J Antimicrob Chemother 2022; **77**: 1202–1204
https://doi.org/10.1093/jac/dkac006
Advance Access publication 25 January 2022

Precise classification of antimicrobial resistance-associated IncP-2 megaplasmids for molecular epidemiological studies on *Pseudomonas* species

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The incompatibility group P-2 (IncP-2) of plasmids defined by Bryan *et al.*[1] includes many antimicrobial resistance (AMR)-associated large plasmids (megaplasmids, ≳400 kb) found in *Pseudomonas* species,[2] including *Pseudomonas aeruginosa*. In a recently published article, Jiang *et al.*[3] proposed a novel incompatibility group of plasmids, $\mathrm{Inc}_{\mathrm{pRBL16}}$, and listed 17 $\mathrm{Inc}_{\mathrm{pRBL16}}$ plasmids, including pRBL16 (accession no. CP015879), in Figure 1 of that article. The *repA* gene that encodes a replication initiation protein (RIP) for the $\mathrm{Inc}_{\mathrm{pRBL16}}$ plasmid, $repA_{\mathrm{pRBL16}}$, was located between 303 463 and 304 650 nt in pRBL16. In Figure 2 of that article, Jiang *et al.*[3] classified another set of 12 plasmids, which were previously proposed as IncP-2 plasmids based on the nucleotide sequences of the *repA* gene. The authors analysed five of them and reported that pSx1 (accession no. CP013115) and pCP017294 (PA83 plasmid unnamed1, accession no. CP017294) contain a single RIP gene, $repA_{\mathrm{IncP\text{-}2}}$, whereas pOZ176 (accession no. KC543497), pTTS12 (accession no. CP009975), and pJB37 (accession no. KY494864) contain another RIP gene, $repA_{\mathrm{pRBL16}}$ ($repA_{\mathrm{IncP\text{-}2}}$ and $repA_{\mathrm{pRBL16}}$ in pOZ176 were *pOZ176_301* and *pOZ176_183* genes, respectively), as the primary RIP gene, in addition to the auxiliary RIP gene $repA_{\mathrm{IncP\text{-}2}}$.[3–5]

We agree with the authors that they found two types of RIP genes in pRBL16 and pOZ176 (according to their nomenclature, $repA_{\mathrm{pRBL16}}$ in pRBL16, and $repA_{\mathrm{pRBL16}}$ and $repA_{\mathrm{IncP\text{-}2}}$ in pOZ176[3]). However, we would like to highlight that they identified the true RIP gene of IncP-2 plasmids as $repA_{\mathrm{pRBL16}}$, designated here as *repP-2A*, and not necessarily a novel replicon named $repA_{\mathrm{pRBL16}}$. In addition, we propose that their $repA_{\mathrm{IncP\text{-}2}}$ is not the primary RIP gene of IncP-2 plasmids. They also described that the above three plasmids (pOZ176, pTTS12, and pJB37) were misidentified as IncP-2 plasmids.[3–5] However, one of them, pOZ176, could not be stably maintained in the same bacterial cell with another IncP-2 plasmids in plasmid incompatibility tests, strongly indicating that pOZ176 is a member of the IncP-2 plasmids.[6] Subsequently, Xiong *et al.*[7] determined the complete nucleotide sequence of pOZ176 containing two RIP genes (*pOZ176_183* and *pOZ176_301* in accession no. KC543497), and proposed one of two *repA* genes (*pOZ176_301*, i.e. the auxiliary RIP gene in the plasmid) as a candidate RIP gene of IncP-2. Plasmids with this misidentified *repA* (*pOZ176_301* gene), not *repP-2A* (*pOZ176_183* gene), have been misrecognized as IncP-2 plasmids in some later studies, including ours.[8]

In this study, we determined the complete nucleotide sequence of the *Pseudomonas aeruginosa* plasmid Rms139 (accession no. LC653116),[9] which has been classically identified as a member of IncP-2 through plasmid incompatibility tests.[2] This plasmid contains a sole RIP gene (*repP-2A*, located between 1 and 1188 nt in Rms139) whose nucleotide sequence showed 100% identity with that of $repA_{\mathrm{pRBL16}}$ in pRBL16. Cazares *et al.*[10] proposed the pBT2436-like family as a group of megaplasmids, including pBT2436 (accession no. CP039989), in *Pseudomonas* species. Of note, each of them contained a conserved RIP gene (*FC629_32540* gene in pBT2436),[10] showing high identity with $repA_{\mathrm{pRBL16}}$ (92%–100% identity at the amino acid sequence level). This shows that $repA_{\mathrm{pRBL16}}$ is the primary and true RIP gene (*repP-2A*) of IncP-2 plasmids. Therefore, the nucleotide-sequence-based classification of IncP-2 plasmids should be updated based on the sequence of $repA_{\mathrm{pRBL16}}$.[3]

