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High Diversity and Novel Species of *Pseudomonas aeruginosa* Bacteriophages

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The diversity of *Pseudomonas aeruginosa* bacteriophages was investigated using a collection of 68 phages isolated from Central Mexico. Most of the phages carried double-stranded DNA (dsDNA) genomes and were classified into 12 species. Comparison of the genomes of selected archetypal phages with extant sequences in GenBank resulted in the identification of six novel species. This finding increased the group diversity by ~30%. The great diversity of phage species could be related to the ubiquitous nature of *P. aeruginosa*.

Most of the described bacteriophages that infect *Pseudomonas aeruginosa* are members of the *Caudovirales,* i.e., they have head-and-tail morphology and contain double-stranded DNA (dsDNA). These phages belong to the three known varieties: the *Siphoviridae* (with a long, flexible tail), the *Myoviridae* (with a contractile tail), and the *Podoviridae* (with a short, stubby tail). The minority groups are polyhedral, filamentous, or pleomorphic and may contain single-stranded/double-stranded DNA (ss/dsDNA) or single-stranded/double-stranded RNA (ss/dsRNA) as genetic material (2). A catalog of 23 species based on viral particle morphology and DNA-DNA hybridization of 113 individual phages has been proposed (28). Comparative DNA sequence analysis of 10 phages from this catalog with 8 phages of other collections resulted in the absence of homology among them, confirming the wide diversity of the *P. aeruginosa* phages (30). Currently, GenBank lists 40 complete genome nucleotide sequences of tailed *P. aeruginosa* phages. Recent studies based on genome sequences and architecture, as well as possible gene function, have categorized 36 of these phages into 20 species (12). In addition, phage sequences have been identified within various *P. aeruginosa* genomes (38, 41, 45). Although the use of the term "species" to describe phage variants has been questioned (11), like other researchers in the *P. aeruginosa* field, we use it in this work to refer to groups of phages that share morphological and genomic features.

Almost all studies concerning *P. aeruginosa* phage species have been done with collections from European, Australian, and Asian countries (13, 23, 34, 42), leaving a void regarding the species found in unexplored locations. The aim of this study was to investigate the diversity of *P. aeruginosa* phages in Central Mexico. To this end, 68 phage isolates were characterized and compared to those described in the literature. In the process, novel species were discovered and classified according to rules used by authors in the field.

Phage isolation and phenotypic characterization. Sixty-eight phages infecting *P. aeruginosa* (each named PaMx plus a number) were isolated from environmental and sewage water samples collected from four states in Central Mexico, as reported previously (6). The host strains were obtained from three hospitals in Mexico City. The phages were propagated on five clinical strains (Ps17, Ps25, Ps26, Ps33, and Ps53) and two strains from culture collections (PAO1 and Ps9), which did not show evidence of vegetative phages in cross-plating tests. Most of the phages produced clear lytic plaques ranging from 0.5 to 5 mm in diameter. Of the phages PaMx73, PaMx1, and PaMx7, which produced turbid plaques, only PaMx73 was proved to be temperate (data not shown).

Morphological diversity. To analyze phage morphologies, phage particles purified by CsCl gradient centrifugation (39) were observed under a JEM-2000 transmission electron microscope at 80 Kv. These studies revealed that 65 of the 68 phages examined were members of the *Caudovirales*, and the other 3 were small polyhedral phages belonging to the family *Leviviridae* (Fig. 1; see also Fig. S1 in the supplemental material). Among the *Caudovirales*, all three families—*Myoviridae*, *Siphoviridae*, and *Podoviridae*——were distributed in proportions similar to those observed for phages in other bacterial species (1). We identified 20 members of the *Siphoviridae* with elongated heads, or morphotype B2 (5). The head sizes varied between 63 and 91 nm long and 49 and 64 nm wide, and the tail lengths ranged between 110 and 170 nm. Twenty other members of the *Siphoviridae*, as well as 12 *Myoviridae* and 13 *Podoviridae*, showed isometric heads (morphotypes B1, A1, and C1, respectively) between 54 and 88 nm in diameter and with tail lengths that ranged between 10 and 302 nm. The *Leviviridae* phages PaMx54, PaMx60, and PaMx61 had the smallest isometric heads (morphotype E1), with a diameter of 25 to 27 nm (Fig. 1). The lytic plaque formation by these phages on their host's lawns was inhibited by RNase A in the top agar, confirming that they contain ssRNA genomes (8). The International Committee on Taxonomy of Viruses (ICTV) database showed only three records of leviphages for *P. aeruginosa*. For this study, we report in addition the presence of three new leviviruses for this bacterial species.

