The Concerted Action of Two B3-Like Prophage Genes Excludes Superinfecting Bacteriophages by Blocking DNA Entry into *Pseudomonas aeruginosa*

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ABSTRACT In this study, we describe seven vegetative phage genomes homologous to the historic phage B3 that infect *Pseudomonas aeruginosa*. Like other phage groups, the B3-like group contains conserved (core) and variable (accessory) open reading frames (ORFs) grouped at fixed regions in their genomes; however, in either case, many ORFs remain without assigned functions. We constructed lysogens of the seven B3-like phages in strain Ps33 of *P. aeruginosa*, a novel clinical isolate, and assayed the exclusion phenotype against a variety of temperate and virulent superinfecting phages. In addition to the classic exclusion conferred by the phage immunity repressor, the phenotype observed in B3-like lysogens suggested the presence of other exclusion genes. We set out to identify the genes responsible for this exclusion phenotype. Phage Ps56 was chosen as the study subject since it excluded numerous temperate and virulent phages. Restriction of the Ps56 genome, cloning of several fragments, and resection of the fragments that retained the exclusion phenotype allowed us to identify two core ORFs, so far without any assigned function, as responsible for a type of exclusion. Neither gene expressed separately from plasmids showed activity, but the concurrent expression of both ORFs is needed for exclusion. Our data suggest that phage adsorption occurs but that phage genome translocation to the host's cytoplasm is defective. To our knowledge, this is the first report on this type of exclusion mediated by a prophage in *P. aeruginosa*.

IMPORTANCE *Pseudomonas aeruginosa* is a Gram-negative bacterium frequently isolated from infected immunocompromised patients, and the strains are resistant to a broad spectrum of antibiotics. Recently, the use of phages has been proposed as an alternative therapy against multidrug-resistant bacteria. However, this approach may present various hurdles. This work addresses the problem that pathogenic bacteria may be lysogenized by phages carrying genes encoding resistance against secondary infections, such as those used in phage therapy. Discovering phage genes that exclude superinfecting phages not only assigns novel functions to orphan genes in databases but also provides insight into selection of the proper phages for use in phage therapy.

KEYWORDS lysogenic conversion, superinfection resistance, gene function assignment, phage genome library, comparative genomics, phage B3 family

Pseudomonas aeruginosa is a Gram-negative bacterium that behaves as an opportunistic pathogen in humans. Clinical isolates of *P. aeruginosa* present resistance to a broad spectrum of antibiotics, survive disinfection protocols (1–5), and are frequently isolated from immunocompromised cystic fibrosis patients (6), where they cause acute

Citation Carballo-Ontiveros MA, Cazares A, Vinuesa P, Kameyama L, Guarneros G. 2020. The concerted action of two B3-like prophage genes excludes superinfecting bacteriophages by blocking DNA entry into *Pseudomonas aeruginosa*. J Virol 94:e00953-20. <https://doi.org/10.1128/JVI.00953-20>.

Editor Rozanne M. Sandri-Goldin, University of California, Irvine

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Received 15 May 2020

Accepted 15 May 2020

Accepted manuscript posted online 27 May 2020

Published 16 July 2020

lung infections (7, 8). Currently, bacteriophages (phages) are considered a promising alternative to treat multidrug-resistant *P. aeruginosa* infections (9).

Depending on their interaction with bacteria, phages are classified as virulent when they only generate progeny and lyse the host and temperate when their genetic material enters a latent state (prophage) within the host (lysogen). In many instances, when the lysogenic host suffers DNA damage, prophage repression is lifted to start the lytic cycle, which ends with the release of a new phage progeny after lysis of the cell. In latency, prophages may induce phenotypic traits that improve the fitness of their host (10), such as host resistance to secondary infections (11). One such mechanism, inherent to every prophage, is the expression of its repressor protein, which confers infection immunity to the lysogens against homologous phages, as has been described in detail for phage lambda of *Escherichia coli* (12, 13). For *P. aeruginosa* prophages, immunity exclusion has been studied for D3112-like phages (14–16) and D3 (17, 18). In other exclusion events, *P. aeruginosa* prophages may alter the receptors used by heterologous superinfecting phages to impede infection (11, 19, 20). For instance, phages D3 and JBD30 alter the structure of the O antigen (21–24), phage 2 modifies both polysaccharides and lipopolysaccharides (25), JBD26 and JBD44 compromise the expression of the bacterial flagellum (24), and D3112, JBD24, JBD26, JBD30, and JBD44 affect type IV pili (24, 26).

Many phage coding sequences lack an assigned function; around 20% to 25% of the open reading frames (ORFs) in the *Pseudomonas* phage genomes are uncharacterized and show no detectable similarity to other sequences reported in databases (27). Although it remains unclear as to whether these sequences are related to bacterial or viral nonessential processes, they represent a large and diverse source of genetic information. Among these sequences, diverse exclusion genes are likely to be uncovered due to the double advantage they provide to lysogens: bacterial viability maintenance and preservation of the resident prophage lineage (28). Few studies address this topic; however, there are unanalyzed phage genomes. This is the case of phages related to B3, which is a temperate transposable *P. aeruginosa* phage isolated from an Australian strain (29, 30) that is related to other transposable phages such as B39 and D3112 (*P. aeruginosa*) and Mu (*E. coli*) (31–34). Comparative genomics revealed that B3 replicates like coliphage Mu by replicative transposition (35), in which phage DNA is inserted into the host DNA and the phage-host structure is resolved by target-primed replication, leaving heterogeneous host sequences at the ends of the phage genome (36, 37). However, the B3 genome presents genetic rearrangements that distinguish it from Mu and other Mu-like phages (35), and it also encodes transposition proteins related to transposon Tn552 of *Staphylococcus aureus* (38). Moreover, prophages with a similar transposase have been found in the genomes of several *Enterobacteria*, *Spirochaetes*, and *Pseudomonas* species (39). Over the last decade, genomic information on B3-like phages has accumulated in databases but has gone unreported for them as a group.

