RESEARCH ARTICLE

The Widespread Multidrug-Resistant Serotype O12 *Pseudomonas aeruginosa* Clone Emerged through Concomitant Horizontal Transfer of Serotype Antigen and Antibiotic Resistance Gene Clusters

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ABSTRACT **The O-specific antigen (OSA) in *Pseudomonas aeruginosa* lipopolysaccharide is highly varied by sugar identity, side chains, and bond between O-repeats. These differences classified *P. aeruginosa* into 20 distinct serotypes. In the past few decades, O12 has emerged as the predominant serotype in clinical settings and outbreaks. These serotype O12 isolates exhibit high levels of resistance to various classes of antibiotics. Here, we explore how the *P. aeruginosa* OSA biosynthesis gene clusters evolve in the population by investigating the association between the phylogenetic relationships among 83 *P. aeruginosa* strains and their serotypes. While most serotypes were closely linked to the core genome phylogeny, we observed horizontal exchange of OSA biosynthesis genes among phylogenetically distinct *P. aeruginosa* strains. Specifically, we identified a "serotype island" ranging from 62 kb to 185 kb containing the *P. aeruginosa* O12 OSA gene cluster, an antibiotic resistance determinant (*gyrA*^{C248T}), and other genes that have been transferred between *P. aeruginosa* strains with distinct core genome architectures. We showed that these genes were likely acquired from an O12 serotype strain that is closely related to *P. aeruginosa* PA7. Acquisition and recombination of the "serotype island" resulted in displacement of the native OSA gene cluster and expression of the O12 serotype in the recipients. Serotype switching by recombination has apparently occurred multiple times involving bacteria of various genomic backgrounds. In conclusion, serotype switching in combination with acquisition of an antibiotic resistance determinant most likely contributed to the dissemination of the O12 serotype in clinical settings.**

IMPORTANCE **Infection rates in hospital settings by multidrug-resistant (MDR) *Pseudomonas aeruginosa* clones have increased during the past decades, and serotype O12 is predominant among these epidemic strains. It is not known why the MDR phenotype is associated with serotype O12 and how this clone type has emerged. This study shows that evolution of MDR O12 strains involved a switch from an ancestral O4 serotype to O12. Serotype switching was the result of horizontal transfer and genetic recombination of lipopolysaccharide (LPS) biosynthesis genes originating from an MDR taxonomic outlier *P. aeruginosa* strain. Moreover, the recombination event also resulted in acquisition of antibiotic resistance genes. These results impact on our understanding of MDR outbreak strain and serotype evolution and can potentially assist in better monitoring and prevention.**

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Lipopolysaccharide (LPS) of the Gram-negative cell envelope is a first point of contact with the external environment, and it is a major virulence factor of *Pseudomonas aeruginosa*. LPS has been reported to be a target for antibiotics, bacteriophages, pyocins, and other extracellular molecules affecting the cell (1–5). Hence, we hypothesize that there is selective pressure on LPS and related biosynthesis in order to adapt to the external environment. Evidence that would support this hypothesis include the observation of increased mutational frequency in single LPS-related genes, such as *wbpM* (encoding a dehydratase and key enzyme in the biosynthesis of O-specific antigen [OSA]) and *waaL* (O-antigen ligase), and the contribution of phosphorylation in the core oligosaccharide toward intrinsic resistance of *P. aeruginosa* to antibiotics (1, 6, 7).

P. aeruginosa simultaneously produces two forms of LPS that differ in their O antigen, named common polysaccharide antigen (CPA) and O-specific antigen (OSA). CPA is a shorter and less immunogenic polysaccharide than OSA, yet CPA has been linked to chronic lung infections in patients infected with *P. aeruginosa* (8, 9). CPA biosynthesis is orchestrated by a cluster of 13 contiguous genes (PA5447 to PA5459) as well as *algC* (PA5322) (8, 10, 11). However, CPA production has been detected in only 14 of the

20 International Antigenic Typing Scheme (IATS) serotypes (not produced in serotypes O7, O12, O13, O14, O15, and O16) (12, 13).

Unlike CPA, OSA is constitutively produced by all strains of *P. aeruginosa* and is highly diverse. The differences that arise from inter- or intramolecular bond orientation, O-repeat lengths, and side groups allow *P. aeruginosa* to be classified into 20 distinct serotypes (O1 to O20) in the IATS (14–16). The OSA clusters of the 20 serotypes can be divided into 11 highly divergent gene clusters with variation in genetic contents (17). The localization of the cluster in the *P. aeruginosa* genome is nonetheless conserved and clearly marked by its being flanked by the *himD* (also called *ihfB*) (PA3161) and *wbpM* (PA3141) genes (18–20). The high diversity of the LPS cluster makes it difficult to investigate whether the LPS-OSA genes are involved in adaptation, as genes in this region will be neglected in the algorithms used for conventional analysis of conserved genes from the core genome.

Population studies of *P. aeruginosa* have revealed the dynamics of the serotyping characteristics. As a case in point, a recent study showed that 35% of the *P. aeruginosa* isolates could not be serotyped, particularly among cystic fibrosis (CF) isolates, whereby 33/43 strains examined were nontypeable using a commercial typing kit (21). Another study that included 145 isolates found that 9.6% could not be serotyped, with 11 being polytypeable and 3 being nontypeable. Of the isolates that were assigned specific serotypes, serotype O11 was the most prevalent in population studies (representing 20.1 to 35.1%), followed by O6 (13.1 to 14.2%), O1 (11 to 11.9%), and O12 (6.2 to 7.9%). The relatively high frequency of O12 might be caused by sample bias, due to the prevalence of O12 strains with many hospital outbreaks of *P. aeruginosa* infections. Nonetheless, the worldwide occurrence of persistent MDR multilocus sequence type 111 (ST111) clones and known O12 strains, provides the rationale for investigating whether there is a correlation between a specific serotype and MDR strains in this species (21–25). In comparison, a study involving non-CF clinical isolates showed a greater variation of serotypes, with the most prevalent serotypes being O11 (26.8%) and O12 (16.2%), both of which are known to be associated with a higher frequency of MDR clones (26).

