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Short Communication

Acquisition of a second multi-drug resistance-encoding element by IncM1 plasmid pACM130 abolished conjugative transfer

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ABSTRACT

Within the IncM plasmid family there is a lineage that has a transposon Tn*1721*-based multiple-resistance island inserted in the backbone gene *mucB*. So far, this group includes R1215, p202c, pIGT15, pARM26, and pACM1, from Europe and the USA. A new member of this group, pACM130, was isolated at the same American hospital as pACM1 and has a similar resistance island, but also carries a copy of Tn*1331* that interrupts the *traY* gene in the conjugation operon. The conjugative phenotype of this plasmid has been abolished, though pACM130 could be mobilized by an intact *traY* cloned into a laboratory vector and transformed into the same donor bacterium.

Short Communication

1. Introduction

pACM130 is one of a group of plasmids from an Albany, NY, USA, hospital that share a common IncM backbone and a set of resistance, integron, and insertion sequence markers, as determined initially by dot-blot hybridization (Preston et al., 2003). The key characteristic of this group was the production of the SHV-5 extended-spectrum β-lactamase (ESBL), which was responsible for a cephalosporin-resistant phenotype. The prototypical plasmid of the group was pACM1, isolated in 1993 from a strain of *Klebsiella oxytoca* that caused a clonal outbreak in a neonatal intensive care unit (Venezia et al., 1995). The sequence of pACM1 (Preston et al., 2014) revealed a 32 kb resistance island with a Tn*1721* foundation, and internal insertions of Tn*1696*, Tn*2003* [carrying *bla*(SHV-5)], and two integrons. The resistance island was inserted into the gene *mucB*, in the backbone of an incompatibility group M (IncM) plasmid. As more plasmid genomes have been added to the GenBank database, it has become evident that pACM1 is one example of a lineage of plasmids carrying related resistance islands in the same location in the IncM backbone (Blackwell et al., 2016).

pACM130 was found during a hospital-wide survey of cephalosporin-resistant isolates that followed the *K. oxytoca* outbreak and continued through the year 2000 (Preston et al., 2003). The plasmid was obtained from a *Citrobacter freundii* isolate in 1999. In addition to being positive for the *bla*(SHV) and IncM markers, pACM130 DNA hybridized with a gene probe for the TEM-family of β-lactamases (not present on pACM1), and isoelectric focusing of proteins demonstrated that it encoded two β-lactamases with isoelectric points (pI) of 5.4 and 6.9, in addition to the SHV-5 ESBL (pI 8.2). The resistance phenotype encoded by pACM130 was essentially the same as that encoded by pACM1, except that pACM130 did not express trimethoprim resistance.

Except for two fragment size changes, the *Eco*RI fingerprint of pACM130 was indistinguishable from that of pACM1 (Preston et al., 2003). Because the termini of the Tn*1721*-based resistance island contain *Eco*RI sites (Preston et al., 2014), an *Eco*RI digest of pACM1 produces fragments belonging either to the backbone, or to the resistance island. Relative to pACM1, pACM130 has a resistance island fragment (fragment A) that has decreased in size from 21 kb to 14.8 kb and a backbone fragment (fragment B) that has increased in size from 4.9 kb to 12.9 kb. Both fragments A and B were subcloned into pACYC184 (Chang and Cohen, 1978), a vector which encodes no β-lactamases of its own, by selecting *Eco*RI library transformants on ampicillin agar (Preston et al., 2003). We have now sequenced the cloned fragments in order to identify the source of the additional β-lactamases and compare these regions of pACM130 to the relevant portions of pACM1 and similar plasmids.

2. Materials and methods

2.1. Nucleotide sequencing and analysis

Sanger sequencing, primer-walking, contig assembly and annotation were performed as previously described (Preston et al., 2014). The initial sequences of fragments A and B were obtained with primers KEP685 (5′-ACCGTTCAGCTGGATATTACGG) and KEP686 (5′-

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Table 1
Summary of conjugation experiments with *E. coli* DH5α strains containing plasmids.

