IS*Aba32* in pD36-4 (GenBank accession number CP012956) revealed that it was also inserted 5 bp from an XerC binding site in a p*dif* site. A number of unnamed ISs with sequence identity to IS*Aba32* of 89% or higher were found in seven entries in GenBank. In each of these sequences, the IS is again located 5 bp away from a XerC binding site and always in the same orientation. It appears that the variants of IS*Ajo2* and IS*Aba32* both target the same sequence, a XerC binding site, although how this targeting is achieved is unclear; thus, further investigation of the IS*Ajo2* group of insertion sequences is warranted. Nonetheless, these ISs can serve to pinpoint p*dif* sites that may otherwise be difficult to find.

In most publications to date, only a limited number of p*dif* sites (or Re-27 repeats) or none of them have been recorded, and whether other sites are in the broader vicinity has not been investigated. This is likely due to the difficulty in finding them. A feature of regions containing one or more *dif* modules that emerged from our analysis of all p*dif* sites in pS30-1 and in several other plasmids is the fact that, in most cases, the p*dif* sites are inversely oriented. This inevitably leads to two types of *dif* modules, one flanked by a XerC-D and a XerD-C site (i.e., the XerD binding sites are both closer to the internal segment) and the other type flanked by a XerD-C and a XerC-D site, making the XerC sites internal. In cases where more than one *dif* module is present, this feature

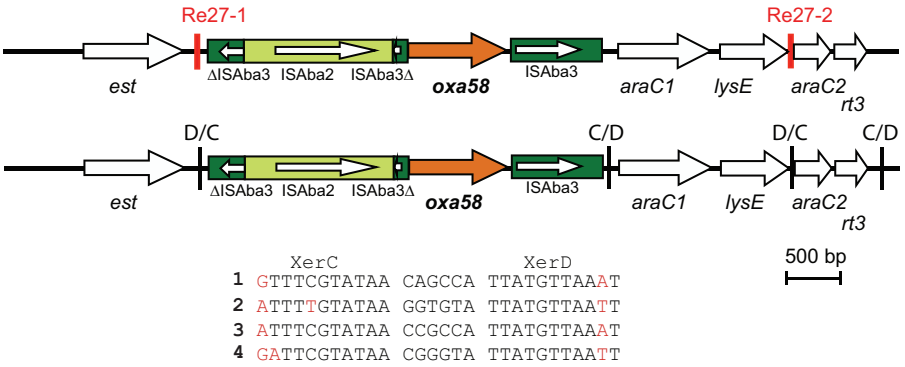


FIG 3 Location of p*dif* sites in the region surrounding the *oxa58* gene in a fragment from a plasmid of *A. baumannii* MAD. Arrows indicate the extents and directions of genes, and the *oxa58* gene is shown in dark orange. The light green box indicates IS*Aba2*, while the dark green box indicates IS*Aba3*; the internal arrows represent the transposase gene. Vertical red and black bars indicate the Re-27 and p*dif* sites, respectively, and the orientation of the p*dif* site is shown above the bars. The figure is drawn to scale based on GenBank accession number AY665723. In the sequences of the p*dif* sites, the numbers 1 to 4 correspond to the sites from left to right, and the bases that vary are highlighted in red.

dictates that D type and C type modules should alternate, and this is usually seen (Fig. 1 and 2). As the Re-27 sites surrounding the *oxa58* gene were identified as direct repeats (9, 16, 18), we examined the original sequence of the fragment from isolate MAD (GenBank accession number AY665723) for additional p*dif* sites. As shown in Fig. 3, two additional p*dif* sites were found, both in opposite orientation to the Re-27 sites. One creates a smaller *dif* module containing *oxa58* and an adjacent module containing the genes usually annotated as *araC* and *lysE*. The second p*dif* site discloses an additional adjacent *dif* module. Similarly, inversely oriented p*dif* sites were found surrounding *oxa58* in pOXA_58_882 and pABIR. We found 15 *dif* modules (8 D type and 7 C type) in pOXA_58_882 and 12 *dif* modules in pABIR (6 D type and 6 C type). In both cases, D type and C type modules alternate. However, two regions of pS30-1 are surrounded by directly oriented p*dif* sites, and searches did not reveal an additional site within them. Whether these are fusions of two *dif* units may become apparent as other sequences become available, but a clear fusion can be seen in the case of the *ser* module (Fig. 2), and the incomplete *msrE-mphE* gene module in Tn*1548* suggests that such events do occur.

A pair of *dif* modules was recently found in *Proteus mirabilis* (20), and this represents the first time that *dif* modules have been found outside the *Acinetobacter* genus. One of the modules is related to the smaller *oxa58* module described above, and the second is a novel module that includes an *ampC* gene that confers resistance to amoxicillin. In this case, the pair of *dif* modules, *oxa58* with XerC sites internal and *ampC* with XerD sites internal, was present three times in a single array. This configuration clearly should preserve the alternation of an XerC-D and an XerD-C site seen in *Acinetobacter* plasmids and raises the possibility that *dif* modules may usually move in pairs.