In this work, we present a comparative genomic study of seven new *P. aeruginosa* temperate phages (Fc02, Fc22, Ps56, Ps59, Ps60, H71, and H72) that are homologous to the archetype B3. We show that despite the extensive genomic resemblance among the phages of this group, here referred as B3-like, they display a broad variety of exclusion patterns when their lysogens are exposed to a collection of superinfecting temperate and virulent phages. Among the B3-like phages, Ps56 was analyzed further to identify the genes responsible for the exclusion phenotype. The characterization of a region in the Ps56 genome, conserved in the B3-like genomes, showed that it contains a pair of genes responsible for the superinfection exclusion phenotype. Interestingly, exclusion manifests only when the genes are expressed together from independent constructs and not when the constructs express separately in different cells. Our results provide evidence for an exclusion system in which adsorption by the superinfecting phage occurs normally but phage DNA translocation from the virion to the cell cytoplasm is blocked.

FIG 1 Transmission electron microscopy of the seven new B3-like siphophages. Measurements (diameter of capsid/length of tail) are as follows: Fc02, 62.4 ± 2.9 nm/193.7 ± 9.7 nm; Fc22, 64.9 ± 2.6 nm/203.8 ± 12.04 nm; Ps56, 66.1 ± 2.7 nm/197.2 ± 12.6 nm; Ps59, 65.3 ± 1.7 nm/204.4 ± 9.05 nm; Ps60, 69.6 ± 4.4 nm/219.2 ± 15.3 nm; H71, 64.2 ± 1.4 nm/200.9 ± 7.14 nm; and H72, 59.6 ± 3.8 nm/215.1 ± 32.5 nm.

RESULTS

Host strains and virion morphology. Seven new temperate phages (Fc02, Fc22, Ps56, Ps59, Ps60, H71, and H72) were isolated from a set of *P. aeruginosa* clinical isolates from Mexico City (40). All the phages produce turbid plaques between 3 and 5 mm in diameter when infecting their corresponding bacterial hosts, i.e., strain PAO1 for phage Fc02 and clinical isolate Ps33 from our collection for the other six phages. Morphologically, the phage virions consist of an isometric capsid of morphotype B1 (41, 42) attached to a long, flexible, noncontractile tail typical of the *Siphoviridae* family (43). The capsid diameter measurement varied from 59.6 to 69.6 nm, and the tails ranged from 193.7 to 215.1 nm in length (Fig. 1).

Genome sequencing and comparative analysis. To evaluate the degree of similarity among the new phages, their genomes were sequenced (Table 1). Comparison of the genomes at the nucleotide level revealed overall sequence identity values ranging from 18% to 93% (Fig. 2). Additional sequence homology searches in GenBank identified other related genomes in addition to B3 (35): JBD18, JBD25, JBD67 (44), and PM105 (45). The transposable phage B3 represents this group of temperate phages, as it was the first to be characterized (14, 30, 34). Consistent with the homology to B3, we identified heterogeneous DNA fragments attached to the phage genome ends during the assembly of our collection of phage sequences (see Fig. S1 in the supplemental material), a typical feature of phages with Mu-like replicative transposition (36, 46). Further analysis of the heterogeneous sequencing reads recovered from the Ps56 genome assembly, used as a representative of the group, confirmed that they correspond to fragments of the host genome. Comparison of more than 250 reads retrieved from both phage genome ends against the genomes of *P. aeruginosa* PAO1 (accession number NC_002516) and the host strain Ps33 (see below) identified multiple matches displaying at least 97% nucleotide sequence identity (see Data Set S1 in the supplemental material). In all cases, the match against the bacterial genome started immediately after the phage portion of the read (Fig. S1; Data Set S1). More than 160 different insertion sites scattered across the host genome in both intragenic and intergenic

TABLE 1 Sequencing and genomic features of the new B3-like phages^a

Phage	Avg coverage (fold)	Genome size (bp)	No. of predicted ORFs	GC content (%)	GenBank accession no
Fc02	14.32	38,122	54	62.81	MH719189
Fc22	13.01	38,255	53	62.75	MH719191
Ps56	178.63	39,816	57	62.62	MH719192
Ps59	33.50	39,020	54	61.74	MH719195
Ps60	26.34	39,676	56	63.15	MH719194
H71	8.68	38,223	54	62.65	MH719190
H72	15.01	38,579	55	62.66	MH719193

^aAll phage genomes start with the trinucleotide 5'-TGA at the left end and terminate in ACA-3' at the right end. Both sequences were recognized after elimination of host DNA attached to the ends. All the genomes were assembled up to one final contig.

FIG 2 Nucleotide sequence identity among B3-like phages. Percent identity between pairs of sequences was calculated as the number of matches divided by the length of the shortest sequence. Numbers were color coded using a gradient from red (less identity) to green (more identity).

regions were recognized in the analysis, thus confirming the transposable nature of phage Ps56.

