Epidemic Territorial Spread of IncP-2-Type VIM-2 Carbapenemase-Encoding Megaplasms in Nosocomial *Pseudomonas aeruginosa* Populations

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ABSTRACT In 2003 to 2004, the first five VIM-2 metallo- β -lactamase (MBL)-producing *Pseudomonas aeruginosa* (MPPA) isolates with an In4-like integron, In461 (*aadB-bla_{VIM-2}-aadA6*), on conjugative plasmids were identified in three hospitals in Poland. In 2005 to 2015, MPPA expanded much in the country, and as many as 80 isolates in a collection of 454 MPPA (~18%) had In461, one of the two most common MBL-encoding integrons. The organisms occurred in 49 hospitals in 33 cities of 11/16 main administrative regions. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) classified them into 55 pulsotypes and 35 sequence types (STs), respectively, revealing their remarkable genetic diversity overall, with only a few small clonal clusters. S1 nuclease/hybridization assays and mating of 63 representative isolates showed that ~85% of these had large In461-carrying plasmids, ~350 to 550 kb, usually self-transmitting with high efficiency (~10⁻¹ to 10⁻² per donor cell). The plasmids from 19 isolates were sequenced and subjected to structural and single-nucleotide-polymorphism (SNP)-based phylogenetic analysis. These formed a subgroup within a family of IncP-2-type megaplasms, observed worldwide in pseudomonads from various environments and conferring resistance/tolerance to multiple stress factors, including antibiotics. Their microdiversity in Poland arose mainly from acquisition of different accessory fragments, as well as new resistance genes and multiplication of these. Short-read sequence and/or PCR mapping confirmed the In461-carrying plasmids in the remaining isolates to be the IncP-2 types. The study demonstrated a large-scale epidemic spread of multidrug resistance plasmids in *P. aeruginosa* populations, creating an epidemiological threat. It contributes to the knowledge on IncP-2 types, which are interesting research objects in resistance epidemiology, environmental microbiology, and biotechnology.

KEYWORDS IncP-2, MBL, *Pseudomonas aeruginosa*, VIM, carbapenemase, megaplasmid, metallo- β -lactamase, plasmid

Pseudomonas aeruginosa is a frequent cause of nosocomial infections, affecting mainly the respiratory tract, skin and soft tissue, urinary tract, and bloodstream (1, 2). Their treatment is difficult owing to common multidrug resistance (MDR) of the pathogen, combining intrinsic and acquired antimicrobial resistance (AMR) mechanisms (3, 4). Among the latter, metallo- β -lactamases (MBLs) are especially relevant, hydrolyzing all penicillins, cephalosporins, and carbapenems and not being inhibited by any β -lactamase inhibitor of clinical use (5, 6). *P. aeruginosa* has been the main host of the “older” worldwide-spread MBL types, IMPs and VIMs, encoded by gene cassettes in highly diverse, usually class 1 integrons (3, 4, 6). Acquired AMR determinants,

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TABLE 1 Geographic regions and years of isolation of the MPPA isolates with In461

Administrative region	No. of isolates	No. of centers	No. of cities	Yr(s) ^a
Mazowieckie	46	23	11	2003 ^b –2015
Warsaw urban area	32	16	4	2003 ^b –2015
Śląskie	9	8	8	2007, 2011–2014
Łódzkie	8	5	4	2008–2015
Kujawsko-Pomorskie	5	2	2	2009–2013
Podkarpackie	4	4	1	2006, 2014–2015
Opolskie	2	2	2	2011, 2015
Podlaskie	2	1	1	2013–2014
Lubelskie	1	1	1	2007
Świętokrzyskie	1	1	1	2015
Warmińsko-Mazurskie	1	1	1	2014
Zachodniopomorskie	1	1	1	2008

^aYears or periods of isolation of the In461 MPPA isolates; in some regions the year of the 1st identification was separated from the period of regular isolation.

^bIn Mazowieckie and Warsaw, the first identifications of the In461 MPPA occurred in 2003 to 2004 and were reported previously (9).

including MBL integrons, circulate in *P. aeruginosa* populations with plasmids and other transmissible platforms, like integrative and conjugative elements (ICEs), and relatively often incorporate into the chromosome (3). Several pandemic clones, e.g., sequence type 235 (ST235) and ST111, have been largely accumulating these, and clonal spread of their lineages has been the main method of AMR dissemination in *P. aeruginosa* (3, 4).