While there is now clearly sufficient evidence to conclude that *dif* modules are discrete mobile elements and some of the features associated with them are emerging, further work will be needed to investigate the mechanism of movement of *dif* modules. A catalogue all of the *dif* modules present in other *Acinetobacter* plasmids, such as those carrying the *oxa24* and *oxa58* genes, compiled by careful examination of sequence surrounding other *dif* modules should provide additional useful insights. Experimental investigation of module movement will be needed to understand the mechanism and demonstrate that XerC and XerD are involved.

MATERIALS AND METHODS

Bacterial isolate. SGH0823, a GC2 *A. baumannii*, was isolated in 2008 from Singapore General Hospital and was kindly supplied by Li Yang Hsu. The isolate was screened for resistance to 30 antibiotics using a disc diffusion method as described previously (21). Colistin susceptibility was determined by Etest (bioMérieux, Durham, NC).

DNA isolation, sequencing, and PCR amplification. Genomic DNA for sequencing was prepared as described previously (22) and sequenced using Illumina MiSeq. Paired-end reads (read depth of 67.6) were assembled into contigs using SPAdes (version 3.5.0) (23), yielding 62 contigs ranging from 202 to 462,003 bp, with an N_{50} of 262,554. Primers tet39-R (10), RH2502 (5′-AACAGGGATGTTCGGGCTAT-3′), RH2503 (5′-TGTCGCTTTGAGAGTTAGGC-3′), and RH2504 (5′-TCGTATTGGTTCGCTCGGTA-3′) were used to assemble pS30-1. PCR conditions used to detect short and long amplicons were as described previously (24), and amplicons were sequenced. Sequencher, version 3.2.3 (Gene Codes Corporations, Ann Arbor, MI, USA), was used for final sequence assembly, and Gene Construction Kit, version 4 (Textco, West Lebanon, NH, USA), was used to draw figures to scale.

Sequence analysis. Multilocus sequence typing (MLST) in the Institut Pasteur and Oxford schemes was performed via the *A. baumannii* MLST database (http://pubmlst.org/abaumannii). The coverage of plasmid contigs relative to the average coverage for contigs containing the genes used in both MLST schemes was used to assess copy number. Contigs containing antibiotic resistance genes were identified using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) (25). Contigs containing fragments of IS*26* and an IS*Ajo2*-like insertion sequence were recovered using stand-alone BLAST (26). DNA and amino acid sequences were compared to those in the GenBank nucleotide and protein databases using nucleotide and protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the sequences used for comparison are listed in Table 1. The ISFinder database (https://www-is.biotoul.fr) (27) was used to compare ISs. The p*dif* sites in plasmids were located by first identifying a sequence matching the less variable XerD binding site (5′-ATTTAACATAA-3′) and then examining the sequence 6 bp in either direction for a site resembling the XerC binding site (5′-TTATGCGAAAT-3′) of *Acinetobacter*.

Transformation and conjugation. ATCC 17978 cells (tetracycline susceptible) were grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.7 and made electrocompetent with a series of washes with 10% glycerol. Chromosomal DNA (2 μl of 2,940 ng/μl) was transformed by electroporation into competent cells using a 0.2-cm cuvette and the following parameters: 2.5 kV, 25 μF, and 200 Ω. Following recovery in LB medium, transformants were selected on L-agar containing 4 mg/liter of tetracycline and were screened for the presence of the *tetA39* gene (10). Transformants were tested for resistance to tetracycline, doxycycline, and minocycline using a disc diffusion method as described previously (21). Resistance to erythromycin was determined by patching ATCC 17978 and transformants on Muller-Hinton agar (cation adjusted) containing various concentrations of erythromycin (2, 8, 16, 32, 64, and 128 μg/ml).

A spontaneous rifampin-resistant mutant of the sulfonamide-resistant *A. baumannii* strain ATCC 19606 was isolated for use as a recipient in conjugation experiments. Matings were performed as described previously (4), and transconjugants were recovered on L-agar plates containing rifampin (100 mg/liter) to select for the recipient and with ticarcillin (100 mg/liter) to select for the transfer of the *repAci6* plasmid which carries the *oxa23* gene. Potential transconjugants were purified and screened on L-agar containing kanamycin (20 mg/liter), to which the donor is resistant and the recipient is susceptible, to eliminate spontaneous rifampin-resistant derivatives of the donor. To detect mobilization, transconjugants were selected on L-agar containing tetracycline (4 mg/liter), ticarcillin (100 mg/liter), and rifampin (100 mg/liter).

Accession number(s). The sequence of pS30-1 has been submitted to GenBank under accession number KY617771.

ACKNOWLEDGMENTS

We thank Li Yang Hsu from Singapore General Hospital for supplying the isolate SGH0823 used in this study. We also thank Ian Grainge for providing the predicted sequences of the XerC and XerD binding sites of *Acinetobacter*.

Funding for this study was received from National Health and Medical Research Council grant 1079616. G.A.B. is supported by an Australian Postgraduate Award.

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