Our understanding of the relationship between serotype and the properties of an isolate is limited, and analysis of genomic diversity of the serotypes has not been performed. Such an analysis will be affected by the sequence diversity of the *P. aeruginosa* genome being very low both on a local and global scale (27, 28), and some distinct LPS genes may be located in the pangenome of *P. aeruginosa*. The sequence diversity in conserved genes has been found to be 0.3%, making it 1 order of magnitude lower than those in organisms such as *Salmonella*. Despite studies designed for identifying niche-specific clones within the *P. aeruginosa* species, no link has been identified between ecological setting and genotype. The same clones have been isolated across a wide range of unrelated habitats with the same variant being able to predominate in several unrelated niches (27, 28).

To gain further knowledge of the diversity of serotypes among *P. aeruginosa* bacteria, this study uses comparative genomics to investigate the genomic evolution of the *P. aeruginosa* population in relation to LPS structure and determine whether LPS structures and serotype play significant roles in the evolutionary trajectory of *P. aeruginosa* clones.

RESULTS

***In silico* serotyping of *P. aeruginosa* isolates.** To investigate the genomic structure of LPS biosynthesis genes in the *P. aeruginosa* population, the OSA and CPA gene clusters were analyzed in 83 *P. aeruginosa* isolates (see Table S1 in the supplemental material). These isolates include the 20 IATS serotype strains (O1 to O20) and 10 published isolates from a range of environments or hospital outbreaks, including serotype O12 and nontypeable isolates (21). A group of 52 isolates previously used by Stewart et al. (29) for investigation of the *P. aeruginosa* population structure were used as representatives for a wide range of environments and times (Table S1). In addition, a known O12 MDR transmissible ST111 isolate was included as a control together with a genetically distinct O12 isolate from the PA7 group (included in the population used by Stewart et al. [29]). For all the isolates, the CPA and OSA gene clusters were identified and annotated based on whole-genome annotation using the RAST server (30). The gene clusters were then compared across isolates to define subgroups among the population. The CPA cluster structure was found to be relatively conserved among the 20 IATS serotypes, while the OSA cluster demonstrated a high degree of diversity as anticipated (17). Fourteen of the 20 serotypes share the same organization in the CPA gene cluster, and the strains that differ from this group do so by divergence in single genes. The only exception is the O12 IATS strain, which lacks the operon of five genes (PA5455 to PA5459) that is contiguous to the eight-gene cluster (see Fig. S1 in the supplemental material).

The variations in the *P. aeruginosa* OSA gene cluster structure were outlined by Raymond et al. (17) and showed 11 distinct OSA cluster structures among the 20 IATS serotypes. In the present study, the observed OSA cluster structures were confirmed, and these distinct gene clusters were used for determining the genetic serotype of all the isolates (see Table S1 in the supplemental material). This *in silico* serotyping revealed 22 serotype O6 isolates, 14 O2+ (which comprises O2, O5, O16, O18, and O20) isolates, 8 O11/O17 isolates, 7 O12 isolates, 6 O10/O19 isolates, 5 O1 isolates, 4 O3 isolates, 3 O4 isolates, 2 O13/14 (only the type strains) isolates, and 1 O15 (only the IATS) isolate. Four isolates were found to be nontypeable by this approach, meaning that a single definitive hit to an entire OSA cluster could not be identified (Table S1). As such, all except three serotypes were represented among the analyzed isolates, and over half of the population was represented by 8 of the 20 serotypes. The most predominant serotype is O6 (~27%), which is consistent with observations reported in other population studies (21).

Four CF isolates described as nontypeable by Pirnay et al. (21) were also examined. These isolates were serotyped *in silico*, and it was found that one isolate belonged to the O9 *in silico* serotype (PR42), and the remaining three belonged to the O6 *in silico* serotype (PR195, PR196, and Li010). For the remaining six isolates from the Pirnay collection, four were identified as O12 serotype isolates (O12-4, O12-17, O12-1709, and PR317 isolates), and the remaining two were found to be O2+ (PR276) and O11 (PR233) (see Fig. S1A and S1B in the supplemental material).

Genomic evolution of *P. aeruginosa* is linked to serotype. To correlate the *in silico* serotypes with the genomic architectures of the *P. aeruginosa* population, a maximum likelihood phylogeny based on single nucleotide polymorphisms (SNPs) identified in the core genome was constructed (Fig. 1). The phylogenetic orga-