More recently, there have been several reports on AMR-associated IncP-2 megaplasmids in *Pseudomonas* species clinical isolates. Urbanowicz *et al.*[11] showed endemic spread of pBT2436-like megaplasmids carrying the carbapenemase gene $bla_{\mathrm{VIM\text{-}2}}$ using 19 plasmids, including pPUV-1 (accession no. MT732179), in *P. aeruginosa* isolated in Poland, which formed a subgroup within a family of IncP-2 megaplasmids. Zhang *et al.*[12] showed that 16 IncP-2 megaplasmids [9 plasmids in *P. aeruginosa* isolated in China, including pHS17-127 (accession no. CP061377), and 7 plasmids in *Pseudomonas* species in the NCBI database] carry the carbapenemase gene $bla_{\mathrm{IMP\text{-}45}}$ and this IncP-2 plasmid subgroup contributed to the worldwide spread of $bla_{\mathrm{IMP\text{-}45}}$.

AMR genes are often carried on plasmids and spread among bacteria via conjugation. Precise classification of AMR-associated plasmids by phenotyping methods based on plasmid incompatibility and genotyping methods based on RIP sequences are crucial for molecular epidemiological studies on clinically relevant bacterial pathogens, including *Pseudomonas* species. Indeed, we confirmed that IncP-2 megaplasmids in recent

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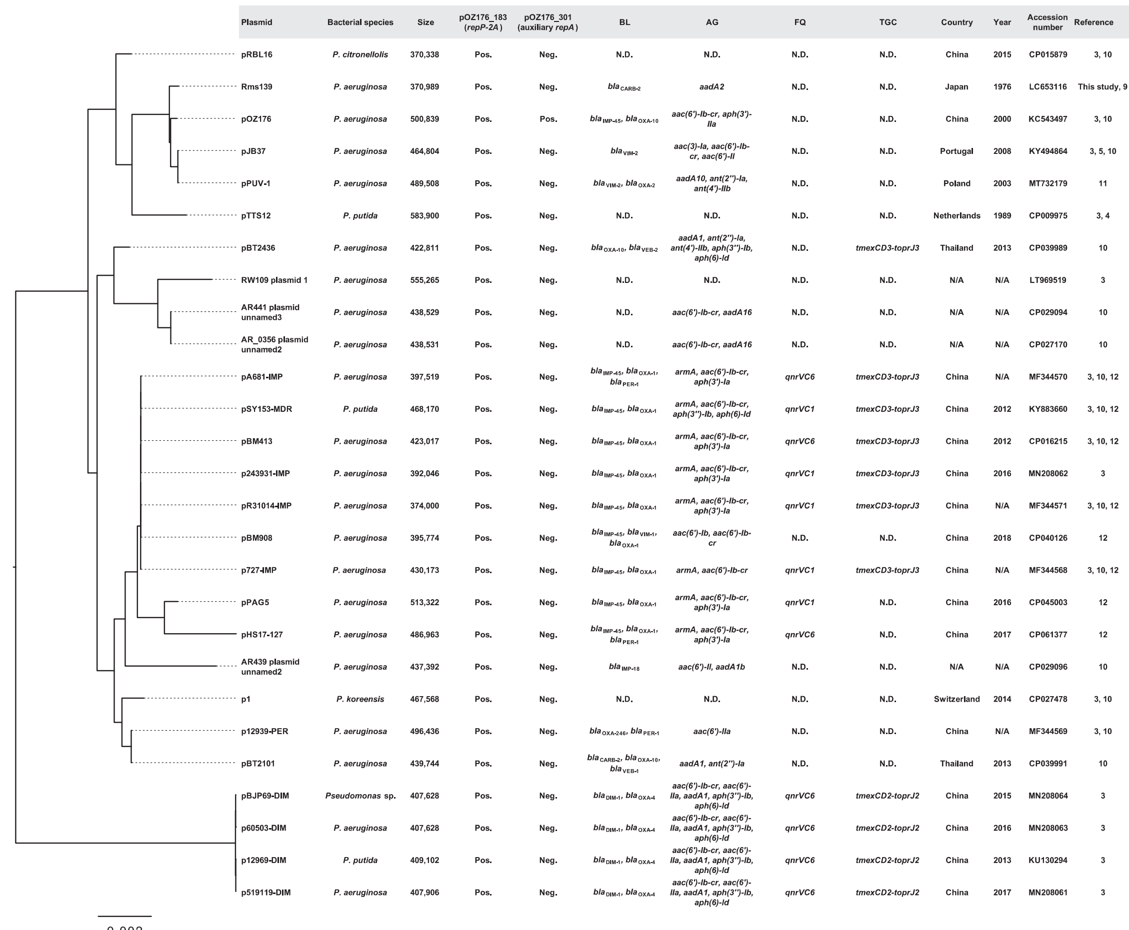