Experiment	Donor plasmid(s) conjugative ability to be tested		Recipient plasmid(s) known or shown to be non-conjugative		Transconjugants	
	Name(s)[a]	Relevant Properties for experiment, backbone type	Name(s)[a]	Relevant Properties for experiment, backbone type	Selective Antibiotics (medium)[b]	Colonies/donor cfu
1 Control	pACM1	WT-*traY*, AMP^R, IncM1	pACYC184	CM^R, p15A	AMP + CM (LB)	3.4×10^{-4}
1 Exptl	pACM130	Δ*traY*, AMP^R, IncM1	pACYC184	CM^R, p15A	AMP + CM (LB)	None
2	(pACM127 pACM130)	WT-*traY*, CM^R, pUC19 Δ*traY*, TET^R, IncM1	pACM44	TMP^R, pUC19	TET + TMP (MHII)	2×10^{-3} [c]
3 Control	pACM1	WT-*traY*, TMP^R, IncM1	pACYC184	CM^R, p15A	CM + TMP (MHII)	1.8×10^{-4}
3 Exptl	pACM1	WT-*traY*, TMP^R, IncM1	(pACYC184 pACM130)	CM^R, p15A Δ*traY*, WT-*excA*, IncM1	CM + TMP (MHII)	5×10^{-6} [d]

[a] Plasmids in brackets were co-transformed into the same *E. coli* strain.
[b] AMP, ampicillin 50 μg/ml; CM, chloramphenicol 25 μg/ml; TET, tetracycline 15 μg/ml; TMP, trimethoprim 5 μg/ml; LB, Luria-Bertani agar; MHII, Mueller-Hinton II agar.
[c] No CM^R colonies were found among transconjugants, so pACM127 was not transferred with pACM130 from donor to recipient. The WT-*traY* sequence was an *Eco*RI-*Afe*I fragment of pACM1 and included the 3′ end of *traW*, *traX*, *traY*, and the 5′ end of *excA*. This was not oriented so that *traY* could be expressed from any of the vector promoters, and in fact, several attempts to create such a construct were unsuccessful.
[d] Colony PCR detected the unique *res* gene of pACM130 in all original transconjugant colonies. However, on the selective agar, 50% and 100%, respectively, of first and second passage transconjugant colonies were negative for the *res* gene, indicating loss of pACM130.

GTGGTATTCACTCCAGAGCGATG), which flank the *Eco*RI site in the vector pACYC184. A graphic multiple alignment was drawn with EasyFig 2.2.2 software (Sullivan et al., 2011) downloaded from http://mjsull.github.io/easyfig/.

The sequences of fragments A and B of pACM130 have been deposited as a gapped sequence in GenBank under Accession number KU530222.

2.2. Conjugation experiments

Liquid matings were performed between strains of *E. coli* DH5α containing plasmids encoding at least one unique resistance marker per donor or recipient (Table 1). Recipients contained plasmids known or shown not to be conjugative. Except for the selective media involved (Table 1), the procedure has been described previously (Preston and Venezia, 1999). To add a unique resistance marker or gene to an *E. coli* strain for use as a conjugation donor or recipient, it was sometimes necessary to add two plasmids to one aliquot of *E. coli* DH5α competent cells, and select co-transformants with drugs that would insure the presence of both plasmids (Table 1). For experiment 3 (Table 1), pACM130 had no phenotypically unique resistance marker for selection, so colony PCR (direct amplification of bacteria suspended in water) with primers KEP713 (5′-TGGCGGCTGGATCGGCTCG) and KEP714 (5′-CACGGAATATCAGGAGTCGG) was used to detect its unique *res* gene in transconjugant colonies.