RFLP analysis. In order to obtain a direct indication of the genetic diversity of the *Caudovirales* in our collection, phage DNA was treated with NdeI, HindIII, and EcoRI enzymes to generate restriction fragment length polymorphisms (RFLPs) (data not

Received 10 January 2012 Accepted 2 April 2012
Published ahead of print 13 April 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.00065-12

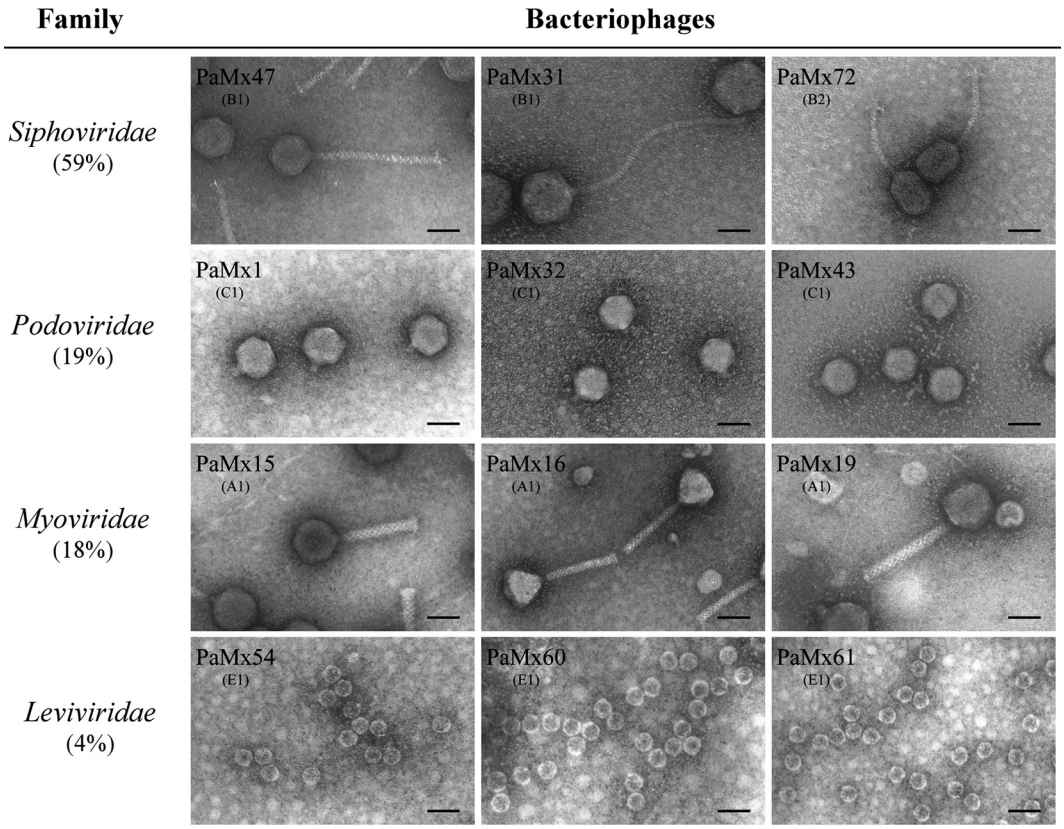

FIG 1 Electron microscope images of selected phages. Each row shows three examples of the morphological family indicated in the left margin. Each percentage corresponds to the proportion of phages in the collection belonging to each family. The upper left corners are marked with the name and morphotype of the phage in each picture. Magnification, ×100,000. Bar = 50 nm.

shown). The results showed that each enzyme restricted a different number of phage genomes; nevertheless, of the 65 phage DNAs assayed, 19 were resistant to restriction by all three enzymes. Interestingly, all the phages containing restriction-resistant DNAs were siphoviruses with elongated heads. However, *in silico* analysis of the DNA sequences (data not shown) revealed the presence of HindIII, EcoRI, and NdeI restriction sites in three of the DNAs. This suggests the presence of base modification, such as the glycosylated residues found in coliphages T2, T4, and T6 (21) or hydroxymethyl-dUMP, found in *Bacillus subtilis* phage SPO1 (40). A putative gene similar to the gp17 gene from *P. aeruginosa* phage YuA, whose product confers resistance to restriction (15), may be present.