Plasmid	Bacterial species	Size	pOZ176_183 (*repP-2A*)	pOZ176_301 (auxiliary *repA*)	BL	AG	FQ	TGC	Country	Year	Accession number	Reference
pRBL16	*P. citronellolis*	370,338	Pos.	Neg.	N.D.	N.D.	N.D.	N.D.	China	2015	CP015879	3, 10
Rms139	*P. aeruginosa*	370,989	Pos.	Neg.	*bla*$_{CARB-2}$	*aadA2*	N.D.	N.D.	Japan	1976	LC653116	This study, 9
pOZ176	*P. aeruginosa*	500,839	Pos.	Pos.	*bla*$_{IMP-45}$, *bla*$_{OXA-10}$	*aac(6')-Ib-cr, aph(3')-IIa*	N.D.	N.D.	China	2000	KC543497	3, 10
pJB37	*P. aeruginosa*	464,804	Pos.	Neg.	*bla*$_{VIM-2}$	*aac(3)-Ia, aac(6')-Ib-cr, aac(6')-II*	N.D.	N.D.	Portugal	2008	KY494864	3, 5, 10
pPUV-1	*P. aeruginosa*	489,508	Pos.	Neg.	*bla*$_{VIM-2}$, *bla*$_{OXA-2}$	*aadA10, ant(2'')-Ia, ant(4')-IIb*	N.D.	N.D.	Poland	2003	MT732179	11
pTTS12	*P. putida*	583,900	Pos.	Neg.	N.D.	N.D.	N.D.	N.D.	Netherlands	1989	CP009975	3, 4
pBT2436	*P. aeruginosa*	422,811	Pos.	Neg.	*bla*$_{OXA-10}$, *bla*$_{VEB-2}$	*aadA1, ant(2'')-Ia, ant(4')-IIb, aph(3'')-Ib, aph(6)-Id*	N.D.	*tmexCD3-toprJ3*	Thailand	2013	CP039989	10
RW109 plasmid 1	*P. aeruginosa*	555,265	Pos.	Neg.	N.D.	N.D.	N.D.	N.D.	N/A	N/A	LT969519	3
AR441 plasmid unnamed3	*P. aeruginosa*	438,529	Pos.	Neg.	N.D.	*aac(6')-Ib-cr, aadA16*	N.D.	N.D.	N/A	N/A	CP029094	10
AR_0356 plasmid unnamed2	*P. aeruginosa*	438,531	Pos.	Neg.	N.D.	*aac(6')-Ib-cr, aadA16*	N.D.	N.D.	N/A	N/A	CP027170	10
pA681-IMP	*P. aeruginosa*	397,519	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$, *bla*$_{PER-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC6*	*tmexCD3-toprJ3*	China	N/A	MF344570	3, 10, 12
pSY153-MDR	*P. putida*	468,170	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id*	*qnrVC1*	*tmexCD3-toprJ3*	China	2012	KY883660	3, 10, 12
pBM413	*P. aeruginosa*	423,017	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC6*	*tmexCD3-toprJ3*	China	2012	CP016215	3, 10, 12
p243931-IMP	*P. aeruginosa*	392,046	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC1*	*tmexCD3-toprJ3*	China	2016	MN208062	3