Pangenome of the B3-like phages. A pangenome analysis of the group of twelve vegetative phages, including five from databases, allowed the operational identification of core ORFs present in all the compared genomes and accessory ORFs shared by only some members of the group (Fig. 3). The organization of the core ORFs across the genomes highlights the synteny among the functional modules of the B3-like phages. Moreover, in the left arms of the genomes, there is a region related to the genome insertion-replication by transposition. Meanwhile, a short middle region bearing the putative repressor and antirepressor genes (ORFs 12 and 11, respectively) is responsible for the gene control of lysogeny and lysis, and the right-arm region mostly corresponds to the virion morphogenesis genes (35). Despite their conserved presence in the

FIG 3 Pangenome analysis of phages of the B3-like group. The upper map corresponds to the ORFs shared (core genome) among all the vegetative phages, which are numbered from 1 to 40 and color coded as follows: ORFs with assigned function, red; ORFs without assigned function, green; and virion morphogenesis and structural ORFs, gray. Above the map are the functions assigned by homology to known phage proteins. Likewise, ORFs that are present in only some members of the group (accessory) are indicated in their corresponding RGPs. In the matrix below, accessory ORFs for each phage are indicated with blue squares. These are designated in lowercase letters from a to k, according to the RGP (A to K) they belong to. Phage genomes were ordered according to the phylogenetic tree reflecting their genome relationship (left side), and purple dots correspond to supporting bootstrap values of 100.

genomes, the core ORFs display a wide range of sequence variation among the members of the B3-like group (see Data Set S2 in the supplemental material). Homologs of the core ORFs 13 and 14, encoding holin and endolysin, were among the most similar in the group, whereas ORFs 1 and 12, encoding the Mor and repressor protein homologs, exhibited the lowest sequence conservation across all the genomes. It is worth noting that the core ORFs 22, 23, and 24 (associated with capsid morphogenesis) are particularly divergent in five members of the group; hence, their presence could be recognized only through synteny and conserved domain searches. Complex patterns of core ORF sequence similarity were identified among the B3-like genomes. Some phages shared high sequence similarity between all their core ORFs, but others featured blocks of divergent ORFs in discrete regions of their genomes (Data Set S2), thus highlighting the mosaicism of the group. Additionally, the accessory ORFs, i.e., those absent in one or various genomes, were identified at eleven discrete loci named regions of genomic plasticity (RGPs) and were designated A to K along the genomes (Fig. 3) following the rules used previously (46). Some RGPs (B and E) contain complex arrays of accessory genes across the compared genomes, whereas other RGPs (A and C) showed the presence/absence of only an ORF. Unlike the core ORFs, the homologs of most accessory ORFs shared high levels of sequence identity, suggesting a more recent acquisition in the genomes. A phylogenomic analysis of the twelve B3-like genomes portrayed a diverse group featuring at least two major clusters, where B3/PM105 and Ps59/JBD25 were the most distant clades (Fig. 3). Interestingly, the presence/absence of some accessory ORFs (e.g., RGPs G and I) reflected the genome cluster distribution when observed in the phylogenomic tree. We did not find a clear association between the geographic region of isolation of the B3-like phages and their phylogenetic relationships, although the number of analyzed cases was small. Due to the high diversity within the B3-like phages, reflected in both their variable and constant ORFs, the search for differences among them proceeded to a phenotypic level, analyzing the exclusion pattern of superinfecting phages by lysogens of the different B3-like phages.

Exclusion of superinfecting phages. Our analysis focused on superinfection exclusion by Ps33 lysogens for Fc22, Ps56, Ps59, Ps60, H71, H72, and JBD18 challenged for infection with a collection of temperate and virulent phages (see Table S1 in the supplemental material). We made sure that the genome of strain Ps33 did not harbor prophages homologous to phages of the B3-like group. The assembled chromosome, featuring ~6.29 Mb and 66.5% GC content, harbors three prophage regions of 56.6, 18.4, and 14.3 kb (coordinates 1359918 to 1416579, 5258102 to 5276572, and 5348284 to 5362629, respectively). None of these regions showed extensive sequence similarity at the nucleotide level to the B3-like phages analyzed in this work. Further, Ps33 lysogens for each of the selected phages were exposed to infection by two different sets of phages: 32 temperate phages isolated from our collection of *P. aeruginosa* clinical strains (data not shown) and 16 virulent phages described previously (47). Four of the seven tested lysogens displayed exclusion to more than half of the superinfecting temperate phages. It is noteworthy that the lysogen carrying Ps59 prophage was infected by almost the whole set of temperate phages tested (Fig. 4A). To infer which prophages share immunity, we applied the criterion of cross-infection mutual inhibition. The arrangement of exclusion events of the B3-like phages in the infection matrix revealed the presence of four immunity groups, i.e., (i) JBD18/Ps56/H71, (ii) H72, (iii) Fc22/Ps60, and (iv) Ps59 (Fig. 4A), where the immunity is shared among the prophages of each group. However, the exclusion events detected outside the immunity groups should be independent of repressors but dependent on other exclusion genes. This was tested by exposing the lysogens to a set of virulent phages. It was found that prophages JBD18 and Ps56 showed the broadest exclusion activity by blocking the infection of 11 of the 16 phages tested (Fig. 4B).