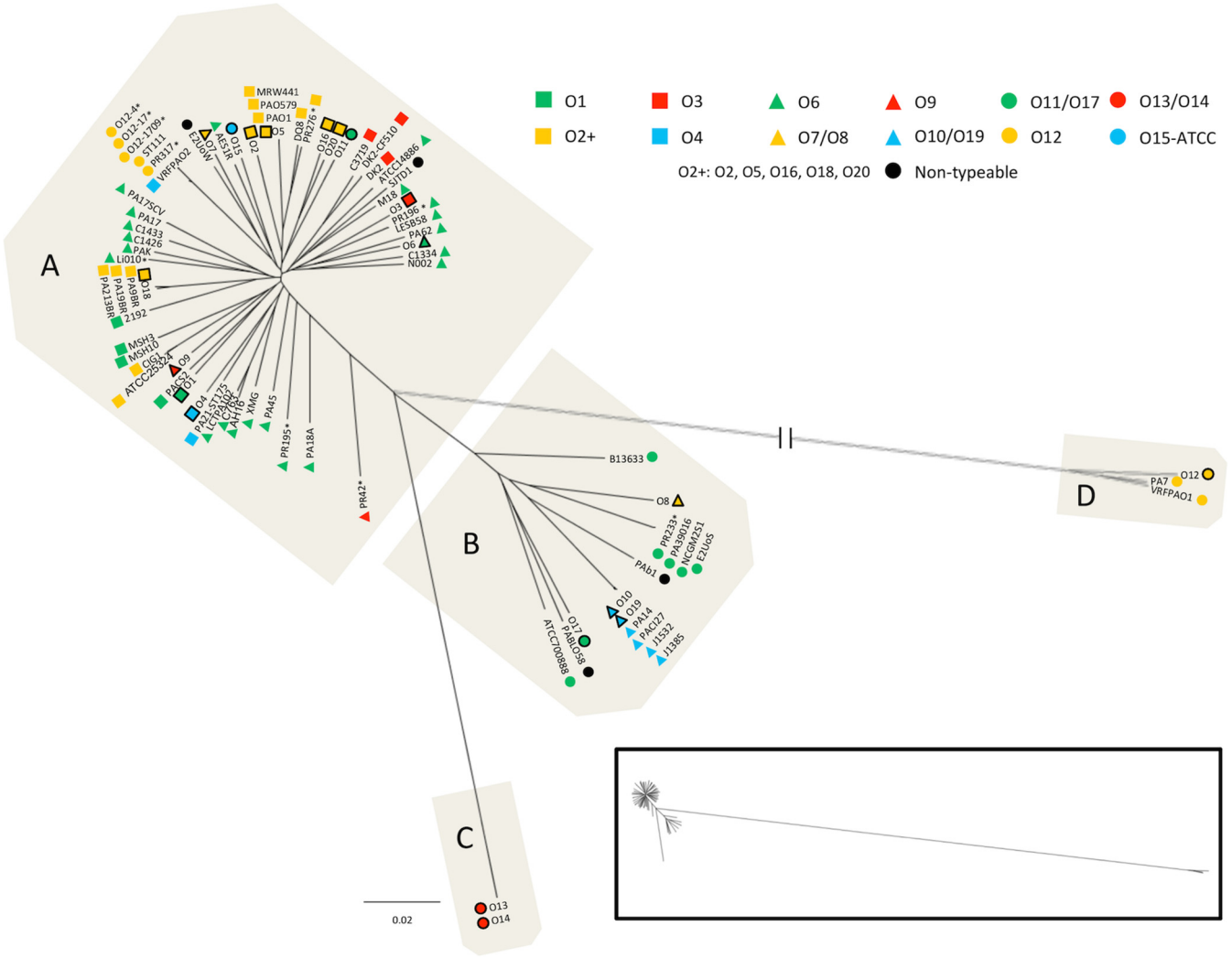

FIG 1 Maximum likelihood phylogeny based on SNPs in the core genomes of 81 *P. aeruginosa* isolates. The phylogeny was estimated using the parsnp software program (45). All isolates are marked according to their *in silico* serotype by the colors and shapes outlined in the key. For example, isolates marked with a green box have been typed to an O1 *in silico* serotype. The groups referred to in the article are shaded and named groups A, B, C, and D. IATS serotype reference isolates are indicated by a black line outlining the *in silico* serotype symbol. The tree is represented with the branch for group D shown to scale in the black box or inset. Strains marked by an asterisk represent the 10 isolates sequenced in this study from the Pirnay collection (21). The scale bar indicates the relative distances of the branches in the phylogenetic tree.

nization shows four distinct groups, groups A to D (Fig. 1). The majority of the isolates analyzed (77/83) fall into two groups, a major one (group A), and a minor one (group B) (Fig. 1). Furthermore, a very distantly related group containing the O12 IATS serotype isolates PA7 and VRFPAO1 was found (group D in Fig. 1). Most of the strains examined could be distributed into three phylogenetic groups; this corresponds well with the structure of the core genome phylogeny reported by Stewart et al. (29). Interestingly, an additional group closely related to group A and group B was also identified, and this new group was populated by two isolates belonging to O13 and O14 IATS serotypes (group C in Fig. 1). The 20 IATS serotype strains analyzed were distributed across the phylogeny (Fig. 1). Projection of the *in silico* serotypes of all the sequenced *P. aeruginosa* isolates back onto the maximum likelihood phylogeny revealed that within the four phylogenetic groups, there was often a clear clustering of the isolates sharing *in silico* serotype with their corresponding IATS serotype isolate (Fig. 1). This was observed for the O10/O19, O2+, and O1 *in silico* serotype isolates (Fig. 1). On the other hand, the O6 *in silico* serotype isolates were distributed across group A of the maximum likelihood phylogeny, while the O11 *in silico* serotype isolates were found within group B (Fig. 1). In most cases, a certain serotype found in one phylogenetic group was not found in others. However, there are exceptions, and deviations from this trend include the IATS serotype pairs O7/O8 and O11/O17. Each pair of these serotypes share highly similar OSA gene structures, suggesting that each pair shares a common ancestor. Nevertheless, for each of the two pairs, we found one isolate in group A and the other in group B of the maximum likelihood phylogeny (Fig. 1).

It was intriguing to observe that the seven O12 *in silico* serotype strains identified among the 83 isolates were located in two distinct groups in the maximum likelihood phylogeny (Fig. 1). The O12 IATS serotype strain was grouped with strains PA7 and VRFPAO1, thus defining group D of the phylogeny, while the other

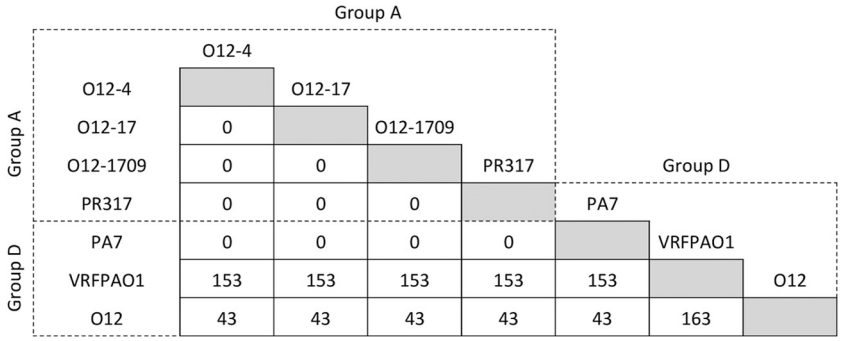

FIG 2 Sequence homology matrix of the serotype O12 OSA gene cluster showing the number of sequence differences. Identity is determined based on an ungapped sequence alignment. Isolates from group A and group D are marked in the figure for comparison.

five O12 strains, ST111, O12-4, O12-17, O12-1709, and PR317, were placed together on a branch in group A. Based on their genomic structures, these five strains were most related to the VRFPAO2 strain, which was determined to be an O4 *in silico* serotype (Fig. 1). These results demonstrate that strains with similar genomic architectures can exhibit different serotypes (e.g., ST111 versus VRFPAO2) and point toward a potential role for horizontal transfer of serotype-defining genes.