MBL-producing *P. aeruginosa* (MPPA) emerged in Poland in the period from 1998 to 2000 (7, 8). Studies on all 53 MPPA isolates collected by 2005 unveiled parallel clonal outbreaks caused by different organisms with chromosomal new VIM-encoding integrons in several parts of the country (9, 10). The central region Mazowieckie, with Warsaw as its main city, was peculiar in that nonrelated *Pseudomonas* strains from different hospitals from 2003 to 2004 had a unique integron, In461 (*aadB-bla_{VIM-2}-aadA6*), on conjugative plasmids, indicating territorial horizontal MBL transmission (9). From 2005 to 2015, the National Reference Centre for Susceptibility Testing (NRCST) recorded 1,314 MPPA cases from all of Poland, recently subjected to comprehensive studies on major factors driving this spread (11). This analysis was aimed at the characterization of the In461-carrying plasmids and addressing their role in MPPA epidemiology.

(Parts of the data described here were presented orally at the 28th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID] Madrid, Spain, 2018 [11], and as a poster at the 29th ECCMID, Amsterdam, The Netherlands, 2019 [12].)

RESULTS AND DISCUSSION

MPPA with In461—basic epidemiological and microbiological data. Since 2000 the NRCST has been conducting MPPA surveillance in Poland. Of the all nonduplicate 1,314 MPPA isolates confirmed from 2005 to 2015, a collection of 454 isolates was selected, representing at least one organism per medical center and year of recovery (11). The isolates were first typed by pulsed-field gel electrophoresis (PFGE) and then were screened for *bla_{VIM/IMP}* integron variants by PCR fingerprinting (9), followed by sequencing these for each pulstotype/integron fingerprint combination. In461 was found in 80 isolates (sequenced in 54), which constituted ~18% of the collection, indicating the element to be one of the two most common MBL integrons (11). The isolates were recovered in 49 hospitals in 33 cities of 11/16 major administrative regions (Table 1; see also Fig. S1 in the supplemental material). The most affected county was Mazowieckie, where In461 had occurred first in 2003 to 2004 (9) and where at least 46

isolates (56%) were recovered in 23 centers and 11 towns in 2005 to 2015 (mainly in the Warsaw urban area). Łódzkie and Śląskie were the two next regions with recurring isolations of In461-bearing MPPA in multiple hospitals and locales, since 2007 to 2008. Most of the isolates were from infections ($n = 75$), almost equally representing those of the urinary tract ($n = 21$), skin and soft tissue ($n = 20$), and bloodstream and the respiratory tract ($n = 17$ each). Eight genetically diverse isolates selected for susceptibility testing (criteria reported below) showed resistance to carbapenems and to penicillins and cephalosporins plus their β -lactam inhibitor combinations (Table S1). Resistance to aminoglycosides was common, though it varied for individual drugs. The isolates differed in resistance to fluoroquinolones but were susceptible to colistin. With some diversity in AMR patterns and levels, all of the isolates revealed the MDR phenotype.

Clonality of the In461-bearing MPPA. By PFGE the 80 In461 isolates were classified into 55 pulsotypes (Table S2), representatives of which were then subjected to multilocus sequence typing (MLST). Thirty-five STs were discerned, 27 of which grouped isolates with In461 only and 8 of which had isolates with various integrons in the collection (Table S2) (11). A large majority of the types, 29 STs and 49 pulsotypes, comprised 1 or 2 isolates each. Of the pandemic MDR clones (3, 4, 13), ST111 was represented by 13 In461 isolates, though split into eight pulsotypes, with just one arising from a limited clonal spread in Śląskie. Others, like ST235, ST253, and ST395, had a few isolates each. Noteworthy was ST244, identified originally in 2003 to 2004 in Warsaw with PER-1 extended-spectrum β -lactamase (ESBL) (14) and then observed worldwide (https://pubmlst.org/bigdb?db=pubmlst_paeruginosa_isolates&page=profiles). It included 12 isolates with In461, mainly of two pulsotypes spreading in the Warsaw area. In summary, MPPA with In461 showed high diversity, typical for populations with the dynamic horizontal transmission of a key genotypic trait, and a reduced role of clonal dissemination. Numerous “minor” clones were observed plus a limited presence of globally or locally relevant lineages.