Identification of the phage exclusion genes. Next, we searched for the genes involved in the broad exclusion phenotypes observed in some of the lysogens. To this end, we selected Ps56 as the representative of the B3-like phages because of its

FIG 4 Exclusion patterns of B3-like prophages in the Ps33 strain. (A) Infection assay of thirty-two temperate phages on lysogens for each of the seven B3-like phages analyzed. The first seven columns correspond to the B3-like lysogens, arranged by the number of temperate phages excluded (top row). (B) Infection assays of sixteen virulent phages (top row) on the seven lysogens. In both matrices, lysogens were ordered from the highest (top) to the lowest (bottom) by number of different excluded phages. Note that the order of exclusion events for the lysogens is similar in both matrices. The sets of temperate and virulent phages in the matrices were ordered from the most (left) to the least (right) excluded phages. As a control of infection, strain Ps33 was included (bottom rows). The outcome of every infection assay in drops of 5 μ l of phage suspensions, containing about 10^6 PFU, was recorded and classified according to the relative frequency of PFU detected as exclusion ($<10^{-4}$), low infection (10^{-4} to 10^{-2}), or high infection ($>10^{-2}$).

extensive range of exclusion. We focused on the regions with the largest number of ORFs without functional homologs reported in databases (Fig. 3). The Ps56 genomic DNA was restricted with different enzymes to generate discrete fragments encompassing different regions across the genome (see Fig. S2 in the supplemental material). However, only three fragments of the left arm of the Ps56 genome (A.1, B.1, and B.2) were individually cloned in the expression plasmid pHERD30T (48). Infection assays were performed to determine whether the cloned fragments exerted exclusion activity similar to that of the Ps56 prophage. The fragment B.2, comprising five core ORFs (ORFs 8 to 11, three without known function) and one accessory ORF (c, with unknown function), was the only one displaying the exclusion phenotype (see Fig. S3 in the supplemental material). Hence, the remaining ORFs were evaluated by generating different gene combinations of the B.2 restriction fragment (Fig. S2). The expression of the B.2 Δ NcoI fragment indicated that ORFs 8 and c were not related to the exclusion phenotype (Fig. S3). Fragment B.2 Δ Sall, harboring ORFs 10 and 11 and $\sim$ 64% of the ORF 9 sequence, also retained the phenotype. However, the exclusion activity disappeared when the fragment was cloned in the reverse complement direction (Fig. S3). This indicated that the observed phenotype is not driven by an ORF in the complementary strand. Thus, we designed primers to separately clone the genes present in the shortest fragment displaying exclusion activity (see Table S2 in the supplemental material). However, ORF 11 was ruled out of the study because it predictably encodes a Ner-like protein whose function has been already described (49). Surprisingly, the exclusion phenotype toward virulent phages was reproduced when ORFs 9 and 10 of Ps56 were cloned and expressed jointly but not when they were expressed separately (Fig. S3). To confirm that ORFs 9 and 10 act together to produce the exclusion phenotype, they were individually cloned into two compatible plasmids, pHERD30T (48) and pSEVA55*, and these were cotransformed in strain Ps33. Figure 5 shows that

FIG 5 Evaluation of the exclusion phenotype of ORFs 9 and 10 cloned individually in plasmids pHERD30T and pSEVA55*. Ps33/pHERD30T+pSEVA55* and Ps33/pHERD30T/ORFs_10_9+pSEVA55* were used as infection and exclusion controls, respectively. All the constructs were exposed to the set of virulent phages. Note that exclusion occurs only in transformants containing ORFs 10 and 9, either in the same construct or in separate constructs.

only the Ps33 cotransformant with pHERD30T/ORF_9 and pSEVA55*/ORF_10 presented the exclusion phenotype as also observed in the cotransformant pHERD30T/ORFs_10_9 + pSEVA55* when exposed to the virulent phage set. Detailed comparison of the ORFs homologous to ORFs 9 and 10 exposed sequence variation among the members of the B3-like group. However, the patterns of similarity recognized did not correlate with the differential exclusion observed between the lysogens tested (see Fig. S4 in the supplemental material). The comparative analysis was extended to the ORFs and intergenic regions flanking ORFs 9 and 10 in order to identify potential genetic elements associated with the broad exclusion phenotype detected in the Ps56 and JBD18 lysogens. However, none of the individual trees inferred from the comparison of the different flanking regions showed the corresponding Ps56 and JBD18 sequences as the closest in the alignment (Fig. S4), thus implying a complex regulation of the core ORFs 9 and 10 to drive superinfection exclusion or that more than one exclusion system is at play in some of the lysogens.

Stage of superinfecting phage exclusion by prophage genes 10 and 9. Subsequently, we analyzed the adsorption stage, which is the starting point in the phage infection process (50). To evaluate whether adsorption occurs, cells harboring the exclusion-driving pHERD30T/ORFs_10_9 plasmid construction were exposed to the virulent phage PaMx53. No significant differences in the percentage of phage adsorption were found between the cells carrying the plasmid construction and the control strain Ps33/pHERD30T (Fig. 6A). However, much less phage was adsorbed by Ps33(Ps56)/pHERD30T than by Ps33/pHERD30T (Fig. 6A). This implies adsorption inhibition in the lysogen, possibly due to a second exclusion phenomenon encoded in the prophage. We then tested if the phage DNA uptake by the cell was affected by ORFs 9 and 10. Previous work with coliphages T4 and T5 showed that translocation of the virion DNA into the cell is accompanied by the release of K^+ from the cell (51, 52). We

