



Application of Whole-Genome Sequencing Data for O-Specific Antigen Analysis and *In Silico* Serotyping of *Pseudomonas aeruginosa* Isolates

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Accurate typing methods are required for efficient infection control. The emergence of whole-genome sequencing (WGS) technologies has enabled the development of genome-based methods applicable for routine typing and surveillance of bacterial pathogens. In this study, we developed the *Pseudomonas aeruginosa* serotyper (PAst) program, which enabled *in silico* serotyping of *P. aeruginosa* isolates using WGS data. PAst has been made publically available as a web service and aptly facilitates high-throughput serotyping analysis. The program overcomes critical issues such as the loss of *in vitro* typeability often associated with *P. aeruginosa* isolates from chronic infections and quickly determines the serogroup of an isolate based on the sequence of the O-specific antigen (OSA) gene cluster. Here, PAst analysis of 1,649 genomes resulted in successful serogroup assignments in 99.27% of the cases. This frequency is rarely achievable by conventional serotyping methods. The limited number of nontypeable isolates found using PAst was the result of either a complete absence of OSA genes in the genomes or the artifact of genomic misassembly. With PAst, *P. aeruginosa* serotype data can be obtained from WGS information alone. PAst is a highly efficient alternative to conventional serotyping methods in relation to outbreak surveillance of serotype O12 and other high-risk clones, while maintaining backward compatibility to historical serotype data.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and a major cause of mortality and morbidity among hospitalized and compromised patients, including those with cystic fibrosis (CF). *P. aeruginosa* is well known for its ability to cause chronic and extensively drug-resistant infections (1). The outer membrane lipopolysaccharide (LPS) layer is a major virulence factor of *P. aeruginosa* (2). LPS has been linked to antibiotic resistance and immune evasion. Furthermore, LPS is one of the receptors that determines the susceptibility of the bacterium to bacteriophages and pyocins (2–4). Our ability to control *P. aeruginosa* infections depends on the availability of accurate typing methods. Previously, serotyping was a benchmark typing method for *P. aeruginosa*. In the 1980s, the International Antigenic Typing Scheme (IATS) was established to classify the species *P. aeruginosa* into 20 serotypes (O1 to O20) (5–7). Today, serotyping is infrequently used in the clinic for typing purposes, mainly because of the time-consuming protocol, the need for a continuous supply of serotype-specific antisera, and a high prevalence of polyagglutinating or nontypeable isolates.

The loss of *P. aeruginosa* typeability has been known for decades and has often been linked to bacteria isolated from chronic infections, where typeability is lost over time during the course of infection (8, 9). A study performed by Pirnay et al. (10) showed that 65% of all *P. aeruginosa* isolates examined were either non- or multitypeable and therefore assigning a particular serotype to these strains would be difficult. The occurrence of these non- or multitypeable isolates was higher when isolates sampled from CF infections exclusively were evaluated (10). Multitypeability has been associated with poor prognosis for CF patients and is a trait of persistent or chronic infection. This correlates with the observation that *P. aeruginosa* isolates from chronic CF infections are initially resistant to human serum but evolve to becoming serum sensitive over time, likely due to the loss of production of O anti-

gen, which protects the bacterial cell from the human serum (8). The mechanism underlying the loss of typeability over time is not fully understood but is most likely due to modifications of LPS structures over extended periods of bacterium–host interactions as a means to improve fitness in the host and to evade the host immune system, bacteriophages, and antibiotic therapy.