In461-carrying plasmid mating and profiling. Sixty-three In461 isolates representing all pulsotypes (and multiple subtypes) were used in conjugation tests; transconjugants were obtained for 46 isolates, usually with high efficiency ($\sim 10^{-1}$ to 10^{-2} per donor cell). The S1-PFGE profiling of the same 63 isolates revealed that 54 isolates, including all mating and eight nonmating ones, had plasmids of ~ 350 to 550 kb (Table 2). Hybridization of the S1 patterns, done for 20 selected isolates, assigned In461 to the large plasmids. The remaining nine nonmating isolates had either smaller, *bla*_{VIM-2}-non-hybridizing plasmids (~ 150 to 200 kb) or no visible plasmids at all. The analysis proved that like the In461 isolates from 2003 to 2004 (9), the vast majority of those from 2005 to 2015 ($\sim 85\%$) had this integron on large, usually transmissible plasmids. Therefore, plasmid diffusion has been the main factor in In461 MPPA expansion in Poland.

Sequencing of pPUV plasmids and their comparative analysis. Full sequences of In461-carrying plasmids varying in size and mating ability were obtained for 18 study isolates plus 1 early strain from 2003 (9). The isolates represented different years and sites (hospitals, cities, and regions) of identification and were clonally diverse. Seventeen sequences, named pPUV-1 to pPUV-17, were determined in a specific long-read whole-genome sequencing (WGS) analysis, and two remaining ones, pPUV-18 and -19, were obtained by short-read WGS during a pPUV plasmid screening experiment described below (Table 2). The 19 circular contigs (~ 400 to 490 kb) were compared in detail within the sample and against GenBank. They matched 27 sequenced IncP-2-type plasmids (as of 24 June 2020), of which 22 with the highest BLAST score were chosen for the analysis (Table S3). These megaplasmids (~ 370 to 580 kb) had been identified in clinical, environmental, or industrial pseudomonad strains identified worldwide, and conferred resistance/tolerance to various stress factors, like antibiotics, heavy metals, or organic solvents (15–27). Fifteen of these were analyzed recently by Cazares et al. as “pBT2436-like megaplasmids” (16). A noncontinuous core genome was discerned in these (~ 200 kb; 42 to 57% of a molecule), with genes/operons or regions providing plasmid backbone-associated functions (16), all of which were found also in pPUVs (described below).

TABLE 2 STs, pulsotypes, and plasmid content of the 63 In461 MPPA isolates selected for the detailed plasmid analysis