FIG 6 Assays to determine the stage of exclusion by ORFs 9 and 10 in the infection process. (A) Adsorption assay of virulent phage PaMx53. Note that the phage seems to adsorb equally to Ps33/pHERD30T compared to Ps33 harboring ORFs 10 and 9. Ps33(Ps56)/pHERD30T was used as an exclusion control, and PaMx53 without bacteria (mock) was used as a control of phage stability, as samples were taken before and after the centrifugation step in the adsorption assay. (B) K⁺ efflux assay. Notice that K⁺ was not released either from the lysogens for prophage Ps56 or from cells carrying the construct harboring phage ORFs 10 and 9. Ps33/pHERD30T and Ps33(Ps56)/pHERD30T were used as infection and exclusion controls, respectively. All the samples were measured with and without infection with the virulent phage PaMx53 at 0 and 30 min. One-way ANOVA with Bonferroni's posttest was performed to calculate statistical differences in both assays using GraphPad Prism 5 software. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

measured the K⁺ efflux in Ps33 strains harboring prophage Ps56 or construct pHERD30T/ORFs_10_9 upon infection with phage PaMx53. A significant percentage of K⁺ (~35%) was released from control Ps33+pHERD30T cells after 30 min (Fig. 6B), as expected from a successful phage DNA translocation into the host's cytoplasm. In contrast, strains Ps33(Ps56)/pHERD30T and Ps33/pHERD30T/ORFs_10_9 did not show K⁺ release even after 30 min of incubation with phage PaMx53 (Fig. 6B). These results indicate that expression of ORFs 9 and 10 excludes superinfecting phages at the cell envelope level by blocking DNA translocation from adsorbed superinfecting phage.

DISCUSSION

B3-like family of phages. Comparative analysis of twelve phage genomes from the data banks that share size and ORF organization with phage B3 showed that they could be grouped as a family. This similarity allows for the identification of core ORFs present in all the specimens and accessory ORFs of variable presence and frequency among the genomes (Fig. 3). The genomes of the B3-like phages contain core and accessory genes, named the pangenome, like other bacteria (53, 102) and phages, including those that infect *P. aeruginosa* (46). At fixed specific locations, the homologous genomes show clusters of accessory ORFs named regions of gene plasticity (RGPs). In the D3112-like group, RGP C and G clusters contain eight and nine accessory ORFs, respectively (46); for the B3-like group, the distinctive cluster is RGP B, including thirteen variable or accessory ORFs. In addition to their genomic resemblance, the twelve phages presented similar morphological features, independent of their different temporal and spatial isolation. These identity features have been observed for other families of *P. aeruginosa* phages, such as the ϕ KMV-like (54–56), ϕ KZ-like (57), PB1-like (58), D3112-like (46), and 119X-like (59) phages, as well as for phages infecting other bacterial genera, such as *Brucella* spp. (60–62). Therefore, we propose establishing the B3-like phages as a formal group whose members had not been organized until now.

Superinfection exclusion. We analyzed the ability of B3-like lysogens to exclude superinfecting virulent and temperate phages. Among the seven lysogens assayed, four immunity repressor groups were detected (Fig. 4A, red), implying that shared immunity is common among the B3-like phages. Since we were focused on exclusion mediated by genes different from immunity repression genes, exclusion of virulent phages was assayed. Overall, about 37% of exclusion not assigned to immunity occurred in the lysogens (Fig. 4A, black boxes). When the seven lysogens for each of the B3-like phages were challenged with virulent phages, exclusion occurred at about 29% (Fig. 4B). Although the nucleotide identity of the B3-like group is diverse (Fig. 2), phages with a low percentage of identity, especially Ps56 and JBD18, presented a similar exclusion phenotype. The ranking of lysogens for exclusion of virulent phages was similar to that found for temperate phages (JBD18 > Ps56 > H71 > Fc22 > Ps60). Therefore, the prophage genes responsible for exclusion of temperate and virulent superinfecting phages should be the same. Exclusion events against *P. aeruginosa* phages, mediated by D3112-like prophages, have been previously reported (63). To our knowledge, studies of virulent phage exclusion mediated by prophages are scarce (64). The present study adds novel genes to the superinfection exclusion topic.

Two genes cooperate in superinfection exclusion. The nature of the superinfection exclusion by lysogens for B3-like phages was further investigated in Ps56, a phage that showed one of the broadest exclusion profiles. Using the reductionist approach of finding cloned fragments of phage DNA that retained the exclusion phenotype, the results pointed to ORFs 9 and 10 together as responsible for the phenotype of superinfecting phage exclusion (Fig. 5). We noticed that a construct carrying ~64% of ORF 9 still was active in exclusion (see Fig. S3 in the supplemental material). This result suggests that the N-terminal segment of ORF 9 retains its function in collaboration with ORF 10. Moreover, the genomic architecture of ORFs 9 and 10, although different in phages Ps56 and JBD18 (see Fig. S5 in the supplemental material), does not significantly affect the exclusion phenotype (Fig. 4). In most of the described cases, a single gene was required to provoke the exclusion phenotype (11). In phage D3112, the *gp05* or *tip* gene, which is responsible for exclusion against superinfecting phages, is shared between MP22 and other Mu-like phages (26, 46). Other D3112-like phages (JBD24, JBD26, JBD26, and JBD44) contain genes that confer superinfection exclusion independent of immunity (24). However, superinfection exclusion in *P. aeruginosa* by phage D3 requires three genes. The genome of D3 carries three adjacent genes (*iap*, *wzy_β*, and *oac*) whose joint function is to modify the bacterial O-antigen structure, altering the adsorption of phages D3 and E79 (22, 23, 65).