The knowledge concerning the serotype of an isolate is important for monitoring outbreaks and for understanding the structures of the LPS expressed on the surface of these bacteria. O11 and O12 are more predominant than other serotypes in the clinic, and, intriguingly, these serotypes have been associated with multidrug resistance (MDR) (10–13). This implies that these particular LPS structures improve fitness within the hosts and the hospital environments in ways that we currently do not understand. Specifically for the O12 serotype, it has been shown that horizontal gene transfer of LPS genes has resulted in MDR isolates and the switching of a certain serotype to O12 (14). To continuously monitor LPS structure and evolution, serotyping can help to improve our understanding of the isolates that successfully in-

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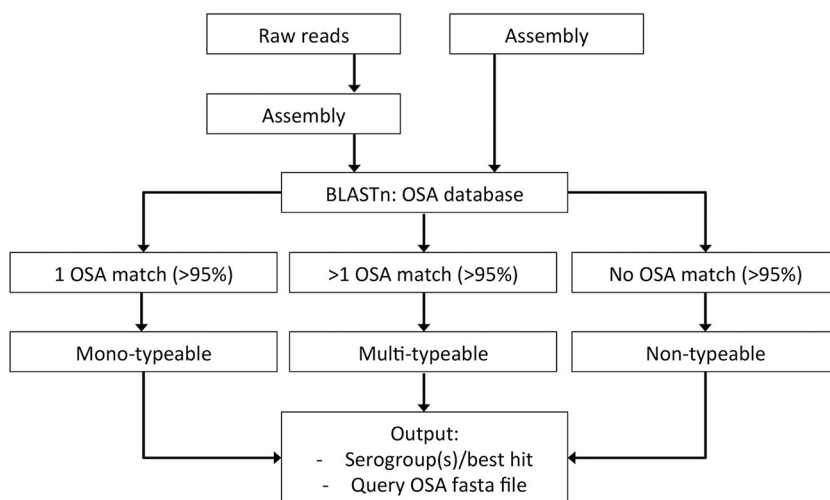


FIG 1 Workflow illustrating the *in silico* serotyping of the *Pseudomonas aeruginosa* serotyper (PAst).

fect patients. The continued collection of these data will also enable retrospective population analysis, as the serotype has also been recorded for decades prior to the emergence of other DNA-based typing methods such as multilocus sequence typing (MLST) and PCR.

P. aeruginosa LPS is composed of three domains: lipid A, core oligosaccharide, and O antigen (2). Most *P. aeruginosa* isolates produce two forms of O antigen simultaneously: common polysaccharide antigen (CPA) and O-specific antigen (OSA). While CPA is relatively conserved, OSA is variable and defines the serotype of an isolate (2, 15). OSA is encoded in a gene cluster varying in size from just <15 kb to >25 kb. The OSA gene cluster is flanked by the genes *ihfB/himD* and *wbpM*. The 20 serotypes harbor 11 distinct OSA gene clusters, each with a high number of unique genes (16). With the emergence of whole-genome sequencing (WGS) methods, it is now possible to assign an isolate to one of 11 serogroups based on the sequence and structure of the OSA gene cluster (11, 14, 17).

The present study presents a program that our group has developed for fast and reliable *in silico* serotyping of *P. aeruginosa* isolates using WGS data: the *Pseudomonas aeruginosa* serotyper (PAst). The program has been made publically available as a web service and can enable high-throughput serotyping analysis based on analysis of the OSA gene cluster. Using PAst, issues with typeability of clinical isolates can be overcome, and serotyping can be performed in a rapid and cost-effective way in the clinic as whole-genome sequencing of isolates become accessible.

MATERIALS AND METHODS

PAst verification and isolates included in the study. To evaluate the efficiency of the *in silico* serotyping using PAst, all available *P. aeruginosa* genomes were acquired and analyzed. These *P. aeruginosa* genomes were downloaded from NCBI and included 1,120 genome assemblies (extracted 18 August, 2015; see Table S1 in the supplemental material). An exclusively CF-related *P. aeruginosa* data set was constructed, due mainly to the documented high level of nontypeability in persistent infecting clones. The isolates described by Marvig et al. (475 genomes) (18) were used as the initial data set. These were assembled using SPAdes (19) prior to analysis. Additional CF isolates were recovered by searching for *P. aeruginosa* genome assemblies related to CF in PATRIC (54 genomes) (20). It was verified that frequently observed CF-specific strains such

as DK2 and LES were part of the data set. The final data set included 529 CF-related *P. aeruginosa* genome assemblies. *In silico* serotyping of both data sets was performed using PAst in order to evaluate the typeability of the program. Nontypeable isolates (i.e., isolates in which the percent coverage of reference OSA was <95%) were manually examined for either biological or technical explanations of the lack of typeability.