Horizontal gene transfer drives serotype switch to the O12 serotype. To test the hypothesis that the serotype O12 OSA gene cluster has been horizontally transferred among distinct clone types, we first examined the relationship between the OSA genes from the different O12 *in silico* serotype isolates. No sequence variations in the OSA gene cluster among the O12 group A isolates could be discerned (Fig. 2). Importantly, the genes within the OSA cluster of these O12 group A isolates were identical to those found in isolate PA7 from group D (Fig. 2).

To investigate whether horizontally transferred DNA extends beyond the OSA gene cluster, we characterized the genomic differences between the serotype O12 strains from groups A and D. Inspection of the number of SNPs in the core genomes in all seven O12 *in silico* serotype isolates revealed that the three group D isolates differed from group A isolates by >200,000 SNPs. The number of SNPs was estimated relative to the core genome of the O12-4 isolates, including O12-17 (30 SNPs), O12-1709 (41 SNPs), ST111 (85 SNPs), PR317 (190 SNPs), VRFPAO2 (2343), PA7 (201.599 SNPs), VRFPAO1 (202.507 SNPs), and O12 (204.506) (see Fig. S2 in the supplemental material). With such a high number of SNPs in their core genome, the mean sequence identity between isolates from each of the two clusters would not be expected to exceed 97%. The regions flanking the OSA gene cluster in the five group A isolates were examined for sequence identity with isolate PA7. On the basis of these analyses, we identified an ~70-kb genomic region that showed close to 99% identity with the same region in PA7, whereas the flanking regions showed only 93 to 94% identity (Fig. 3A). The ~25-kb OSA gene cluster was localized centrally in the PA7-like genomic region (Fig. 3A). In summary, these results demonstrate that a large genomic region en-

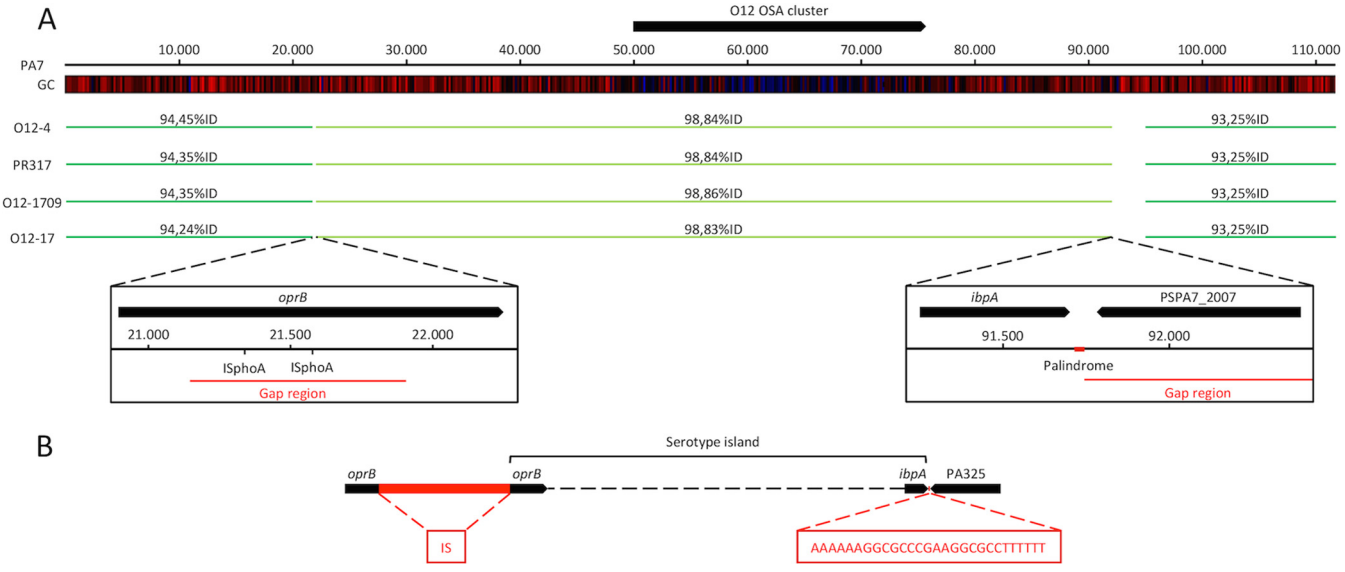

FIG 3 Outline of the genomic region in isolate PA7 containing the serotype island. (A) The PA7 backbone is shown with the corresponding GC skew and placement of the O12 OSA cluster. Below the PA7 backbone, the size and identity (percent identity [%ID]) of the sequence from the four O12 *in silico* serotype isolates and a higher-magnification view into the two gap regions, where the serotype island is proposed to have inserted into the genome, are depicted. (B) The proposed architecture of the serotype island in the O12 serotype-switched isolates is depicted with the recombination sites highlighted.

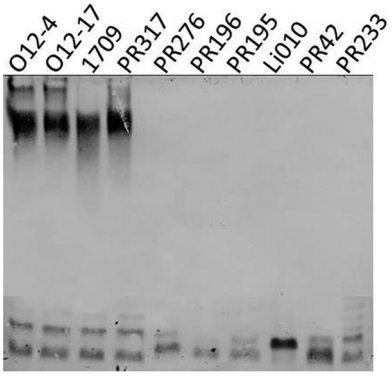

FIG 4 Serotype screen of *P. aeruginosa* isolates using *in vitro* methods. Western immunoblotting probed with MAbs specific to the O12 OSA (MF35-4) and inner core D (5c7-4). The O12 OSA antibody reacts strongly in the lanes of the identified O12 isolates. The appearance of the inner core MAb reactivity demonstrates equal loads for all samples.

compassing the OSA genes has been horizontally transferred from a member of the PA7 group to a serotype O4 strain, which has evolved into the group represented by the five O12 isolates in group A (ST111, O12-4, O12-17, O12-1709, and PR317 isolates). Acquisition and recombination of the "serotype island" resulted in displacement of the native OSA gene cluster in the recipient strain.