Isolate ^a	Yr of isolation	Region of isolation ^b	Source	MLST	PFGE ^c	Mating ^d	Plasmid S1 profile ^e	pPUV-like plasmid analysis
804/03	2003	Mazowieckie	Urine	ST244	ND	+	ND	pPUV-1 (489,508) ^f
17/09	2008	Zachodniopomorskie	Urine	ST111	CF	+	~440	+ ^h
6106/09	2009	Kujawsko-Pomorskie	Blood	ST111	CE1	+	~450, ~190	+ ^h
9152/11	2011	Śląskie	Urine	ST111	AA1	—	~150	— ^h
9794/11	2011	Śląskie	Bronchial exudate	ST111	BV4	+	~420	+ ^h
601/12	2012	Łódzkie	Urine	ST111	BW1	—	—	— ^h
1897/13	2013	Śląskie	Bronchoalveolar lavage	ST111	BV1	—	—	— ^g
3231/13	2013	Śląskie	Wound	ST111	AY	—	—	— ^g
700/14	2014	Podlaskie	Urine	ST111	C	+	~400	pPUV-16 (398,692) ^f
1814/15	2015	Opolskie	Urine	ST111	K8	—	—	— ^h
259/06	2006	Mazowieckie (WUA)	Nasal swab	ST244	BH3	+	~460	pPUV-2 (471,725) ^f
981/06	2006	Mazowieckie (WUA)	Urine	ST244	BH5	+	~450, <50	pPUV-3 (458,868) ^f
1466/06	2006	Mazowieckie	Bronchoalveolar lavage	ST244	BO1	+	~470, <50	+ ^h
2498/07	2007	Lubelskie	Urine	ST244	BH1	+	~460	+ ^h
408/12	2012	Mazowieckie (WUA)	Wound	ST244	CJ	+	~500	+ ^h
6124/12	2012	Mazowieckie	Wound	ST244	CA	—	~430	pPUV-14 (421,847) ^f
505/11	2011	Mazowieckie (WUA)	Wound	ST27	CI	+	~430	+ ^g
5418/12	2012	Łódzkie	Urine	ST27	BM	+	~450	+ ^h
3606/13	2013	Podlaskie	Blood	ST27	B1	+	~350	+ ^h
1483/14	2014	Śląskie	Wound	ST27	F	+	~430	+ ^h
2785/12	2012	Mazowieckie	Wound	ST17	M4	+	~420	pPUV-19 (425,512) ^g
4927/14	2014	Podkarpackie	Tracheostomy tube	ST17	CR2	—	—	— ^g
3989/15	2015	Podkarpackie	Urine	ST17	CR1	—	—	— ^h
1185/06	2006	Mazowieckie (WUA)	Nasal swab	ST253	DX	+	~440, ~280	pPUV-4 (433,698) ^f
2407/06	2006	Podkarpackie	Urine	ST253	CS	+	~480	+ ^h
162/10	2010	Mazowieckie	Tracheostomy tube	ST253	BZ1	+	~440, ~180	pPUV-11 (436,091) ^f
8635/11	2011	Łódzkie	Sputum	ST108	BJ	+	~440	pPUV-12 (440,659) ^f
239/15	2015	Mazowieckie (WUA)	Blood	ST108	DR	+	~430	+ ^h
2418/06	2006	Mazowieckie	Blood	ST235	CO	—	~440	pPUV-5 (435,868) ^f
3459/07	2007	Śląskie	Urine	ST235	E	+	~420	pPUV-7 (413,916) ^f
5751/12	2012	Kujawsko-Pomorskie	Blood	ST381	DK3	—	~200	— ^h
5352/14	2014	Mazowieckie (WUA)	Blood	ST381	DK1	—	~500, ~180, <50	+ ^g
333/14	2014	Mazowieckie (WUA)	Wound	ST390	AI	+	~450	+ ^g
521/15	2015	Mazowieckie (WUA)	Blood	ST390	DL	—	~470	+ ^h
202/07	2007	Mazowieckie (WUA)	Bronchial exudate	ST500	AK1	+	~460	+ ^h
2635/08	2008	Łódzkie	Sputum	ST500	AK2	+	~500	pPUV-8 (489,682) ^f
42/05	2005	Mazowieckie (WUA)	Urine	ST694	DN1	+	~450	+ ^h
3438/07	2007	Śląskie	Bronchoalveolar lavage	ST694	DN2	+	~500	pPUV-6 (489,681) ^f
84/11	2011	Mazowieckie (WUA)	Bronchial exudate	ST697	AM	+	~520	+ ^h
8769/11	2011	Opolskie	Tracheostomy tube	ST697	BP	+	~400, ~170	pPUV-13 (398,622) ^f
133/15	2015	Mazowieckie	Decubitus ulcer	ST1020	CN3	+	~400	pPUV-17 (398,641) ^f
4054/15	2015	Mazowieckie	Ulcer	ST1020	CN1	—	~400	+ ^h
3005/09	2009	Mazowieckie (WUA)	Tracheostomy tube	ST41	DW	+	~460	pPUV-10 (470,075) ^f
5557/09	2009	Mazowieckie (WUA)	Ulcer	ST155	AL	+	~550	+ ^h
4793/14	2014	Mazowieckie	Urine	ST164	Y2	+	~450	+ ^h
1412/07	2007	Mazowieckie (WUA)	Blood	ST198	CG	+	~430	+ ^h
724/13	2013	Kujawsko-Pomorskie	Urine	ST207	R	+	~450	+ ^h
347/11	2011	Kujawsko-Pomorskie	Wound	ST245	DY	—	~450, ~160	+ ^h
30/15	2015	Mazowieckie (WUA)	Blood	ST313	DJ	+	~460	+ ^h
5912/09	2009	Mazowieckie (WUA)	Wound	ST360	DQ	—	~430	pPUV-18 (426,903) ^g
2098/13	2013	Mazowieckie (WUA)	Rectal swab	ST395	DP	+	~460	+ ^h
2541/15	2015	Mazowieckie (WUA)	Decubitus ulcer	ST446	CM1	+	~350	+ ^h
18/09	2008	Mazowieckie (WUA)	Blood	ST589	CH	+	~400	+ ^g
3364/08	2008	Mazowieckie (WUA)	Wound	ST611	—	+	~450	pPUV-9 (452,776) ^f
3872/10	2010	Mazowieckie	Urine	ST792	DF	+	~400	+ ^h
4157/10	2010	Mazowieckie (WUA)	Urine	ST794	DG	+	~450	+ ^g
3671/10	2010	Mazowieckie (WUA)	Urine	ST815	DS	+	~450	+ ^h
3209/12	2012	Mazowieckie (WUA)	Wound	ST931	EN	+	~430	+ ^h
236/11	2011	Łódzkie	Bronchial exudate	ST1028	CQ	—	—	— ^h
2529/15	2015	Łódzkie	Bronchoalveolar lavage	ST1197	P	—	~450	+ ^h