Because EcoRI restricted the majority of DNAs, the RFLPs generated with this enzyme were resolved by agarose gel electrophoresis, and the gels were digitized using the molecular imager GelDoc XR System (Bio-Rad Laboratories) and analyzed with the software program Gelcompar II v.6.5 (Applied Maths, Sint-Martens-Latem, Belgium) to identify homologous patterns. Cluster analysis divided the phages into eight restriction groups (Fig. 2) using the Dice similarity coefficient and the unweighted pair group method with arithmetic averages (UPGMA) for tree construction. An archetypal phage for each RFLP group was selected at random, and the group was named after the selected phage. Phages within each group shared both morphology and genome size. The DNAs of four known phages (F8, D3, D3112, and F10) were also restricted, and their RFLPs were analyzed. Phage F8, a lytic myovirus, shared life cycle style, DNA homology, morphology, and genome size with 10 phages from the PaMx13 group (Fig. 2). Since all known *P. aeruginosa* myoviruses are in the same homology group or species (16, 28, 37) and they share 61% RFLP identity, we took this threshold to define the groups shown in Fig. 2. This limit seems appropriate because it results in homogeneous groups of phages that share physical properties. Phage PaMx73, the only temperate phage, was grouped with the temperate transposable phage D3112 (7, 9, 24, 43). The temperate phages D3 and F10 did not match any of our phages, and neither did lytic phage PaMx62. Some of the phage DNAs showed identical restriction patterns (boxes in Fig. 2). With few exceptions, these phages came from the same water samples, but they are included separately in Fig. 2 because they have different host ranges (see below).

Phage homology by DNA hybridization. To assess diversity in the entire collection of phages, including those whose DNAs were resistant to restriction enzymes, phage-phage homology by DNA hybridization was analyzed by using the dot blot technique (39). All the phage DNAs in the collection were fixed on a nylon membrane and probed with radioactively labeled DNAs from phages selected as archetypal of RFLP groups and those with nonrestrictable DNA (see Fig. S2 in the supplemental material). Among the restricted phage DNAs, the results showed that each probe recognized only the phage DNAs belonging to the RFLP groups they represent. Three of the nonrestricted DNA probes hybridized only to the 19 nonrestricted phage DNAs (see Fig. S2 in the supplemental material). No cross-reaction was detected with the DNA of