When further analyzing the breakpoint regions of the ~70-kb "serotype island" sequence, we identified multiple IS insertion sites in the upstream region of the island (Fig. 3A). The potential downstream breakpoint coincided with an intergenic palindrome sequence found in all *P. aeruginosa* isolates on the *Pseudomonas* Genome Database (31) (Fig. 3A). Both of these sequence motifs have been reported to be prone to homologous recombination. A closer examination of the flanking sequences in four of the five serotype-switched O12 isolates (O12-4, O12-17, O12-1709, and PR317) revealed that they are identical and all have the serotype island inserted in the *oprB* gene upstream and at an intergenic palindrome between *ibpA* and PA325 downstream (Fig. 3B). Due to a reduced sequencing quality of ST111, that particular O12 isolate could not be included in this analysis.

Genetic and immunochemical screens of LPS characteristics of O12 isolates. To determine whether the OSA structure is expressed on the cell surfaces of these isolates, genetic and immunochemical experiments were performed. The OSA cluster of each of the isolates, O12-4, O12-17, O12-1709, and PR317 isolates, and an IATS O12 control strain was amplified using O12-specific primers, yielding a single band at 0.8 kb. In contrast, the OSA clusters of non-O12 isolates PR276, PR196, PR195, LiO10, PR42, and PR233 did not amplify (see Fig. S3A in the supplemental material). To screen the LPS produced on the cell surfaces of these *P. aeruginosa* isolates, LPS prepared from each of the O12 isolates as well as that from the O12 control were subjected to analysis by Western immunoblotting. The monoclonal antibody (MAb) specific to serotype O12 (MF35-4) (32) reacted to LPS prepared from isolates O12, O12-4, O12-7, O12-1709, and PR317 (Fig. 4). To confirm the total absence of O4 serotype genes, the candidate O12 samples were probed with a MAb specific to serotype O4 (MF31-4), resulting in no discernible signal (see Fig. S3C in the supplemental material). To standardize the sample loading, an inner-core-specific MAb (5c7-4) was used, and it recognized a low-molecular-weight LPS band near the dye front of the gel and blot, and the intensity of this band was consistent among the samples, regardless of whether the OSA was expressed or not (Fig. 4).

Annotation of the "serotype island." The number of new genes transferred via insertion of the "serotype island" was evaluated by annotation of the island (based on the annotation available from isolate PA7) and identification of orthologous genes (see Table S2 in the supplemental material). The "serotype island" comprises 63 genes (PSPA7_1944 to PSPA7_2005), including *gyrA* and three genes encoding an efflux pump of unknown function (PSPA7_1991 to PSPA7_1993). Interestingly, isolate PA7 contains a *gyrA*^{C248T} (C-to-T change at position 248 encoded by *gyrA*) allele known to contribute to the high level of ciprofloxacin resistance observed in this strain (MIC of>128 μg/ml [33]). All five O12 serotype isolates in group A (ST111, O12-4, O12-17, O12-1709, and PR317) contained this C248T mutation in their respective *gyrA* gene, and these five isolates share a near-complete sequence identity with *gyrA* from isolate PA7, therefore substantiating the acquisition of this island from a PA7-like isolate (Fig. 5). In addition, the *gyrA* genes in these five isolates share high

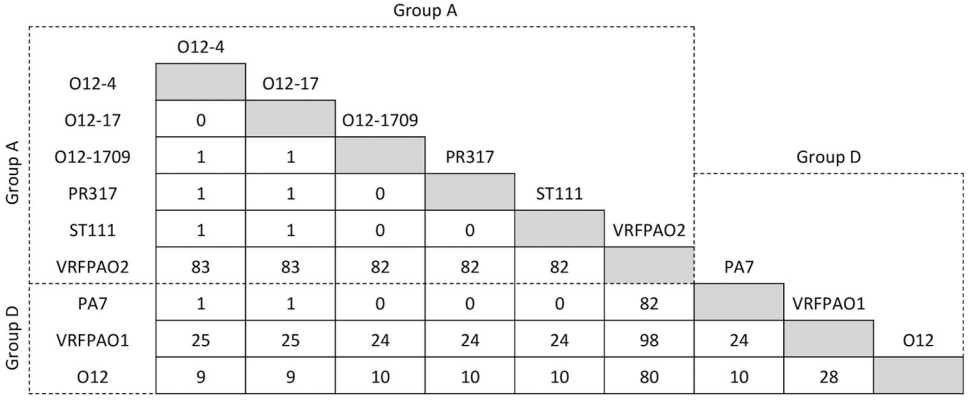

FIG 5 Sequence homology matrix of the *gyrA* gene showing the number of sequence differences. Isolates originating from group A (serotype switched) and group D (PA7 like) are shown in the table. All included isolates are of the O12 *in silico* serotype except for isolate VRFPAO2 (O4 *in silico* serotype) from group A.

or complete sequence identity with the *gyrA* gene from isolate PA7, further substantiating the fact that the island has been acquired from a PA7-like isolate (Fig. 5). In contrast, the other O12 isolates of group D (IATS O12 and VRFPAO1) and the O4 strain from group A (VRFPAO2) are devoid of the C284T mutation. The replacement of the native *gyrA* allele with $gyrA^{C248T}$ correlates well with elevated ciprofloxacin resistance (MIC of >8 μg/ml) observed in ST111 (22). We note that isolate PA7 has a MIC of 128 μg/ml, implying that other factors might affect fluoroquinolone resistance in this isolate. The five O12 serotype-switched strains were isolated between 1988 and 2011, which is the period after ciprofloxacin was introduced into the market to treat bacterial infections (Fig. 6).