(Continued on next page)

TABLE 2 (Continued)

Isolate ^a	Yr of isolation	Region of isolation ^b	Source	MLST	PFGE ^c	Mating ^d	Plasmid S1 profile ^e	pPUV-like plasmid analysis
773/10	2010	Mazowieckie (WUA)	Wound	ST2238	DT	+	~460	+ ^h
5515/10	2010	Łódzkie	Bronchial exudate	ST3213	CT	+	~420	+ ^h
846/06	2006	Mazowieckie (WUA)	Blood	ST3214	DU	+	~430	+ ^h
1389/13	2013	Mazowieckie (WUA)	Wound	ST3212	CK	+	~ 450	pPUV-15 (433,807) ^f

^aIsolate 804/03, shown in the first position, is an archival strain from the previous report (9), included in this study only for the plasmid long-read sequencing; the following isolates are ordered according to representation of their STs; in case of equally represented STs these are shown in the ascending manner.

^bWUA, Warsaw urban area within the Mazowieckie region.

^cUppercase letters designate pulsotypes, followed by numbers symbolizing subtypes; designations are according to those assigned to the entire baseline MPPA collection ($n = 454$) (11), to which the In461 isolates belonged. —, not identified due to DNA degradation. ND, not determined in this study.

^d+ and —, positive and negative results of the conjugation test, respectively.

^eValues represent sizes of plasmids in kilobases, roughly calibrated on S1/PFGE gels. Underlined size values indicate 20 MPPA isolates selected for hybridization of their S1 plasmid profiles with the *bla*_{VIM-2} probe; values in bold refer to the plasmids that hybridized with the probe. ND, not determined because this archival isolate (9) was not included in the S1 analysis. —, no plasmids visible in the S1 analysis.

^fSeventeen pPUV plasmid variants sequenced by the PacBio long-read WGS technology (Pacific Biosciences); their sizes, in base pairs, are in parentheses.

^gTen isolates selected for the short-read WGS (MiSeq, Illumina) pPUV-like plasmid mapping. pPUV-18 and -19 variants with sizes, in base pairs, in parentheses are those for which full circular contigs were obtained with MiSeq. + and —, pPUV presence and absence, respectively.

^hThirty-seven isolates selected for the PCR pPUV-like plasmid mapping. + and —, pPUV presence and absence, respectively.

The single-nucleotide-polymorphism (SNP)-based phylogenetic analysis of the pBT2436-like plasmids, using the variant pPUV-2 (471,725 bp) as a reference, revealed pPUVs to form a tight cluster with 0 to 10 SNPs between each other (Fig. 1, Fig. S2, and Table S4). All but one of the other plasmids differed by 993 to 4,594 SNPs from pPUV-2; the exception was pJB37 from a Portuguese clinical *P. aeruginosa* isolate (15) which fell into the pPUV cluster (4 SNPs). The actual range of its colinearity with pPUVs extended beyond the core genome, up to ~80 to 90% of its sequence with 99.6 to 100% nucleotide identity (Fig. S3). This indicated that pJB37 and pPUVs shared also some accessory genome regions (addressed below), the variety of which, usually larger mosaic assemblies of genes and mobile elements, were identified in pBT2436-like molecules by Cazares et al. and others (16, 20, 23, 27). Interestingly, pJB37 was the only “non-Polish” pBT2436-like plasmid with the *bla*_{VIM-2} MBL gene (15); however, dissimilar integrons (In58 versus In461) and their mobile context (described below) proved separate recent evolution of these closely related molecules, comprising independent *bla*_{VIM-2} acquisitions.