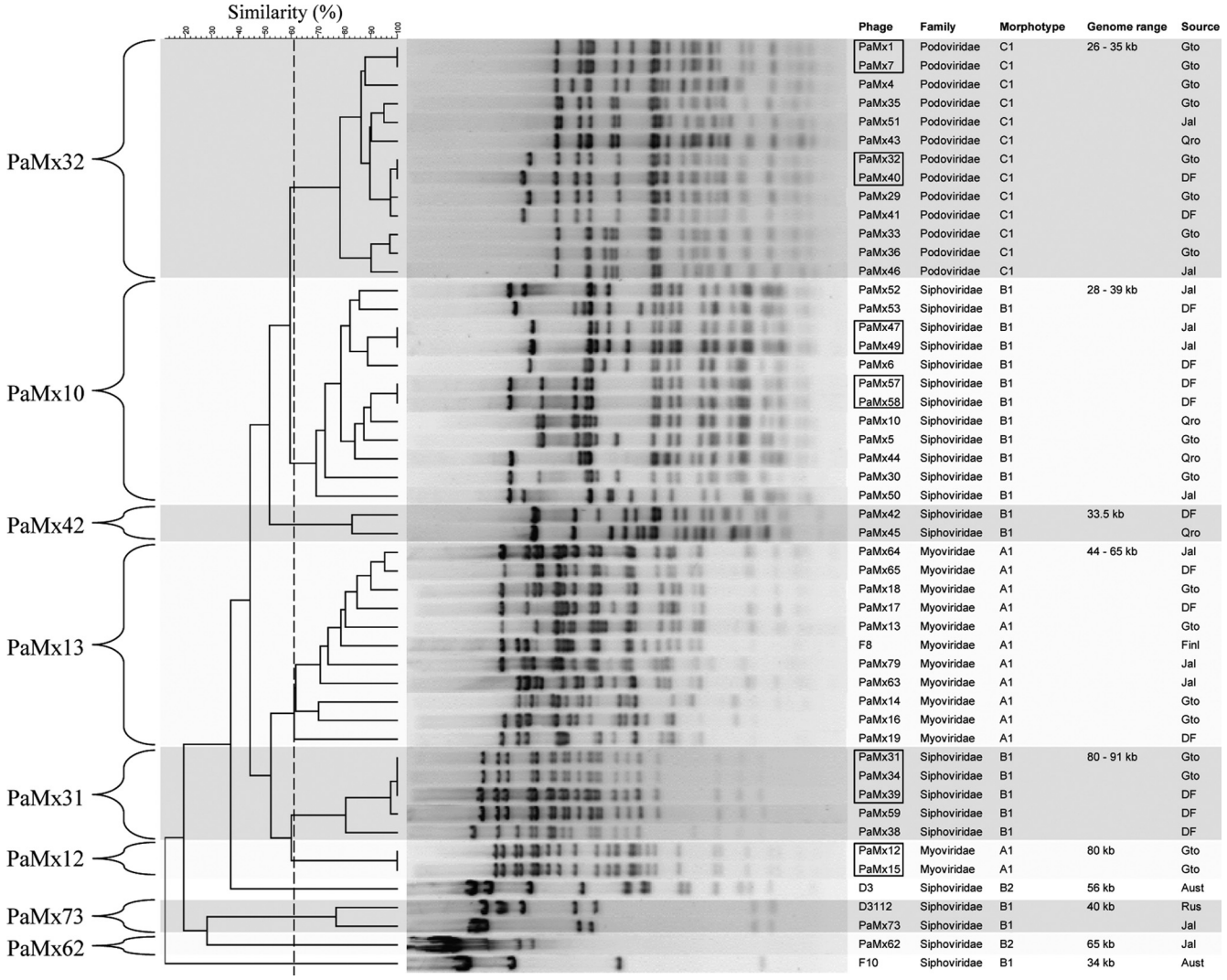

FIG 2 Similarity dendrogram of EcoRI-generated RFLPs of 46 phages with restrictable DNAs. The RFLPs of four reference phages (F8, D3, D3112, and F10) were included in the groups within which they showed similarity. The dashed vertical line denotes the similarity threshold (61%) used to define the groups. All the branches to the right of the threshold were included in the same group. The names of the archetypal phages for each group are shown in the left margin. Columns to the right of the RFLP pictures indicate phage characteristics and sample sources. Boxed phage names indicate 100% RFLP similarity. Morphology was based on electron microscopy. Genome lengths were estimated by adding the sizes of individual restriction fragments. Sources: Gto, Guanajuato; Jal, Jalisco; Qro, Queretaro; DF, Distrito Federal; Finl, Finland; Aust, Australia; Rus, Russia.

phages from different groups, except a weak cross-reaction between probe PaMx57 and the DNAs from PaMx42 and PaMx45 and between the nonrestrictable probe PaMx75 and the nonrestrictable DNAs from PaMx74, PaMx76, PaMx77, and PaMx78 (see Fig. S2 in the supplemental material). No cross-hybridization was found with DNAs from the control phages lambda, D3, and F10 (see Fig. S2 in the supplemental material). As was the case for other authors, DNA homology was not observed among phages belonging to different morphological families (4, 26, 28, 44). Therefore, 12 species were identified among the 65 dsDNA phages analyzed: 2 *Myoviridae*, 9 *Siphoviridae* (4 of which harbored nonrestrictable DNA), and 1 *Podoviridae* species (Table 1).

Host range. Next, the ability of each phage to infect 142 clinical *P. aeruginosa* strains was investigated. Phage suspensions at 10^6 PFU/5 μl were spotted onto the appropriate bacterial lawns and incubated overnight at 37°C. The lysis patterns were recorded for 112 strains susceptible to phage infection, and they were digitally analyzed using the software program Bionumerics v.6.5 to cluster phages and bacteria by host range similarity (see Fig. S3 in the supplemental material). The results showed a high correlation between phage species and host range; however, each phage had a different pattern of host infection. Thus, this phage collection could be suitable for bacterial typing or in the control of clinical infections. The phage species PaMx10, PaMx32, PaMx13, and PaMx31, which include the largest number of individual phages, infected the most strains. The two phages in species PaMx42 have host ranges similar to those in PaMx10 species, reflecting the close relationship between them as observed by RFLPs (Fig. 2) and DNA-DNA hybridization (see Fig. S2 in the supplemental material). Some phages with similar RFLPs had different host ranges for up to five bacterial strains, probably due to the presence of point mutations in the gene encoding the antireceptor, which does