PAst specifications. The PAst program was developed using the programming language Perl for *in silico* serotyping of *P. aeruginosa* isolates using WGS data. It is based on a BLASTn analysis of the assembled input genome, against an OSA cluster database. OSA clusters with >95% coverage in the query genome represent a positive hit for a serogroup. Since *P. aeruginosa* isolates which harbor either multiple OSA clusters or no clusters at all have been described, the program accommodates multi-, mono-, and nontypeability based on analysis of the number of positive OSA hits and coverage (Fig. 1). Compared to other studies (11, 14, 17), PAst optimizes *in silico* serotyping further by distinguishing members of the O2 serogroup through identification of the acquired phage-related *wzy_β* within serotypes O2 and O16 (21, 22). This enables typing into 12 serogroups as opposed to the 11 described by Raymond et al. (16). Together with a summary of the best hit(s) from the analysis and the BLAST report, the user receives a multi-FASTA file containing the sequence(s) of the OSA cluster from the isolate analyzed for use in future analysis.

The *P. aeruginosa* OSA cluster database. The database was constructed using the WGS data of the 20 *P. aeruginosa* IATS serotype reference isolates (14). The genomes were assembled using SPAdes (19), and the OSA clusters were extracted via identification of the *ihfB/himD* gene flanking the cluster upstream and the *wbpM* gene flanking the cluster downstream. The clusters were aligned within their serotypes, described by Raymond et al., and their shared structure was confirmed (16). A representative cluster of each serotype was selected for the database (Table 1). Also included in the database was the *wzy_β* gene for distinguishing the O2 and O5 serotypes, as the two serogroups share the OSA cluster organization, but only the O2 and O16 serotypes harbor the *wzy_β* gene present on a prophage.

***In silico* serotyping of *P. aeruginosa* isolates using PAst.** PAst has been implemented as a simple and user-friendly Web tool available on the Center for Genomic Epidemiology (CGE) service platform (<https://cge.cbs.dtu.dk/services/PAst-1.0/>). The tool accommodates raw reads, draft assemblies (contigs or scaffolds), and complete genomes from all WGS platforms. Raw read data are processed and assembled as previously described for other CGE tools (23). Following analysis of the input data, the

TABLE 1 Serogroup definition in the PAsT OSA database

Serogroup	Reference OSA cluster	Reference gene	Size (bp)	Serotype(s) within serogroup
O1	O1		18,368	O1
O2	O2	<i>wzy_β</i>	23,303	O2, O16
O3	O3		20,210	O3, O15
O5	O2		23,303	O5, O18, O20
O4	O4		15,279	O4
O6	O6		15,649	O6
O7	O7		19,617	O7, O8
O9	O9		17,263	O9
O10	O10		17,635	O10, O19
O11	O11		13,868	O11, O17
O12	O12		25,864	O12
O13	O13		14,316	O13, O14

Web tool outputs the predicted serogroup of the query genome, the percent coverage of the reference OSA cluster, and the OSA cluster sequence in multi-FASTA format, for the user to continue exploring the OSA genes (Fig. 1). If multiple positive hits are found (multitypeability), all of the identified OSA clusters are written for the user (Fig. 1). In the case of a nontypeable query genome (where no OSA cluster has >95% coverage), the best hit identified is written for the user together with the sequence of this hit (Fig. 1).

For batch analysis of larger data sets (only applicable for assembled genomes) the PAsT Perl program has been made available on Github (<https://github.com/Sandramses/PAsT>).

RESULTS

The PAsT web server tool identifies and analyzes the nucleotide sequences of the O-specific antigen (OSA) gene clusters within the genomes provided and places them in 1 of 12 serogroups defined in Table 1. These serogroups are defined by sequence similarities between the 20 IATS serotypes (16) as well as by the absence/presence of the discriminatory *wzy_β* gene (21, 22) and are as such different from previous groupings of serotypes on the basis of *in vitro* serotyping data (11, 14, 17). All serogroups contained ≤3 of the 20 IATS serotypes (Table 1).