Characterization of pPUV plasmids. The in-sample analysis of the 19 pPUV plasmids demonstrated these to share ~330 kb with each other (~67 to 82%) (Fig. 2). This common part comprised the pBT2436-like core genome with the IncP-2-specific determinants of replication (*repA*), partition (*parAB*), conjugation (*traGBV*, *dnaG*, and type IV pilus/type II secretion system genes), chemotaxis (*cheBARZWY*), and tellurite resistance (*terZABCDEF*), as reported by Cazares et al. (16). Our manual annotation allowed identification of three more putative core conjugation genes, *traK* and *traC*, in the *traGBV* region, and *traU*, between *repA* and *cheB*. The role of *traC* (*virB4* [16]) and *traU* was supported by two nonconjugative variants, pPUV-14 and pPUV-5, respectively, in which these genes are disrupted by an ISPa97-like element (IS66 family) and IS*Ppu23*, respectively. The pPUV common part (and the pBT2436-like core genome) also contains the *slvAT* new toxin-antitoxin system genes, recently found in the pTTS12 plasmid from The Netherlands (Table S3), contributing to plasmid stability and organic solvent tolerance (28).

The comparison also revealed seven accessory regions (~23 to 50 kb), variably present in pPUVs (Table S5). A region of ~47 kb occurred in 14 variants and was found entirely also in pJB37 (15) and partially in pTTS12 (17, 29) and pOZ176 (21) from China (Table S3). The fragment contained additional, nonrelated replication (*repA*), partitioning (*parAB*), and conjugation (*trbK*, *traG*, and *trbBCDEJLFGI*) genes, together with the *acrAB* and *tolC* genes of the efflux system for small hydrophobic molecules, including acridine derivatives and various drugs (Fig. 2) (30). Possible insights into functionality of some of these were provided by variants pPUV-14 and pPUV-15, carrying the ~47-kb region. pPUV-14's inability to transmit, likely due to the core *traC/virB4*ISPa97-like

Tree scale: 0.1

FIG 1 Phylogenetic tree of the common part of IncP-2 pBT2436-like plasmids, including the pPUV series, using the pPUV-2 variant as a reference, generated with iTOL (43). Detailed information on 19 pPUVs is in Table 2, and that on the 22 pBT2436-like plasmids identified in GenBank is in Table S2.

disruption (discussed above), suggested nonfunctionality of the extra conjugation locus, contrary to previous reports (15, 26, 29). Otherwise, pPUV-15 had the core *repA* interrupted by *ISPpu18*, implying that its role might have been taken by the additional *repA*. A GenBank search revealed 41 entries with the full ~47-kb fragment (>99% identity), being pseudomonal or enterobacterial whole-genomic, chromosomal, or plasmidic sequences. Recently, Jiang et al. interpreted the corresponding related regions of various sizes in pJB37, pTTS12, and pOZ176 to be likely ICEs (27). The remaining accessory regions in pPUVs were specific for this cluster (Table S5). Of these, an ~31-kb insert present in the “oldest” plasmid, pPUV-1 (2003) only, had the operon *tbtABM*, conferring resistance to tributyltin, a toxic component of marine antifouling paints (31). The acquisition of the accessory regions by individual pPUVs, including some with clear biological functions, has been one of the major aspects of their microevolution.

FIG 2 Comparison of the IncP-2 pUV plasmids, using the pPUV-2 variant as a reference (thin, inner black circle). Only the variants pPUV-1 to -17 sequenced by the long-read PacBio technology (Pacific Biosciences) are shown. The plasmids compared are represented by various colors. Outer thick rings depict common parts of all molecules within the group, plus the annotation of the selected regions/genes. The first outer ring of dark gray and light gray fragments shows the pBT2436-like core genome discerned by Cazares et al. (16), and the second ring with light gray fragments shows only the core genome of pPUVs identified in this work. Black fragments in the first outer ring represent variable regions, identified both by Cazares et al. (16) and in this study. The percent sequence identity is reflected by color intensity. The figure was created using BRIG software (44).

AMR elements and loci in pPUVs. In461(*aadB-bla_{VIM-2}-aadA6*) formed the minimal common resistome of the 19 pPUVs; however, their majority carried also the *ant(4')-IIB* gene and/or integron In1893 (*bla_{OXA-2}-aadA6*), and four variants had *tet(C)*. Both In461 and In1893 were of the In4 type (32), and their distal *aadA6* cassettes were fused with 3'CS (33). The 5'CS segments of In461 and In1893 were truncated by IS26 and an ISCR16-like element, respectively. All AMR determinants resided in the same location, defined by Cazares et al. as resistance region 1 (RR1) between pBT2436 loci 00202 and 00267, the family's major segment with AMR genes and mobile elements (16). Its