3. Results and discussion

3.1. The sequence of fragment A shows a new variation in an existing Tn*1721* resistance island

Fragment A (14,852 bp) extends from the *Eco*RI in *merA* (of Tn*1696*) to the *Eco*RI in *recF* (of Tn*2003*), the central portion of the Tn*1721*-derived mosaic resistance island of pACM130 (Fig. 1A and B). (Although not sequenced, *Eco*RI fragments of pACM130 corresponding to the rest of the *mer* operon and the outer Tn*1721* portions of the resistance island are present (data not shown), and pACM130 encodes tetracycline resistance, as expected.) The structure of this region (Fig. 1B) is similar to corresponding *Eco*RI fragments of five other IncM1 plasmids in the GenBank database, including pACM1, pIGT15, pARM26, p202c, and R1215 (Preston et al., 2014; Adamczuk et al., 2015; Carattoli et al., 2015; Blackwell et al., 2016). These plasmids all have Tn*1721*-based resistance islands inserted in the *mucB* gene of the U.V. light-resistance operon, and all but R1215 are carriers of Tn*2003*, the composite IS*26*-based transposon that contains *bla*(SHV-5). R1215, which carries *bla*(TEM-1) instead, is the oldest, having been isolated prior to 1980 and preserved in the *Serratia marcesens* strain NCTC 50331. R1215 or a similar plasmid is the probable progenitor of the other five plasmids as well as the resistance islands of *Acinetobacter baumannii* strains GC1 and GC2 (Blackwell et al., 2016).

All six plasmids (Fig. 1B) have in common the *mer* operon portion of Tn*1696*, with its associated class 1 integron containing 5 cassettes. The cassette composition changed slightly from the ancestral R1215, which has a duplicate copy of *gcuP* but lacks *aacA4*. The end of the 5′-conserved segment of this integron, including the characteristic *Bam*HI site and the 25-bp terminal inverted repeat (Rådström et al., 1994; Stokes and Hall, 1989), is missing from all plasmids except R1215 and pACM130, which suggests that 1) pACM130 is not a direct descendant of pACM1, which lacks this ancestral sequence, and 2) the initial acquisition of the SHV-5 encoding transposon is not the event that trimmed the end of the integron, since pACM130 has both elements.

The region immediately to the right of the 5-cassette integron differs among the six plasmids (Fig. 1B). All except R1215 have a copy of Tn*2003*, but in pACM1 and pACM130, there is intervening sequence. Where pACM1 has a remnant of a second integron (with *dfrA1* and *aadA1* cassettes) and copies of IS*26* and IS*1R*, pACM130 has a coding sequence which, when translated, has a "resolvase" conserved domain, so we have labeled it "*res*." Often found near *IntI1*, this gene has many iterations in the GenBank database, but there is no clear consensus as to its name or function (e.g. invertase, resolvase, recombinase). However, Haines et al. (2005) described this sequence as the transposase (*tnpA*) of IS*Pa15*, and suggested that IS*Pa15* and its neighboring integron were part of a former composite transposon, in the *Pseudomonas aeruginosa* plasmid Rms149. A complete copy of IS*Pa15* is not present in pACM130, due to truncation by the adjacent IS*26*.

In pIGT15, pARM26, and p202c, Tn*2003* immediately follows the 5-cassette integron, but the point of transition from one element to the next is not identical. There is also variation in the orientation of the terminal IS*26*, and even, in the case of p202c, the entire transposon (Fig. 1 B). Portions of the internal sequence have been lost in pACM130 and pIGT15. These observations suggest that this element has rearranged several times after its initial insertion. Both pACM1 and pACM130 are missing the first 57 bases (terminal repeat and ribosomal binding site) of the copy of IS*26* (Fig. 1B), so even if one is not directly descended from the other, they must share a common ancestor with this deletion. Instead of Tn*2003*, R1215 has a series of transposons (most of which lies beyond the *Eco*RI fragment in the figure), including Tn*2670*/Tn*21*, Tn*1*, Tn*6020*, Tn*5393c*, and Tn*1000* (Blackwell et al., 2016). Tn*1* encodes the TEM-1 β-lactamase.