TABLE 1 *Pseudomonas aeruginosa* phage species identified by morphology, RFLPs, and DNA hybridization

Species archetypal phage[a]	Other phages of the same species	Family	Morphotype
PaMx32	PaMx1, PaMx4, PaMx7, PaMx29, PaMx33, PaMx35, PaMx36, PaMx40, PaMx41, PaMx43, PaMx46, PaMx51	*Podoviridae*	C1
PaMx10	PaMx5, PaMx6, PaMx30, PaMx44, PaMx47, PaMx49, PaMx50, PaMx52, PaMx53, PaMx57, PaMx58	*Siphoviridae*	B1
PaMx42	PaMx45	*Siphoviridae*	B1
PaMx13	PaMx14, PaMx16, PaMx17, PaMx18, PaMx19, PaMx63, PaMx64, PaMx65, PaMx79, F8[b]	*Myoviridae*	A1
PaMx31	PaMx34, PaMx38, PaMx39, PaMx59	*Siphoviridae*	B1
PaMx12	PaMx15	*Myoviridae*	A1
PaMx73	D3112[b]	*Siphoviridae*	B2
PaMx62	M6[b]	*Siphoviridae*	B2
PaMx25[c]	PaMx20, PaMx21, PaMx22, PaMx23, PaMx24, PaMx26, PaMx55, PaMx70	*Siphoviridae*	B2
PaMx28[c]	PaMx27, PaMx71, PaMx75	*Siphoviridae*	B2
PaMx74[c]	PaMx72, PaMx76, PaMx77, PaMx78	*Siphoviridae*	B2
PaMx11[c]		*Siphoviridae*	B2

[a] Species archetypal phages selected randomly *a posteriori* from the group of homologues.
[b] Species archetypal phages of species according to the work of Ceyssens and Lavigne (12).
[c] Phage species harboring nonrestrictable DNAs.

not change the RFLP (27). Similar observations have been reported for *Streptococcus thermophilus* phages (22, 46). The species of siphoviruses with elongated heads (morphotype B2), carrying nonrestrictable DNA, infected a small number of unrelated strains (see Fig. S3 in the supplemental material). There were small groups of bacteria (two to five strains) that were infected by the same sets of phages (see boxes in Fig. S3 in the supplemental material). These strains were isolated from different patients in the same hospital, suggesting that they could belong to the same clonal group, as has been proposed in the context of other studies using molecular techniques (35, 36, 40).

Comparative analysis of partial genomic sequences. To further determine the genetic diversity of the phages in the collection and to place them in the context of sequenced *P. aeruginosa* phages, we compared the nucleotide sequences of the archetypal phage species PaMx13, PaMx25, PaMx73, PaMx10, PaMx11, PaMx12, PaMx28, PaMx31, PaMx32, PaMx42, and PaMx74 with the sequences of the 40 *P. aeruginosa* tailed phages reported in GenBank at the time of writing (January 2012). Each sequence was compared against all the other sequences using the software program MUMmer v.3.0 (29) to determine the degree of homology (see Fig. S4 in the supplemental material). This was calculated as the sum of the identical regions divided by the length of the longest sequence. We used a break length of 60 nucleotides (nt) to determine the extent of the identical regions. Since the known species share ≥53% nucleotide sequence homology, this threshold was used to separate the new *P. aeruginosa* phage sequences into species. Altogether, phages previously reported and those described here were classified into 28 species (see Fig. S4 in the supplemental material). Twenty of them were compiled previously (12); another two species included the characterized phages, KPP1, JG004, and PAK_P1 (18, 20), and the other six reported here (PaMx11, PaMx25, PaMx28, PaMx31, PaMx42, and PaMx74) were new because they did not have homologues in GenBank.