More than 97% of the genomes in the *P. aeruginosa* data set are typeable using PAsT. To evaluate the typeability efficiency of PAsT, all *P. aeruginosa* genome assemblies available in NCBI (1,120 genomes on the date of extraction) were analyzed. A total of 97.68% (1,094) of the 1,120 genomes were typed unambiguously to a single serogroup by PAsT (Fig. 2). This means that each genome assembly had a single BLAST hit of >95% OSA coverage to one sequence in our reference OSA database (Fig. 2). No isolates were found to be multitypeable and 2.32% (26 genomes) of the 1,120 genomes were found to be nontypeable (Fig. 2). In these cases, no significant BLAST hit of >95% OSA coverage to one of the sequence in the reference OSA database was identified. PAsT correctly determined the serogroups of the 20 IATS strains as well as those of PAO1 (serotype O5), PA14 (serotype O10), and PAK (serotype O6).

The analysis showed that all serogroups were represented in the 1,120 genomes (Fig. 2). Four of the 12 serogroups represented 70% of the genomes analyzed; these were O3, O6, O11, and O12 (Fig. 2). The smallest serogroup was O13, which contained only 4 genomes. We note that the same clone type might be present multiple times in the data set and that a substantial

sampling bias would therefore be expected. The distribution of serotypes in our analysis thus describes what has been chosen for sequencing and does not necessarily match the distribution of serotypes in the actual *P. aeruginosa* population. This does not affect the high confidence of PAsT, as it shows that unambiguous typing of multiple isolates from the same lineage is possible.

PAsT overcomes nontypeability issues from *in vitro* typing of CF lineages. *P. aeruginosa* isolates from CF infections are often nontypeable with conventional serotyping assays. To explore if our genome-based method enabled acquisition of serotype information in such isolates, we analyzed 529 genome assemblies of *P. aeruginosa* isolates sampled from CF infections. This data set contained multiple examples of isolates of the same lineage that had been sampled during the course of infection. This enabled us to investigate whether *in silico* typeability might be lost over time as has frequently been observed for *in vitro* serotyping of isolates from chronic CF infections. Interestingly, 99.81% of the genomes in the CF-specific data set were typed to single serogroups. More importantly, no multitypeable isolates were observed, and only one isolate was deemed nontypeable (Fig. 3). All serogroups were represented in the data set, except for O12. The absence of O12 serotypes among CF isolates has previously been reported (10). Serotypes O1, O6, and O7/O8 represented ~65% of the CF-specific data set, and the smallest representation of serotypes was the O9 serogroup with only two isolates from these samples (Fig. 3).

Well-known transmissible CF-specific clone types such as *P. aeruginosa* DK1 (24), DK2 (25), and LES (26) are represented in the data set due to multiple isolates being sampled from various patients over several decades. With our PAsT tool, the typing problems documented from *in vitro* typing of such lineages were not observed, and the DK1, DK2, and LES isolates were consistently *in silico* serotyped with PAsT. DK1 and DK2 were found to belong to the O3 serogroup, while the LES lineage belonged to the O6 serogroup.

Complete loss of O-specific antigen defining genes is a rare event. Out of two WGS-based data sets ($n = 1649$) that were *in*