FIG 3 Graphical representation of AMR genes in the resistance region 1 (RR1) in the pUV plasmids, generated with Easyfig v. 2.2.5 (45). Arrows indicate all genes/coding DNA sequence (CDSs) and mobile elements identified, proportionally to their sizes and orientation, and the key genes/elements are marked with colors and/or names. White arrows represent genes or gene fragments not related to AMR. Multiplications of elements are indicated as repeats in representative structures. Plasmid variants pUV-1 to -17 and pUV-18 and -19 were sequenced by the PacBio long-read and MiSeq short-read technologies, respectively.

structure much varied in size, content, and composition. pUVs again formed a cluster, with all but one [*tet(C)*] AMR gene surrounded by *IS26* on one side and *IS6100* plus a *Tn1013*-like transposon on the other (Fig. 3; part of the *Tn1013*-like element is present also in pJB37 [15]). In six pUV variants *In461* was alone in this site. In all others it was followed by the *ISCR16*-like element, and this module then made various configurations with *ant(4')-IIB* plus truncated *ISCR16*-like sequence and *In1893* (sometimes also with the *ISCR16*-like element). The most frequent arrangement, *In461*–*ISCR16*-like–*ant(4')-IIB*– Δ *ISCR16*-like–*In1893*, was in seven plasmids. In several variants the individual elements, including *In461*, were multiplied 2 or 3 times in different combinations, with copies retaining or not the *ISCR16*-like elements. The *tet(C)* gene was always upstream of *IS26*, flanked further by another, directly oriented *IS26*. The analysis has confirmed RR1 as a major locus for AMR genes and its high structural dynamics (16). The results coevidenced pUVs as a specific sublineage of the family, evolving also by acquisition and multiple rearrangements of AMR genes. The origin and mechanism of the *In461* recruitment remain unclear; however, the role of *ISCR16*-like elements in shaping the

region seems to be probable. Cazares et al. observed duplications of AMR genes and ISCR-like elements in RR1 of other pBT2436-like plasmids as well (16).

Eight isolates representing each variant of the pPUV RR1 structure with respect to In461, In1893, and the *ant(4')-IIb* gene plus their transconjugants were tested for the gene dosage/content effect on AMR (Table S1). The organisms with the In461 multiplied (in pPUV-1 and pPUV-13) had higher MICs of β -lactams, including carbapenems, whereas the presence of *ant4'-IIb* correlated with high-level resistance to amikacin and tobramycin.

Screening for pPUVs in the study isolates. A two-step approach was used to detect pPUVs in 47/63 In461 isolates selected for detailed analysis. The first step was short-read WGS of 10 isolates and mapping reads to pPUV-2 as a reference, followed by screening contigs for IncP-2-specific core genes *repA*, *parA*, and *virB4/traC* and chromosomal sequences. Seven isolates were pPUV positive, including two for which the MiSeq data assembly yielded single circular contigs, as mentioned above (pPUV-18 and -19) (Table 2). In461-carrying contigs of three remaining isolates encompassed also chromosome fragments and did not contain the IncP-2-specific genes, and their reads mapped similar to those of a *Pseudomonas* sp. isolate with chromosomally located In461 (Fig. S4); all these results indicated a chromosomal In461 location. The subsequent PCR mapping of the *repA*, *parA*, and *virB4/traC* genes (16) increased the total numbers of pPUV-positive and -negative isolates to 38 and 9, respectively, among the 47 isolates that were not analyzed by long-read WGS (Table 2). The analysis has definitely confirmed pPUVs as predominant platforms of In461 and vehicles of its spread in *P. aeruginosa*.