pR31014-IMP	*P. aeruginosa*	374,000	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC1*	*tmexCD3-toprJ3*	China	N/A	MF344571	3, 10, 12
pBM908	*P. aeruginosa*	395,774	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{VIM-1}$, *bla*$_{OXA-1}$	*aac(6')-Ib, aac(6')-Ib-cr*	N.D.	N.D.	China	2018	CP040126	12
p727-IMP	*P. aeruginosa*	430,173	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr*	*qnrVC1*	*tmexCD3-toprJ3*	China	N/A	MF344568	3, 10, 12
pPAG5	*P. aeruginosa*	513,322	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC1*	N.D.	China	2016	CP045003	12
pHS17-127	*P. aeruginosa*	486,963	Pos.	Neg.	*bla*$_{IMP-45}$, *bla*$_{OXA-1}$, *bla*$_{PER-1}$	*armA, aac(6')-Ib-cr, aph(3')-Ia*	*qnrVC6*	N.D.	China	2017	CP061377	12
AR439 plasmid unnamed2	*P. aeruginosa*	437,392	Pos.	Neg.	*bla*$_{IMP-18}$	*aac(6')-II, aadA1b*	N.D.	N.D.	N/A	N/A	CP029096	10
p1	*P. koreensis*	467,568	Pos.	Neg.	N.D.	N.D.	N.D.	N.D.	Switzerland	2014	CP027478	3, 10
p12939-PER	*P. aeruginosa*	496,436	Pos.	Neg.	*bla*$_{OXA-246}$, *bla*$_{PER-1}$	*aac(6')-IIa*	N.D.	N.D.	China	N/A	MF344569	3, 10
pBT2101	*P. aeruginosa*	439,744	Pos.	Neg.	*bla*$_{CARB-2}$, *bla*$_{OXA-10}$, *bla*$_{VEB-1}$	*aadA1, ant(2'')-Ia*	N.D.	N.D.	Thailand	2013	CP039991	10
pBJP69-DIM	*Pseudomonas* sp.	407,628	Pos.	Neg.	*bla*$_{DIM-1}$, *bla*$_{OXA-4}$	*aac(6')-Ib-cr, aac(6')-IIa, aadA1, aph(3'')-Ib, aph(6)-Id*	*qnrVC6*	*tmexCD2-toprJ2*	China	2015	MN208064	3
p60503-DIM	*P. aeruginosa*	407,628	Pos.	Neg.	*bla*$_{DIM-1}$, *bla*$_{OXA-4}$	*aac(6')-Ib-cr, aac(6')-IIa, aadA1, aph(3'')-Ib, aph(6)-Id*	*qnrVC6*	*tmexCD2-toprJ2*	China	2016	MN208063	3
p12969-DIM	*P. putida*	409,102	Pos.	Neg.	*bla*$_{DIM-1}$, *bla*$_{OXA-4}$	*aac(6')-Ib-cr, aac(6')-IIa, aadA1, aph(3'')-Ib, aph(6)-Id*	*qnrVC6*	*tmexCD2-toprJ2*	China	2013	KU130294	3
p519119-DIM	*P. aeruginosa*	407,906	Pos.	Neg.	*bla*$_{DIM-1}$, *bla*$_{OXA-4}$	*aac(6')-Ib-cr, aac(6')-IIa, aadA1, aph(3'')-Ib, aph(6)-Id*	*qnrVC6*	*tmexCD2-toprJ2*	China	2017	MN208061	3