ORFs 9 and 10 allow adsorption but block DNA translocation. The superinfection exclusion exerted by the joint action of ORFs 9 and 10 expressed from a construct does not affect the proportion of the virulent phage PaMx53 adsorbed to bacteria (Fig. 6A) despite the fact that it excludes superinfection by this phage (Fig. 5). It is worth noting that Ps33(Ps56) showed a lower percentage of adsorbed phage PaMx53 than bacteria carrying the ORFs_10_9 construct. This result indicates that another gene(s) of phage Ps56 may affect exclusion by interfering with the adsorption of superinfecting phages. However, the search for those genes was not pursued in the present study. On the other hand, ORFs 9 and 10 reduced translocation of superinfecting phage DNA almost 8-fold (Fig. 6B). Exclusion by interference with phage DNA translocation has been reported in Gram-positive bacterium-phage systems, such as in phage Tuc2009 of *Lactococcus lactis* by the expression of the *sie2009* gene (66, 67) and for phage TP-J34 of *Streptococcus thermophilus* by the action of the *ltp* gene (68). Gram-negative bacteria prophage gene-mediated superinfection exclusion affecting DNA translocation has been documented in several instances: phage P22 in *Salmonella enterica* subsp. *enterica* serovar Typhimurium through the *sieA* gene (69–71), coliphage T4 for its *imm* and *sp* genes (72), HK97 by its *gp15* gene (73, 74), and mEp167 by the Cor protein, which prevents phage infection through FhuA receptor blockage (75, 76). Further studies are

required to reveal the molecular mechanism behind the ORF 9 and 10 blockage of superinfecting phage DNA entry.

MATERIALS AND METHODS

Phage isolation and propagation. Our temperate phages (see Table S1 in the supplemental material) were isolated from the supernatants of a collection of clinical strains of *P. aeruginosa*, which were generated from samples collected from three hospitals in Mexico City (40). Overnight (O/N) cultures of *P. aeruginosa* clinical strains were grown in Luria-Bertani (LB) medium at 37°C and centrifuged at $\sim 9,000 \times g$ for 10 min. In order to identify infective phages released by productive lysogens, culture supernatants were collected, serially diluted, and dropped over lawns of five sensitive strains (Ps9, Ps26, Ps33, Ps53, and PAO1) to identify a common host (Table S1) (47). The presence of phage in the supernatant of bacterial cultures as turbid plaques in lawns of sensitive bacteria was taken as evidence of prophage spontaneous induction. Turbid plaques were isolated and propagated to generate a collection of temperate phages that were grouped according to the similarity among them and with other phages reported in databases (see below). Table S3 in the supplemental material summarizes the origin of the seven B3-like phages identified in our collection. These phages were individually propagated on the lawns of the sensitive host. The plaques were deposited in sterile 1.5-ml tubes with 1 ml SM buffer (200 mM NaCl, 0.5 mM MgSO₄, 1 mM Tris-HCl, and 0.1% gelatin; the pH was adjusted to 7.4 with HCl) and centrifuged at $\sim 9,000 \times g$ for 10 min. The supernatants were deposited in new sterile tubes and treated with chloroform. A second centrifugation round was done to collect each supernatant as an individual phage stock (with a titer of $\sim 10^9$ PFU/ml). All the stocks were stored at 4°C.

CsCl gradients. The phage preparations were purified as previously described (77). Briefly, phage particles were collected from lysates of five bacterial lawns on petri dishes concentrated with polyethylene glycol (PEG) 8000 and cleaned with chloroform. Each phage preparation was concentrated by centrifugation in Ultra-Clear centrifuge tubes (item number 344060; Beckman Coulter) with CsCl step gradients. The tubes were centrifuged at $\sim 61,000 \times g$ for 2.5 h at 4°C, and the phages were retrieved through a side puncture. The recovered phages were dialyzed with three rounds of buffer (10 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM MgCl₂) for 1 h at 4°C. The purified phages were then stored at 4°C.

Transmission electron microscopy. The purified phages were negatively stained with 2% uranyl acetate over copper grids previously treated with 0.3% Formvar. The phage samples then were observed under a JEM-1400 transmission electron microscope (JEOL) at 80 kV. Each phage sample was observed at magnifications of 50 $\times$, 150 $\times$, and $\times 200$. The diameter of the capsid and the length of the tail were measured using the ImageJ software (v1.47). Ten images of the same phage preparation were used to obtain measurements and their standard deviations.

DNA extraction and genome sequencing. Phage DNA was extracted with phenol-chloroform (1:1) from CsCl-purified phages (77). The genomic DNA of the *P. aeruginosa* clinical strain Ps33 was isolated following the protocol described by Chen and Kuo (78). DNA samples of each phage were sequenced using the Roche/454 system at the Laboratorio Nacional de Genómica para la Biodiversidad (CINVESTAV-Irapuato, Mexico), and the sequencing reads were preprocessed using Newbler assembler software (default values were used). The genomes were *de novo* assembled in one single contig using the Velvet v1.1 algorithm (79). Heterogeneous sequences of bacterial DNA attached to both ends of the final contigs were identified through BLASTn searches (80) against *P. aeruginosa* genomes and trimmed off until finding the first nucleotide shared by all the reads in the assembly (Table 1; see Fig. S1 and Data Set S1 in the supplemental material).