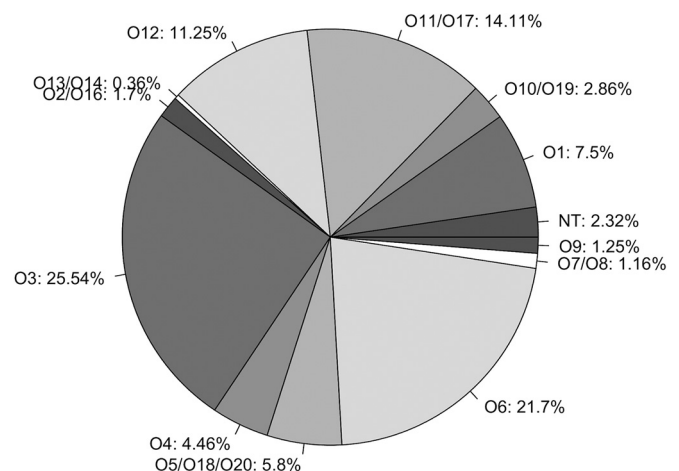


FIG 2 Distribution of the different serogroups (as percentages) identified via *in silico* serotyping of the *P. aeruginosa* data set using PAsT. The analysis is based on all available *P. aeruginosa* genomes assemblies ($n = 1,120$). NT, nontypeable.

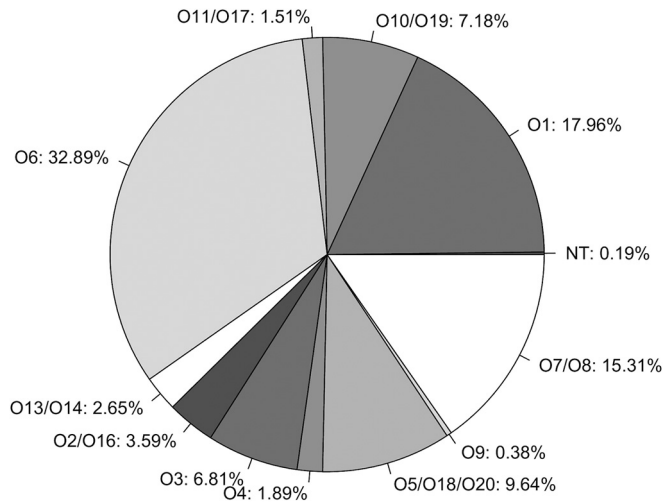


FIG 3 Distribution of the different serogroups (as percentages) identified via *in silico* serotyping of CF-specific *P. aeruginosa* isolates ($n = 529$) using PAST.

in silico typed with PAST, our results yielded a total of 27 nontypeable isolates. The lack of typeability in these 27 genome assemblies was further investigated to resolve whether nontypeability in these cases was due to technical or biological reasons. We found that the percent OSA coverage of the nontypeable isolates ranged from a minimum of 1.91% to a maximum of 93.96% OSA coverage (see Table S2 in the supplemental material). Of the 27 isolates classified as nontypeable, 13 were found to have OSA coverage of 0 to 20%, whereas 7 had OSA coverage of 80 to 95% (Fig. 4). The best hit (serogroup) for each of the nontypeable isolates was then examined to evaluate if certain serogroups were more prone to be problematic in the PAST analysis and why. The 27 isolates were found to distribute across 6 serogroups (O1, O2, O6, O7, O11, and O13), while 15/27 isolates showed a best hit to be typed as the O11 serogroup (Fig. 4).

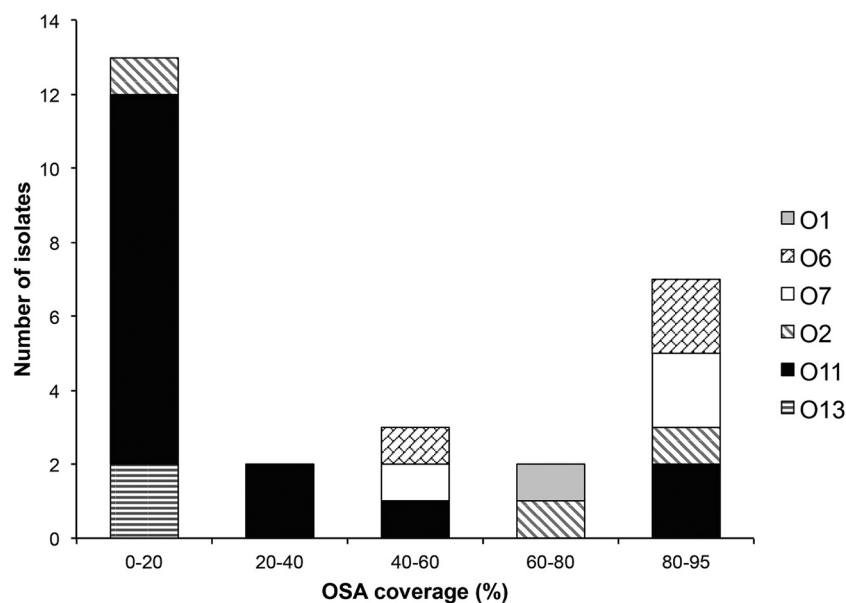


FIG 4 Best-hit serotype distribution of the 27 nontypeable isolates as a function of the OSA coverage.