Figure 1. The phylogeny tree constructed by the pipeline of Bactopia v1.7.1 (https://github.com/bactopia/bactopia) using nucleotide sequences of the indicated IncP-2 plasmids. Bar lengths represent the number of substitutions per nucleotide site. Plasmid names, bacterial species, sizes, replicon types and representative AMR genes (ARGs), including β-lactams (BL), aminoglycosides (AG), fluoroquinolones (FQ), and tigecycline (TGC) resistance genes, detected by Staramr v0.7.2 (https://github.com/phac-nml/staramr) with the custom nucleotide sequence database of plasmid replicons and ARGs, countries and years in which bacteria were isolated, accession numbers and references are shown. N.D., not detected.

clinical isolates of *Pseudomonas* species[3,10–12] actually contain the *repP-2A* gene and have accumulated a number of important AMR genes, such as carbapenemase genes (*bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{DIM}$), 16S ribosomal RNA methyltransferase genes conferring aminoglycoside resistance (*armA*), efflux pump genes conferring fluoroquinolone resistance (*qnr*), and efflux pump gene clusters conferring tigecycline resistance (*tmexCD-toprJ*) (Figure 1).

Recent innovations in long-read sequencing technology and subsequent expansion of the plasmid nucleotide sequence database have enabled us to predict a novel classification of plasmid RIP genes without experimentally examining incompatibility. Therefore, it is important to keep updating the information on plasmid classification from the past for the future.

Acknowledgements

We are grateful to Prof Dr S. Iyobe of Gumma University School of Medicine, Japan and Prof Dr M. Tsuda of Tohoku University, Japan for providing Rms139.

Funding

This work was supported by grants (JP21wm0325022 and JP21wm0225008 to M. Shintani; JP21fk0108093, JP21fk0108139, JP21fk0108133, JP21wm0325003, JP21wm0325022, JP21wm0225004, JP21wm0225008, JP21wm0325037, and JP21gm1610003 to M. Suzuki) from the Japan Agency for Medical Research and Development (AMED), grants (16H06279, 19H02869, and 19H05686 to M. Shintani; 20K10436 and JPMJCR20H1 to H. Suzuki; JP19H05679 and JP19H05686 to H. Nojiri; 20K07509 to M. Suzuki) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and grants (M. Shintani, H. Suzuki, and H. Nojiri) from Consortium for the Exploration of Microbial Functions of Ohsumi Frontier Science Foundation, Japan.

Transparency declarations

None to declare.

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J Antimicrob Chemother 2022; **77**: 1204–1206
https://doi.org/10.1093/jac/dkab483
Advance Access publication 19 January 2022

Impact of changed co-amoxiclav susceptibility testing formats on apparent resistance rates for bloodstream *Escherichia coli* in a long-term surveillance

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Until 2014 the BSAC advocated that co-amoxiclav MICs should be determined using a 2:1 gravimetric ratio of amoxicillin and clavulanate. Subsequently, following adoption of EUCAST breakpoints, this advice changed to using clavulanate at a fixed concentration of 2 mg/L. The effect was to lower the breakpoint for Enterobacterales from systemic infections from 8+4 to 8+2 mg/L. We report on the consequences for apparent resistance prevalence among *Escherichia coli* as recorded in the BSAC Bacteraemia Antimicrobial Resistance Surveillance Programme.

This Programme ran from 2001 to 2019, and has been extensively described.[1] Succinctly, microbiology laboratories across the UK and Ireland sent consecutive isolates, according to a per-species quota, to UKHSA's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for centralized testing. The number of laboratories participating annually was 24–25 from 2001 to 2009 and again from 2016 to 2019, but increased to 38–40 from 2010 to 2015. The Programme sought a total of 250 *E. coli* isolates from these sites annually until 2007 and 500 thereafter; actual numbers collected ranged from 242 to 250 (mean = 247) annually in the 2001–07 period and from 467 to 548 (mean = 508) subsequently.

Species identification used colorimetric agars (CHROMagar™ Orientation, CHROMagar, Paris, France), with API20E strips (bioMérieux, Basingstoke, UK) for any confirmatory tests until 2011 and MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) thereafter. Susceptibility testing was by BSAC agar dilution on Iso-Sensitest agar (Oxoid/Thermofisher, Basingstoke, UK), with a 2:1 amoxicillin:clavulanate ratio until 2013 and clavulanate at a fixed concentration of 2 mg/L subsequently. Parallel tests with both methods were run in 2001 and 2002.

The proportion of *E. coli* isolates found susceptible to co-amoxiclav by year is illustrated in Figure 1(a). In 2001–02, when both formats were tested, resistance rates with clavulanate at a fixed concentration of 2 mg/L were 13.3 percentage points (95% CI = 9.9–16.8; paired data) above those with the 2:1 ratio. Thereafter, from 2003, there were no convincing trends towards more or less resistance during periods when the test format remained constant. However, following the switch to the fixed 2 mg/L format in 2014, with its reduction in the effective breakpoint from 8+4 to 8+2 mg/L, recorded resistance rose by 10.8 percentage points (95% CI = 7.7–13.8; unpaired data, adjusted for centre clustering with robust standard errors, possibly confounded by yearly effects). The resistance rate was under 30% in 7 of the 11 years (2003–13) when only the 2:1 ratio was tested, but exceeded 39% in 5 of the 6 following years, when testing was only with clavulanate at a fixed concentration of 2 mg/L.

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