The genome of strain Ps33 was sequenced at the Biodesign Institute, Arizona State University (AZ, USA) using the Illumina MiSeq instrument (2×300). The resulting sequencing reads were processed for adapter removal and trimming of low-quality bases using Trimmomatic (81) prior to their *de novo* assembly with SPAdes 3.11.0 (82). The assembled contigs were then scaffolded using the PAO1 genome (accession no. NC_002516) with the MeDuSa v1.6 algorithm as a reference (83). Sequence polishing was carried out using BWA (84) and Pilon (85), while genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (86), and prophage regions in the genome were identified through the PHASTER server (87).

Genome annotation and sequence analysis. The phage genomes reported here were annotated as previously described by Cazares et al. (46). Briefly, the prediction of coding sequences was performed with GeneMark (v1.1) using heuristic hidden Markov models (88), and their respective correction with the identification of the ribosome binding sites was performed using rbs_finder.pl (89). The GC content was obtained using Artemis (90). Homologous sequences with the predicted ORFs were identified using BLASTp (80) against nonredundant protein databases to improve annotations. InterProScan (91) and NCBI-CDD (92) were used to identify conserved domains. Additionally, putative promoter sequences were detected while screening noncoding regions of the genomes using BPROM (Softberry Inc.) and Neural Network Promoter Prediction (NNPP) software (93). Specific transcription factor binding sites for *P. aeruginosa* were identified using the promoter analysis tool hosted on the PRODORIC website (94). Subsequently, B3-like genomes deposited in GenBank were identified through BLASTn searches (80) using the Fc02 and Ps60 genomes as queries. The level of sequence similarity at the nucleotide level among the phage genomes was estimated from MUMmer alignments (95) using the all_vs_all_nt.sh script (https://github.com/vflorelo/all_vs_all_nt). A neighbor-joining tree of the genomes, with a bootstrap of 1,000 replicates, was inferred using the Seaview package v4.7 (96) from a multiple-genome alignment obtained with the progressive algorithm of Mauve v2.4.0 (97). The pangenome analysis of the B3-like group was performed following the steps previously reported (46). The presence of B3-like prophages in the genome of the host strain Ps33 was assessed by direct comparison of our phage

sequences against the bacterial genome through BLASTn searches. The prophage regions detected in Ps33 by the PHASTER algorithm were additionally compared against the *Caudovirales* (taxid 28883) genomes available in the GenBank nucleotide collection (nr/nt). Matches covering 10% or less of the query genomes were not considered to assign a phage type.

Lysogen construction. B3-like phages were seeded on bacterial lawns of their sensitive host. From the center of each turbid lysis spot, samples of lysogenic candidates were streaked on LB agar plates. These candidates were purified from isolated colonies thrice before testing for the following. For immunity (test 1), O/N cultures of each candidate in LB were used to prepare lawns on LB agar plates, and a few microliters from a stock of the homologous phage was dropped on top and incubated overnight. Absence of lysis spots on the lawn indicated that the candidate was immune, as expected from a lysogen. For spontaneous induction (test 2), an aliquot of the culture was centrifuged at $\sim 9,000 \times g$ for 10 min, and 5 μ l from the supernatant was dropped over the corresponding propagation strain. After O/N incubation, the presence of turbid lysis spots revealed the candidates that shed phages. Candidates that succeeded in both tests were considered true lysogens and used in further assays.

Infection assays. Five microliters of phage lysates containing $\sim 10^6$ PFU/ml was dropped on lawns of each lysogen to be assayed for exclusion (Table S1). After incubation, the presence or absence of isolated plaques was registered to classify each event of infection in one of three categories: exclusion, low infection, and high infection. The thresholds for each category were defined according to the frequency of the highest and lowest numbers of isolated plaques detected in the whole assay (see Fig. 5 legend). All infection assays were performed in triplicate.

Immunity groups. From the recorded results of the infection assays (see above), among the phages and their Ps33 lysogens, cases in which exclusion from the cross-infection test was reciprocal between the seven phages and their respective lysogens were identified. Phages that fulfilled this criterion were considered to belong to a unique immunity group.

Exclusion gene search strategy. The genome of phage Ps56 was digested with the enzymes BspHI and EcoRI to generate discrete fragments. Those flanked with both restriction sites and harboring ORFs with unknown functions (fragments A.1, B.1, and B.2) (see Fig. S2 in the supplemental material) were cloned in plasmid pHERD30T, which carries a gentamicin resistance marker (48), and electroporated into Ps33 using the procedure and parameters previously reported (98). The ORFs' expression of Ps56 fragments was evaluated by infection assays with virulent phages (Table S1) on LB agar plates supplemented with gentamicin (50 μ g/ml) to identify if any of the fragments conferred the exclusion phenotype as the Ps56 prophage. Once the fragment B.2 was identified as responsible for superinfecting exclusion, it was resected further with NcoI and Sall restriction enzymes, and the candidate ORFs were cloned in pHERD30T to be tested for infection with virulent phages (Fig. S2). To assess if ORFs 9 and 10 are required for the exclusion phenotype, they were cloned into compatible plasmids pHERD30T and pSEVA55* (Table S1). The latter was constructed with the restriction of the RSF1010 replication origin of plasmid pSEVA658 and the amplification of the tetracycline resistance gene of plasmid pSEVA541 (see Table S2 in the supplemental material). Both sequences were joined at the AscI and FseI restriction sites. Plasmid pSEVA55* was named following the nomenclature established for pSEVAs (99, 100) and is recognized because it does not have a cargo module (*). The ORF_10 cloned in pSEVA55* included the addition of the pBAD promoter from plasmid pHERD30T with the MfeI and FseI restriction sites (Table S2). Plasmids pHERD30T/ORF_9 and pSEVA55*/ORF_10 were coelectroporated in the Ps33 strain and tested against the virulent phages on LB agar plates with gentamicin (50 μ g/ml) and tetracycline (80 μ g/ml).