Serotype-switched clones dominate the O12 population. A recent survey (34) of 390 genomes of clinical *P. aeruginosa* isolates sampled between 2003 and 2012 from diverse geographical locations enabled us to inspect additional serotype O12 genomes and to further assess whether serotype switching in combination with acquisition of the particular $gyrA^{C248T}$ allele is important for bacterial fitness and dissemination. Our hypothesis is that the global serotype O12 population would contain a larger proportion of O12 serotype-switched isolates relative to the "native" PA7-like serotype O12 strains. A recent study by Kos et al. (34) identified a total of 32 O12 isolates out of 390 sequenced genomes. Among these O12 isolates, 20 were of the MLST ST111, and five isolates were MLST ST244. The remaining seven isolates belonged to disparate MLSTs, i.e., ST597, ST292, ST1721, ST1722, ST1762, ST1747, and ST1120 (Fig. 6A). All 20 ST111 isolates were able to be grouped with the five previously described O12 serotype-switched isolates as well as the O4 isolate VRFPAO2 in the maximum likelihood phylogeny (Fig. 6A). Significantly, we also found that all 20 ST111 isolates harbored the same 70,000-bp "serotype island" described above. Intriguingly, of the 32 O12 isolates from the study of Kos et al. (34), only 2 isolates were found to cluster with the outliers PA7, VRFPAO1, and the O12 IATS isolate in group D of the maximum likelihood phylogeny. The remaining 10 O12 isolates were placed within or near groups A and B, and as such, they are also distantly related to isolates from group D (Fig. 6A). Inspection of the OSA clusters in these 10 isolates revealed that serotype switching had also occurred in these isolates, but in 8 of 10 strains, the "serotype islands" differed in size from the 70 kbp observed in the ST111 isolates. These islands all contained the serotype O12-defining genes and the $gyrA^{C248T}$ allele but ranged in size from ~62,000 bp to ~185,000 bp (Fig. 6B). Overall, our analysis showed that 35 out of 40 O12 genomes that have been examined are from serotype-switched isolates and that serotype switching to the O12 serotype occurred on multiple occasions involving recipient strains of various sequence types.

DISCUSSION

Acquisition of genes encoding antibiotic resistance determinants and virulence factors through horizontal gene transfer is an important mechanism in the evolution of pathogenic bacteria. LPS is a major virulence factor in *P. aeruginosa*, and in this study, we demonstrate that horizontal exchange of LPS biosynthesis genes among *P. aeruginosa* strains has contributed to the dissemination of the O12 serotype. In the primary case of this study, we document the presence of a 70-kb "serotype island" containing the *P. aeruginosa* OSA gene cluster, an antibiotic resistance determinant ($gyrA^{C248T}$) and a number of other genes originating from the O12 serotype strain PA7 in the genomes of five isolates with a core genome architecture distinct from that of PA7. Interestingly, the genome structures of the five isolates cluster together with strain VRFPAO2 (belonging to O4 *in silico* serotype), and these six strains are all of the sequence type ST111 (Fig. 1). This evidence suggests that the original recipient was an O4 serotype and that uptake and recombination of the O12 serotype-defining OSA gene cluster from a PA7-like strain accounted for serotype switching from O4 to O12. This result was further supported by analysis of 20 additional ST111 serotype O12 isolates that were all found to contain an identical 70-kb "serotype island." This strongly suggests that the majority of O12 serotype-switched ST111 strains in the serotype O12 population (25 out of 40) is the consequence of a single transfer and recombination event followed by clonal expansion.

MDR serotype O12 strains are increasingly widespread in hospital settings across Europe, in particular those with the multilocus sequence type ST111 (21, 22, 35, 36). In the early 1980s, serotype O12 strains were relatively rare in clinical infections, and they were also not associated with MDR. However, from the mid-1980s and onward, a number of studies have reported increased frequencies of MDR O12 strains in patients infected with *P. aeruginosa* (37, 38). It has previously been suggested that this strain population had a clonal origin (24, 25). A recent systematic study of 328 *P. aeruginosa* isolates sampled over 125 years from different countries further supported the model that this clone type emerged very recently (in the 1980s) and has since rapidly disseminated in the hospital environment (21). Despite these observations, the origin and evolution of this clone type are not well understood. Our results show that evolution of the widespread MDR O12 ST111 clone type involved serotype switching from O4 to O12 in combination with acquisition of the particular $gyrA^{C248T}$ allele and that this has been a component of the ecological success of the clone type in relation to infection and dissemination in the human population in Europe.

We also found evidence that serotype switching to the O12 serotype occurred on multiple occasions involving recipient strains of sequence types other than ST111 (Fig. 6). At least four distinct recombination events that resulted in serotype switching to O12 could be identified, and in these cases, the implicated serotype islands were of different sizes than the 70-kb island identified in ST111. Interestingly, these genomic events appear to be geographically restricted, as the 25 serotype-switched ST111 strains were all isolated from Europe and the Americas between 1988 and 2012, while the other serotype-switched strains came from the Americas and/or Asia (Fig. 6). It is clear that sampling size and bias can influence this observation, and analyses of additional strains are required to establish firm conclusions about potential geographical distributions of these events. Importantly, the finding of multiple, independent recombination events provide strong evidence for a selective advantage for this type of genomic recombination.

The precise selective advantage of serotype switching is not known, and this should be addressed in future investigations. We speculate that acquisition of the "serotype island" might have allowed the bacteria to evade the host immune system and the antibodies produced against the previous serotype or to become resistant to bacteriophages (39). A related phenomenon of serotype recombination that confers a fitness advantage in infections has been reported for *Streptococcus pneumoniae* vaccine escape vari-