The group of nontypeable isolates with a best hit to the O11 serogroup were analyzed separately to identify the reason for the lack of typeability. Of the 15 O11 serogroup isolates, 9 had an OSA coverage of 14.94 to 15.84% (see Table S2 in the supplemental material); these corresponded to the presence of only the two flanking genes *himD/ihfB* and *wbpM*. This observation shows that a best hit of a nontypeable isolate to the O11 OSA cluster with a coverage of $\sim 15\%$ is the result of a complete absence of an OSA cluster but the presence of the flanking genes. Two other isolates had an OSA coverage of $< 2\%$ and corresponded to the absence of the entire OSA cluster as well as the flanking genes (see Table S2). In summary, a total of 11 of the 27 nontypeable isolates (or 11 of 1,649 isolates analyzed in total) were nontypeable due to a lack of the OSA cluster sequences.

Genome misassembly accounts for false nontypeability. Since the seven nontypeable isolates with the highest OSA coverage (80 to 95%) in Fig. 4 were all candidates for harboring complete and functioning OSA clusters, we analyzed the cause of nontypeability in this group of isolates. For each of the isolates, we examined whether there were misassembly or assembly gaps within the OSA gene cluster; we also looked for the occurrence of insertion sequence (IS) elements, which often cause gaps in *de novo* assembly. Indeed, five of the seven isolates contained assembly gaps within their OSA cluster, which account for the observed lowered OSA coverage (Table 2). The remaining two isolates had no gaps within their OSA sequence (Table 2). However, both of these isolates had a best type hit to the O11 serogroup, which is known to contain OSA sequences of both the O11 and the O17 serotypes (16) (Table 1). Interestingly, the OSA clusters in these two serotypes differ only by the presence of two IS elements and a deletion in the O17 serotype OSA sequence (16). Alignment of the OSA sequence from the two nontypeable isolates to the O11 and O17 reference OSA sequences, respectively, contained an O17 OSA gene cluster, which had been misassembled into concatenated O11 serotype OSA clusters because of the O17 IS elements.

TABLE 2 Nontypeable *P. aeruginosa* isolates with percent OSA coverage of 80 to 95% with specification of assemblies

Strain	Size (Mb)	Scaffold	% GC	Best hit	% OSA	<i>wbpM</i>	<i>himD</i>	Gap
<i>P. aeruginosa</i> E2	6.36	196	66.4	O7	83.31	+	+	+
<i>P. aeruginosa</i> IGB83	6.48	249	66.4	O2	84.46	+	+	+
<i>P. aeruginosa</i> VRFP04	6.82	1	66.5	O11	86.96	+	+	–
<i>P. aeruginosa</i> DR1	6.28	176	66.1	O6	90.54	+	+	+
<i>P. aeruginosa</i> 148	6.64	128	66.1	O11	90.93	+	+	–
<i>P. aeruginosa</i> ID4365	6.78	172	66.1	O7	91.74	+	+	+
<i>P. aeruginosa</i> C2773C	6.72	200	65.9	O6	93.96	+	+	+

DISCUSSION

The serotyping technique has been one of the standard tools for epidemiological studies and infection controls for many decades. The available historical records of *P. aeruginosa* serotypes offer a vast amount of information about *P. aeruginosa* epidemiology and population structures (27–30). Although problems with nontypeable isolates have been described since the implementation of the method, the serotype information is still applicable today for outbreak tracking, strain typing, and studies of LPS structure and evolution. The present study presents a newly developed web server tool called PAsT, which is user friendly and reliable and has high throughput for *in silico* serotyping of *P. aeruginosa* isolates.