The sequence of PaMx13, the reference phage of a group of 10 phages in the collection (Table 1), showed nucleotide sequence homology with the genomes of PB1 phage species: 14-1, F8, JG024, LBL3, LMA2, and SN (16). These virulent myoviruses are distributed globally and have a broad host range, using the cell lipopolysaccharide layer as their primary receptor (25). No fewer than 42 phages of this species have been isolated and identified across Europe (28, 37); this result evidenced its presence in Mexico. A second myovirus species in our collection was represented by PaMx12 (Table 1). The nucleotide sequence of this phage showed at least 71% homology to the recently isolated and sequenced lytic phages JG004 and PAK-P1 (18, 20) (see Fig. S4 in the supplemental material). The DNA of phage species PaMx10, which includes 12 members (Table 1), was homologous to the DNA of PA73, a phage for which no homologous phage had been found previously (30). We also found nucleotide sequence homology between phage PaMx73, the only temperate phage identified here, and the transposable phage species D3112, additionally comprising phages DMS3, MP22, MP29, and MP38 (12). Vegetative particles of this species are commonly isolated from free-living and clinical strains of *P. aeruginosa* (7). To date, more than100 D3112-related phages have been found in several countries (17, 24). All the podoviruses in the collection were grouped into the PaMx32 species (Table 1). The DNA sequence of this phage showed 90% identity (see Fig. S4 in the supplemental material) with those of the lytic phages 119X and PaP2 (19), defined as one species despite their distant origins in Australia and China, respectively (3, 42). Several studies have shown that podoviruses of the ϕKMV species are widely distributed in Europe (10, 14, 33); however, not a single phage of this species was isolated in this work. Finally, the DNA of PaMx62, which has not been sequenced, hybridized with the DNA of phage M6 (see Fig. S2 in the supplemental material), previously included in the YuA species (12). PaMx62 was the sole representative of this species in the collection (Table 1). The fact that the three previous phages have been isolated in geographically distant locations (Belgium, Australia, and Mexico, respectively) (15) suggests a global distribution of this species.

Conclusions. We have analyzed the diversity of *P. aeruginosa* phages isolated in central Mexico. The phages were characterized, and their features were compared with those of phages from other global locations. The majority of the isolates were tailed phages of dsDNA, but three new ssRNA phages were identified. The morphological distribution of phages was similar to that observed in other bacterial species (1). Tailed phages were split into 12 species,

grouping phages with similar genome sizes, restriction patterns, virion morphologies, and host range. This confirms and expands the congruence between morphology and genomics in *P. aeruginosa* phages (31, 32). Six of the species identified here had been previously described, but the other six were novel species (archetypes PaMx11, PaMx25, PaMx28, PaMx31, PaMx42, and PaMx74) based on the low identity of their nucleotide sequence with those of previously sequenced phages. Previous studies that addressed the diversity of phages infecting *P. aeruginosa* in different global niches were conducted with locally isolated phages grown on native strains. It is possible that the isolation of novel phage species reported here was determined by the number of isolated phages and the geography and nature of the bacterial host. Only clinical bacterial isolates were used during phage isolation, and therefore, the lack of environmental isolates likely introduced bias into the nature of isolated phages. These results suggest either that there is some continental phage endemism (i.e., ϕKMV species) or that too few studies have been conducted to reveal the full diversity of *P. aeruginosa* phages. We intend to use the collection of phages in studies about bacterial typing and therapy of clinical strains.

Nucleotide sequence accession numbers. DNA sequences data of the archetypal phages have been deposited in GenBank under accession numbers JQ067083 (PaMx13), JQ067084 (PaMx25), JQ067085 (PaMx73), JQ067086 (PaMx10), JQ067087 (PaMx11), JQ067088 (PaMx12), JQ067089 (PaMx28), JQ067090 (PaMx31), JQ067091 (PaMx32), JQ067092 (PaMx42), and JQ067093 (PaMx74).

ACKNOWLEDGMENTS

This work was supported by a grant from The Instituto de Ciencia y Tecnología del Distrito Federal (ICyTDF) and a fellowship from The Consejo Nacional de Ciencia y Tecnología (CONACyT) to O.S.-R. (no. 200009).

We are grateful to María de Lourdes Rojas-Morales for her technical assistance with electronic microscopy, to Lidia Caballero-Tapia, Patricia Arzate-Barbosa, and Víctor Rafael Coria-Jiménez for providing the clinical *P. aeruginosa* strains, and to Sergio Vaca-Pacheco for providing strain PAO1. We also thank Víctor Missael Flores-López, Adrián Cazares-López, and Otoniel Maya-Lucas for sequence assemblies and for improving MUMmer v.3.0 for loading and repeating runs automatically. We also thank José Bueno-Martínez, Marco Magos-Castro, and Aurelio González-Hernández for their technical assistance and Verónica Ramírez-Nieves and Gabriela Mora-Macías for clerical support. We thank the Felix d'Hérelle Reference Center for Bacterial Viruses, Quebec City, Canada, for providing the phages F8, F10, D3, D3112, and M6 used in this study.

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