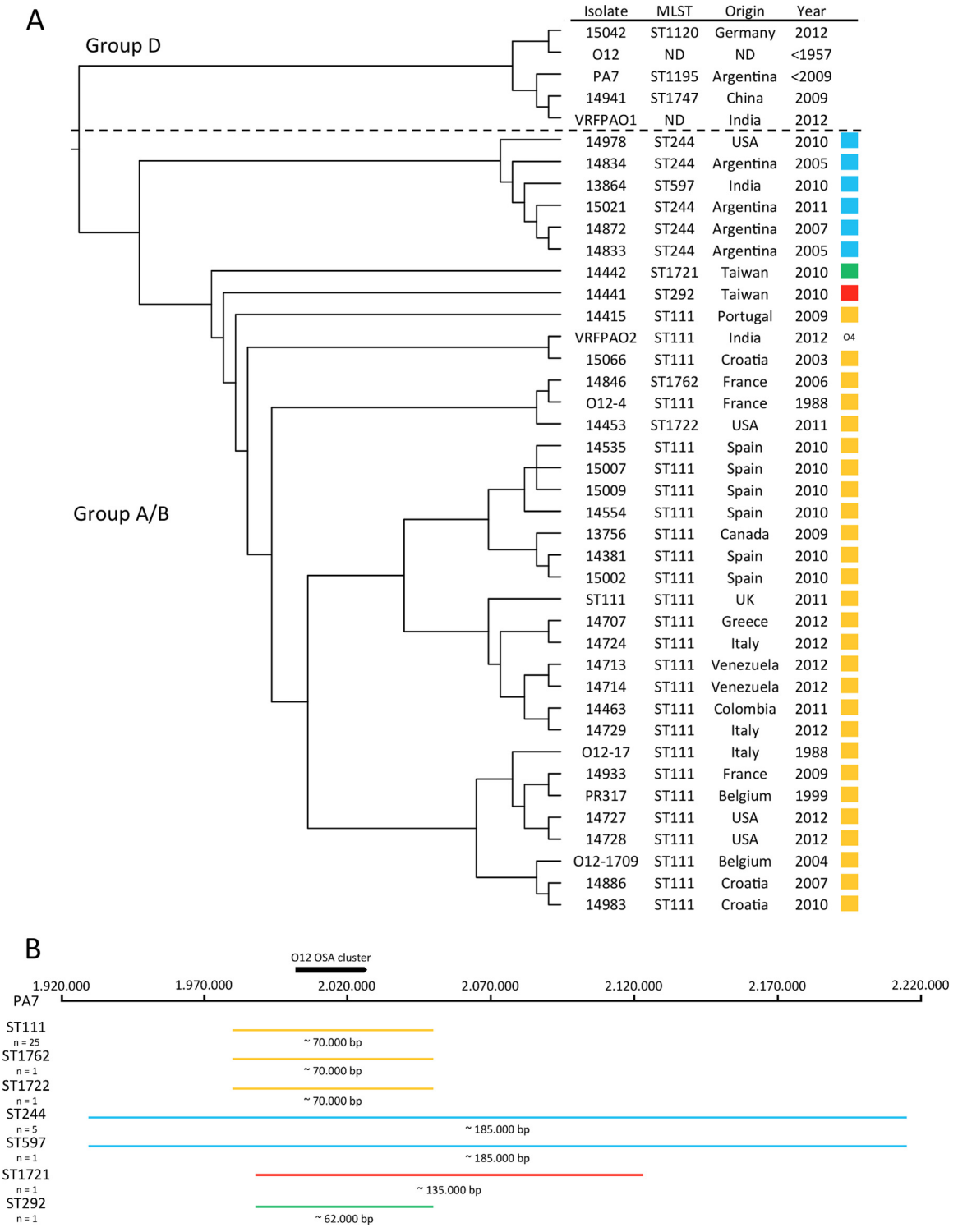

FIG 6 Characteristics and distribution of the O12 population in relation to the maximum likelihood core genome phylogeny. (A) The maximum likelihood phylogeny was constructed using the parsnp (49) software program and is represented as a proportional tree. The tree includes 32 O12 isolates described by Kos et al. (34). These isolates are named according to their identification (ID) number, i.e., isolate 15042 has the original ID AZPAE15042. Also included are the eight O12 isolates described in the present study. The multilocus sequence type (MLST) and country and year of isolation (origin and year) for each isolate are shown. The nature of the "serotype island" (SI) in each of the isolates is indicated by a colored square; blue is a SI of ca. 185 kb, red is a SI of ca. 135 kb, yellow is a SI of ca. 70 kb, and green is a SI of ca. 62 kb. (B) Schematic overview of the size and position of the different "serotype islands" in relation to the PA7 genome. The color scheme is the same as in panel A, and the number of MLST types containing the different islands is shown.

ants (40). The process of serotype switching by recombination has not previously been reported for *P. aeruginosa*. It is a completely different mechanism compared to serotype switching in *P. aeruginosa* due to D3 bacteriophage lysogeny reported previously (41), whereby the switch from *P. aeruginosa* serotype O5 to O16 was mediated by phage-derived elements, including an inhibitor peptide called Iap (inhibitor of cognate O-antigen polymerase Wzy_{α}) and an O-antigen polymerase, Wzy_{β}, that changes the bond between O units from α to β.

To verify that the O12 OSA cluster was not only acquired but also expressed in the isolates, *in vitro* experiments, including PCR amplification and immunochemical studies, were undertaken. A previous study by Raymond et al. (17) has shown that OSA-specific primers can be generated to probe specific genes present within each serotype cluster. However, a set of O12-specific primers was not reported in their study; therefore, we followed their method and designed the primers specific to a region within the O12 OSA cluster. The primers were deemed specific against O12 strains because amplification of LPS genes was observed only against chromosomal DNA prepared from O12 strains but not against DNA prepared from other serotypes (see Fig. S3A in the supplemental material). To substantiate the PCR amplification results, we also showed that the acquired O12 OSA cluster is actively being expressed as shown by LPS from these strains reacting with the O12-specific MAb MF35-4 in Western blots. The presence of high-molecular-weight bands in the blots of LPS samples prepared from the group of O12 *in silico* serotypes revealed that O12 LPS is indeed constitutively expressed among these isolates. The expression of this OSA by non-O12 genetic isolates demonstrates a selective pressure for this specific repeat.