In contrast to conventional serology-based *in vitro* serotyping, PAsT *in silico* serotyping has a very low occurrence of nontypeability. Of the 1,649 analyzed genomes, only 27 nontypeable isolates were detected across two separate *P. aeruginosa* data sets. One data set represents all available whole-genome assemblies of *P. aeruginosa*, while the other specifically represents genomes from CF infections, which are known to contain high occurrences of nontypeability due to the adaptability of the bacteria into a biofilm lifestyle associated with chronicity of the infection (Fig. 1 and 2). Importantly, since the frequency of nontypeability of *in vitro* serotyped *P. aeruginosa* isolates may amount to >65% (10), analysis with PAsT is clearly advantageous and superior compared to conventional *in vitro* serotyping. Importantly, the superiority of the PAsT tool as a reliable and fast typing method is consistent with that of other published tools for *in silico* serotyping (31–35). Similar to SerotypeFinder (*in silico* serotyping of *Escherichia coli*) (31), LisSero (*in silico* serotyping of *Listeria monocytogenes*) (34, 35), and SeqSero (*in silico* serotyping of *Salmonella*) (32), PAsT resolves the OSA cluster information to the most accurate typing possible as a serogroup representing 1 to 3 serotypes.

Interestingly, we observed a high level of conservation of the OSA gene cluster within the *P. aeruginosa* genome. In contrast to certain well-documented difficulties in serology-based *in vitro* serotyping, PAsT identified complete OSA clusters (with >95% sequence being present) in 99.27% of the genomes analyzed. As such, only 12 of the 1,649 isolates examined were found to be devoid of the OSA cluster, and an additional 8 isolates were found to contain only a partial OSA cluster in their genomes (<80% OSA sequence compared to the reference). These findings indicate that the loss of typeability of *P. aeruginosa* isolates during the course of infection either is due to mutations (rather than larger deletions) or is linked to other parts of the LPS biosynthesis, such as regulatory genes or transport of the structure to the cell surface. A study by Bélanger et al. reported that mutation in any of the four *wbp* genes (*wbpO*, *wbpP*, *wbpV*, and *wbpM*) in the OSA gene cluster might disrupt the *P. aeruginosa* O6 OSA biosynthesis (36).

Furthermore, key genes involved in the OSA assembly and translocation through the Wzx/Wzy-dependent pathway not localized within the OSA cluster, for instance, *waalL*, are essential for O-antigen expression (37, 38). It is possible that other OSA-related genes, which have not been discovered yet, might be present in the *P. aeruginosa* genomes. Overall, our study demonstrates that a complete lack of an OSA gene cluster is a rarely observed phenomenon in *P. aeruginosa*.

PAsT will enable further investigations of the diversity, evolution, and variability of the OSA clusters. For example, the sequence of the cluster is part of the output material from the *in silico* serotyping which can then be readily analyzed for sequence variations to provide new knowledge on the mechanisms behind loss of typeability *in vitro* and *in silico*. Furthermore, PAsT will enable systematic analysis of serotype switching by horizontal gene transfer and genetic recombination of the OSA gene cluster among different clone types. This recently described phenomenon has contributed to the evolution of the multidrug-resistant *P. aeruginosa* serotype O12 population that has successfully disseminated across hospitals worldwide (14). It is currently unknown if there are additional cases of such serotype switching by recombination.

The new PAsT web server tool makes *in silico* serotyping of *P. aeruginosa* using WGS data a fast and reliable method. The use of PAsT can play an important role in future surveillance of LPS evolution and possible outbreak detection. With the emergence of rapidly disseminating, high-risk clones of *P. aeruginosa*, such as the O12 ST111 clone, new and reliable typing techniques for improved monitoring and tracking of such outbreaks are becoming increasingly important (13). With the lowered cost of sequencing and the increased focus on WGS of pathogens in clinics and hospital settings, genome-based tools can assist in designing future treatments and containment of outbreaks.

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