Adsorption assay. Strains Ps33/pHERD30T, Ps33(Ps56)/pHERD30T, and Ps33/pHERD30T/ORFs_10_9 were grown on LB agar plates with gentamicin, gently harvested, and suspended in LB as previously reported (101). Subsequently, the cells were diluted to an optical density at 600 nm (OD_{600}) of 1.5 to 1.7 and collected by centrifugation. Each pellet was resuspended with 100 μ l of LB and mixed with virulent phage (PaMx53) at a multiplicity of infection (MOI) of 0.01. The samples were divided in halves: one half was immediately centrifuged, and the second half was incubated at 37°C for 30 min and then centrifuged. Supernatants were collected, diluted, and dropped over Ps33 bacterial lawns. The adsorbed phage was estimated from the difference between the numbers of plaques after incubation and the number of plaques before incubation. Adsorption assays were performed in triplicate.

K⁺ efflux assay. Strains Ps33/pHERD30T, Ps33(Ps56)/pHERD30T, and Ps33/pHERD30T/ORFs_10_9 were grown in LB with gentamicin to an OD_{600} of 0.5 and collected by centrifugation. The pellets were washed twice with 1 ml SM buffer and resuspended in 100 μ l of the same buffer. The samples were infected with the virulent phage PaMx53 at an MOI of 10. All the mixtures were divided into two aliquots; one was immediately centrifuged (0 min), and the other was incubated at 37°C for 30 min and then centrifuged. The pellets and supernatants were brought to a volume of 1 ml with SM buffer and boiled in water for 20 min. Finally, SM buffer was added to a final volume of 2 ml before results were read in a Zeiss PF5 flame photometer. The sum of the pellet and supernatant K⁺ readings for each sample was considered to be 100%. The K⁺ efflux percentage for the infection conditions was obtained by subtracting the amount of K⁺ leaked from uninfected cells from the total K⁺ leaked after infection. K⁺ efflux assays were performed in triplicate.

Statistical analysis. To calculate the statistical differences among the tested conditions, one-way analysis of variance (ANOVA) with Bonferroni's posttest was performed using the GraphPad Prism 5 software.

Data availability. The genome sequences of the seven phages and strain Ps33 reported in this work have been deposited in GenBank. The accession numbers of the phage genomes are as follows: Fc02, [MH719189](#); Fc22, [MH719191](#); Ps56, [MH719192](#); Ps59, [MH719195](#); Ps60, [MH719194](#); H71, [MH719190](#); and

H72, [MH719193](#). The assembly of the Ps33 strain genome is available under BioProject accession number [PRJNA573525](#), BioSample accession number [SAMN12815552](#), and GenBank accession number [CP044533](#).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.8 MB.

ACKNOWLEDGMENTS

This work was supported by grants from the Fondo de Investigación Científica y Desarrollo Tecnológico del CINVESTAV (project no. 37) and the Consejo Nacional de Ciencia y Tecnología-Ciencia Básica (CONACyT-CB, 255255). P.V. acknowledges financial support from DGAPA-PAPIIT-UNAM (grant IN206318). M.A.C.-O. (CVU 374616) was awarded a fellowship from CONACyT.

We thank Lourdes Rojas-Morales (UME, LaNSE-CINVESTAV) for technical assistance with transmission electron microscopy, María Guadalupe Aguilar González (Unidad de Ácidos Nucleicos, DGBM) and Dulce Delgadillo Álvarez (LaNSE-CINVESTAV) for technical support in sequencing the cloned ORFs, Víctor Flores and Otoniel Maya-Lucas for help with the preliminary assembly of the phage genomes, Diana Joancy Muñoz for help with the infection assays, Daniel Cazares (CCG, UNAM) for the isolation of genomic DNA from strain Ps33, and Fanis Missirlis (DFBN, CINVESTAV), Antonio Peña-Díaz, and Martha Calahorra (IFC, UNAM) for advice and assistance in K⁺ efflux assays. The biological material was provided by the following researchers: bacteriophage JBD18 by Alan R. Davidson (University of Toronto, Canada), plasmids pSEVA541 and pSEVA658 by Esteban Martínez-García (Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas, Spain), strain PAO1 by Sergio Vaca-Pacheco (Facultad de Estudios Superiores Iztacala, UNAM), strains Fc1, Fc11, Ps19, Ps33, Ps34, and Ps42 by Víctor Rafael Coria Jiménez and Patricia Arzate-Barbosa (Instituto Nacional de Pediatría), and strains H7 and H11 by Carlos A. Eslava Campos (Facultad de Medicina, UNAM).

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