Conclusions. MPPA dissemination has been one of major AMR-related threats in Poland in last decades, prompting us to study its key factors with reference to the starting period, 1998 to 2004 (9, 10). In 2003 to 2004, several isolates with the specific integron In461 on conjugative plasmids were identified in Warsaw county, and these were found to spread efficiently and interregionally in following years, accounting for a remarkable fraction of all-country MPPA (11). The plasmids represented the IncP-2 type, discerned in the early 1970s and soon identified broadly in pseudomonads from various environments (34, 35). However, it was only recently that their whole sequences started to be analyzed (15–27), including the first comparative study on 15 molecules, verifying and revealing basic aspects of their biology and evolution (16). Despite several works highlighting the IncP-2 plasmids as AMR/MDR platforms (15, 16, 18, 21, 22, 25–27), to our knowledge, this study is the first to practically demonstrate that on a defined time and geographic scale and the first larger analysis of an AMR plasmid epidemic in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial isolates, preliminary typing, and susceptibility testing. According to the NRCST MPPA surveillance procedure, from 2005 to 2015, each *Pseudomonas* species isolate sent by a diagnostic microbiology laboratory as a suspected MBL producer was examined by the EDTA double-disk synergy test (36), followed by PCRs for *bla*_{IMP/VIM}-like genes (9). PFGE typing was performed according to the method of Seifert et al. (37), with visual interpretation of results (38). Class 1 *bla*_{VIM/IMP} integron variants were analyzed by PCR fingerprinting and sequencing as described previously (9). Briefly, variable regions of the integrons were amplified in two parts, from the region 5'CS to a *bla*_{VIM/IMP}-like gene, and from the gene to the region 3'CS. Amplicons were cut with the MboI enzyme for fingerprinting and/or sequenced by the Sanger approach. MICs of 14 antipseudomonal antimicrobials (Table S1) were evaluated by broth microdilution, according to EUCAST recommendations (<https://eucastr.org>).

MLST. MLST was performed according to the method of Curran et al. (39); the database available at <http://pubmlst.org> was used for assigning STs.

Mating and plasmid profiling. Conjugation tests were performed by filter-mating (40), with *P. aeruginosa* PAO1161 resistant to rifampin (41) as a recipient; transconjugants were selected with imipenem (4 μ g/ml) and rifampin (50 μ g/ml). Transfer frequencies were calculated in reference to numbers of donor cells. Plasmid profiling and identification of In461-carrying plasmids were done by S1 nuclease (New England Biolabs, Beverly, MA) analysis, followed by hybridization with a *bla*_{VIM-2} probe (9), using the ECL random-prime labeling and detection system (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Long-read whole-genome sequencing and bioinformatic analysis of IncP-2 plasmids. Long-read WGS was carried out on the PacBio Sequel platform (Pacific Biosciences, Menlo Park, CA). Plasmid contigs were assembled with HGAP4 (Pacific Biosciences), followed by manual annotation based on BLASTp searches against the RefSeq and Conserved Domain NCBI databases. Plasmid sequences were compared

with those deposited in GenBank by BLASTn. SNP-based phylogenetic analysis of plasmids was done with Harvest Tools v.1.2 (42), using a pPUV-2 variant as a reference, and trees were plotted utilizing iTOL (43). BRIG 0.95 was applied for circular alignments (44). Plasmid resistomes were determined with ABRicate (<https://github.com/tseemann/abricate>), using the NCBI database (<https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/>). Resistance regions were visualized by Easyfig v. 2.2.5 (45). Mobile elements were identified with ISFinder (ISs) (46) and BLASTn searches of sequences deposited in GenBank. Putative phage-related regions were indicated by PHASTER (47).

Short-read WGS and IncP-2 plasmid sequence and PCR mapping. Short-read WGS was performed using MiSeq (Illumina, San Diego, CA). Reads were trimmed by Cutadapt 1.16 (<https://cutadapt.readthedocs.io/en/stable/>) and assembled by SPAdes 3.13.2 (48). The trimmed reads were mapped to the reference pPUV-2 plasmid sequence as described by Cazares et al. (16), utilizing BWA-MEM v.0.7.17-r1188 plus SAMtools v.1.10 (49, 50). Illumina reads of pPUV-1-carrying isolate 804/03 and an IncP-2 plasmid-negative *Pseudomonas* sp. isolate with a chromosomally located In461 (P. Urbanowicz, M. Gniadkowski, and R. Izdebski, unpublished results) were used for comparison. Additionally, contigs of the isolates were screened by BLASTn for the IncP-2 plasmid-specific genes *repA*, *parA*, and *virB4/traC* and chromosomal sequences. A multiplex PCR mapping assay, targeting the IncP-2 plasmid genes listed just above, was carried out as proposed previously (16).

Accession number(s). Plasmid nucleotide sequences pPUV-1 to pPUV-19 were assigned GenBank accession numbers [MT732179](#) to [MT732197](#), respectively. The Illumina assembly files and raw sequencing reads were submitted under the GenBank BioProject [PRJNA645026](#) (accession numbers [JACCIC000000000](#) to [JACCIT000000000](#)).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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