Although the precise mechanism of transfer of the 70-kb "serotype island" remains unknown, frequent recombination is a well-documented phenomenon in *P. aeruginosa* (6, 26, 42). Nevertheless, our finding of multiple events of transfer and recombination of large DNA fragments ranging in size from ~62,000 bp to ~185,000 bp is unusual. Our identification of recombination between O12 and non-O12 genetic isolates (e.g., between group A and D strains in Fig. 1) was enabled by our *in silico* serotyping approach and the large sequence differences that exist between these isolates. Interestingly, we note that serotypes other than O12 (such as O7/O8 and O11/O17) also exhibit varied distributions in the phylogenetic tree, and may thus represent related examples of serotype switching by recombination. Furthermore, Dettman et al. (43) identified the *wbpM* gene (which is conserved at the 3′ end of all OSA gene clusters) as a region within the *P. aeruginosa* core genome that exhibits a high recombination rate. While these observations are suggestive of additional examples of serotype recombination, sequencing of a larger panel of *P. aeruginosa* genomes is a logical approach to further our understanding of how frequent serotype recombination takes place and if this phenomenon is relevant for serotypes other than O12. This study is an important step in understanding *P. aeruginosa* distribution and survival, ushering in a new form of serotype identification which could allow better monitoring of outbreaks and tailored treatments of infection.

MATERIALS AND METHODS

Bacterial isolates and genome sequencing. This study analyzes 83 *P. aeruginosa* isolates, 30 of which were sequenced as part of the present work (see Table S1 in the supplemental material). Of the isolates included in this study, 52 isolates were originally sequenced in a previous study (it was not possible to obtain data for isolates 138244, 152504, and NCMG1179) (29). The 20 standard IATS serotype type strains for serotypes O1 to O20 were described earlier (32) and can be retrieved from ATCC: O1 to O17 (ATCC 33348 to ATCC 33364), O18 (ATCC 43390), and O19 and O20 (ATCC 43731 and ATCC 43732). Ten isolates were chosen from the Pirnay culture collection on the basis of these being from a range of locations, environments, and time points; this group included both serotype O12 and nontypeable isolates. A single known hospital outbreak strain of the O12 ST111 type was included (Table S1). The 10 isolates from the Pirnay collection and the serotype isolates O6 and O17 were sequenced at the Plateforme d'Analyses Génomiques (IBIS, Université Laval, Québec, Canada) on an Illumina MiSeq platform generating 300-bp paired-end reads, and the remaining serotype isolates (O1 to O5, O7 to O16, and O18 to O20) were sequenced on an Illumina HiSeq 2000 platform generating 100-bp paired-end reads by BGI Europe (Copenhagen, Denmark).

Construction of the core genome phylogeny. The 20 IATS serotype isolates and the 10 isolates from the Pirnay collection were assembled using the A5 Assembly Pipeline, and the assemblies ranged in scaffolds from 78 to 236 (44). Full assembly statistics are displayed in Table S3 of the supplemental material. The phylogeny was constructed using the parsnp program from the Harvest software suite (45), which defines the core genome across all isolates and builds the phylogeny by the maximum likelihood method using core SNPs across isolates.

Genetic serotyping and exploratory genome analysis. The OSA clusters of all 20 IATS serotype strains were identified and verified by multi-BLASTn analysis against the 21 published OSA clusters (17). Hence, the structure of the OSA gene clusters was confirmed, and it was verified that the chromosome of each isolate contained only a single OSA cluster. The CPA clusters of all 20 serotype isolates and the 10 isolates from the Pirnay collection were identified as marked by *wbpM* and *ihfB* (also called *himD*) as flanking genes of the lusters. The region within the OSA locus was then extracted and analyzed for all isolates. The 30 assemblies were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (30) in order to verify the genes in the OSA and CPA clusters.

Using the same multi-BLASTn approach as for OSA verification, the 10 serotype O12 or nontypeable (NT) isolates and 51 diverse environmental isolates were genetically serotyped based on the structure of their OSA cluster. If no hit covering an entire OSA cluster was found, the strain was deemed nontypeable.

***In vitro* serotyping through genetic screen and LPS analysis. (i) Bacterial growth conditions.** The isolates selected for *in vitro* serotyping are as follows: O12-4, O12-17, 1709, PR317, PR276, PR196, LiO10, PR195, PR42, and PR233. Overnight cultures of each isolate were grown in 5-ml lysogeny broth (LB) (also called Luria-Bertani broth) overnight with shaking (200 rpm) at 37°C. The following morning, each culture was separated: 1 ml was set aside for chromosomal DNA isolation, and the remainder was used for the LPS preparations.

(ii) Chromosomal DNA isolation. The chromosomal DNA was isolated through a combination of the Goldberg and Ohman method (46) and phenol-chloroform extraction (47). The intermediate aqueous phase was treated with 5 μl of 20 mg/ml RNase (Life Technologies) for 10 min at 37°C. RNase was removed with a final chloroform extraction. Primers specific to the O12 OSA cluster were designed following the method described by Raymond et al. (17). The serotype O12-specific primer set is as follows: 5′ ATGAAAAAAGTTTTGGTTACTGGG 3′ and 3′ CCCTCTCG AATCGAGTAGGTAGGCTC 5′. The OSA cluster of the candidate isolates and an O12 IATS control were amplified using the KOD Hot Start polymerase (Life Technologies) and separated by gel electrophoresis.

(iii) LPS extraction and serotyping using monoclonal antibodies. The overnight culture of each isolate and of an O12 IATS control were treated using the proteinase K Hitchcock and Brown method (48). Briefly, cells were equilibrated to an optical density at 600 nm (OD_{600}) of 0.45, resuspended in 250 μl of lysis buffer, boiled for 30 min, and treated with

2 mg/ml of proteinase K (Sigma Aldrich) overnight at 55°C. The LPS was resolved by electrophoresis on 12% SDS-polyacrylamide gels and visualized by ultrafast silver stain. Western immunoblotting was performed using monoclonal antibodies (MAbs) generated by the lab to probe for inner core (MAb 5c-7-4) and serotype O12 (MAb MF35-4). The secondary antibody was goat-anti-mouse Fab_2 conjugated to alkaline phosphatase (Cedarlane).

Sequence data. The assembled genomes of the 20 *P. aeruginosa* IATS serotype isolates which have been sequenced and assembled in this study are available in BioProject PRJNA294638 at NCBI. The 10 Pirnay collection isolates that have also been sequenced and assembled are available in BioProject PRJNA294726.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01396-15/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.9 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.04 MB.

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