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Application of WGS data for O-specific antigen analysis and *in silico* serotyping of *Pseudomonas aeruginosa* isolates

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Running title: *In silico* serotyping of *P. aeruginosa*

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Abstract

Accurate typing methods are required for efficient infection control. The emergence of whole genome sequencing (WGS) technologies has enabled the development of genomics-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 facilitate 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 1649 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 non-typeable isolates found using PAst was the result of either 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.
Introduction

*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 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 1980’s the International Antigenic Typing Scheme (IATS) was established to classify the species *P. aeruginosa* into 20 serotypes (O1-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 non-typeable 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 multi-typeable and therefore assigning a particular serotype to these strains would be difficult. The occurrence of these non- or multi-typeable isolates was higher when evaluating isolates sampled exclusively from CF infections (10). Multi-typeability 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. This is likely due to the loss of production of O-antigen, which protects the bacterial cell from the human serum (8). The mechanism underlying loss of typeability over time is not fully understood, but is most likely due...
to modifications of LPS structures over extended periods of bacteria-host
interactions as a means to improve fitness in the host and to evade 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 multi-drug
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
infect patients. The continued collection of these data will also enable retrospective
population analysis, as serotype has been recorded for decades also prior to the
emergence of other DNA-based typing methods such as MLST and PCR.

P. aeruginosa LPS is comprised 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 under 15 kb
to over 25 kb. The OSA gene cluster is flanked by the genes \textit{ihfB}/\textit{himD} and \textit{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 into 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
\textit{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 1120 genome assemblies (Supplementary Table 1, extracted 18.08.2015). An exclusively CF-related P. aeruginosa dataset was constructed, due mainly to the documented high level of non-typeability in persistent infecting clones. The isolates described by Marvig et al. 2015 (475 genomes) (18) were used as the initial dataset. These were assembled using SPAdes 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 dataset. The final dataset included 529 CF-related P. aeruginosa genome assemblies. In silico serotyping of both datasets was performed using PAst in order to evaluate typeability of the program. Non-typeable isolates (i.e., isolates in which %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 is 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 represents a positive hit for a serogroup. Since P. aeruginosa isolates have been described which either harbor multiple OSA clusters or no clusters at all, the program accommodates multi-, mono- and non-typeability based on analysis of the number of positive OSA hits and coverage (Figure 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 within serotypes O2 and O16 (21, 22). This enables typing into 12 serogroups as opposed to the 11 described by Raymond et al. (16).

on June 6, 2016 by TECH KNOWLEDGE CTR OF DENMARK
http://jcm.asm.org/
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 analyzed isolate 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 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.* 2002 and their shared structure confirmed (16). A representative cluster of each serotype was selected for the database (Table 1). Also included in the database was the *wzy*<sub>ɴ</sub> gene for distinguishing the O2 and O5 serotypes, as the two serogroups share OSA cluster organization, but only the O2 and O16 serotype harbor the *wzy*<sub>ɴ</sub> 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/](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 web-tool outputs the predicted serogroup of the query genome, the %coverage of the reference OSA cluster, as well as 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 (multi-typeability), all the identified OSA clusters are written for the user (Fig. 1). In the case of a non-typeable 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 datasets (only applicable for assembled genomes) the PAst Perl program has been made available on Github: [https://github.com/Sandramses/PAst](https://github.com/Sandramses/PAst)
Results

The PAst web server tool identifies and analyzes the nucleotide sequence of the O-specific antigen (OSA) gene cluster within the provided genomes and place them into one of twelve serogroups defined in Table 1. These serogroups are defined by sequence similarities between the 20 IATS serotypes (16) as well as absence/presence of the discriminatory \textit{wzyN} gene (21, 22) and are as such different from previously groupings of serotypes on the basis of \textit{in vitro} serotyping data (11, 14, 17). All serogroups contained three or less of the 20 IATS serotypes (Table 1).

More than 97% of the \textit{P. aeruginosa} dataset is typeable using PAst

To evaluate the typeability efficiency of PAst all \textit{P. aeruginosa} genome assemblies available in NCBI (1120 genomes on date of extraction) were analyzed. A total of 97.68% (1094) of the 1120 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 multi-typeable and 2.32% (26 genomes) of the 1120 genomes were found to be non-typeable (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 serogroup of the 20 IATS strains as well as PAO1 (serotype O5), PA14 (serotype O10), and PAK (serotype O6).

The analysis showed that all serogroups were represented in the 1120 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 four genomes. We note that the same clone type could be present multiple times in the dataset, 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 \textit{P. aeruginosa} population. This does not affect the high confidence of PAst, as it shows that un-ambiguous typing of multiple isolates from the same lineage is possible.
PAst overcomes non-typeability issues from in vitro typing of CF lineages

P. aeruginosa isolates from CF infections are often non-typeable with conventional serotyping assays. To explore if our genomics-based method could enable acquisition of serotype information in such isolates, we analyzed 529 genome assemblies of P. aeruginosa isolates sampled from CF infections. This dataset 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 dataset could be typed to single serogroups. More importantly, no multi-typeable isolates were observed and only one isolate was deemed non-typeable (Fig. 3). All serogroups were represented in the dataset, 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 dataset and the smallest representation of serotypes was the O9 serogroups 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 dataset due to multiple isolates being sampled from various patients over several decades. Using 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 datasets (n = 1649) that were in silico typed with PAst, our results yielded a total of 27 non-typeable isolates. The lack of typeability in these 27 genome assemblies was further investigated to resolve whether non-typeability in these cases was due to technical or biological reasons. We found that the %OSA coverage of the non-typeable isolates ranged from a minimum of 1.91% to a
maximum 93.96% OSA coverage (Supplementary Table 2). Of the 27 isolates classified as non-typeable, thirteen were found to have OSA coverage of 0-20%, whereas seven isolates had OSA coverage of 80-95% (Fig. 4). The best hit (serogroup) for each of the non-typeable 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).

The group of non-typeable 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, nine had an OSA coverage of 14.94-15.84% (Supplementary Table 2); these corresponded to the presence of only the two flanking genes, himD/hfb and wbpM. This observation shows that a best hit of a non-typeable isolate to the O11 OSA cluster with a coverage of ~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 (Supplementary table 2). In summary, a total of 11 of the 27 non-typeable isolates (or 11 of 1649 isolates analyzed in total) were non-typeable due to a lack of the OSA cluster sequences.

**Genome mis-assembly accounts for false non-typeability**

Since the seven non-typeable isolates with the highest OSA coverage (80-95%) in Figure 4 were all candidates for harboring complete and functioning OSA clusters, we analyzed the cause of non-typeability in this group of isolates. For each of the isolates, we examined whether there were mis-assembly 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 cluster 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 non-typable 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.
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 non-typeable 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, reliable, and 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 non-typeability. Of the 1649 analyzed genomes, only 27 non-typeable isolates were detected across two separate *P. aeruginosa* datasets. One dataset 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 non-typeability due to adaptability of the bacteria into a biofilm life-style associated with chronicity of the infection (Fig. 1 and 2). Importantly, since the frequency of non-typeability of *in vitro* serotyped *P. aeruginosa* isolates may amount to over 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 other published tools for *in silico* serotyping (31–35). Similar to both the SerotypeFinder (*in silico* serotyping of *E. 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 1-3 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 analyzed genomes. As such only 12 of the 1649 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 is either 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 could 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, waal, are essential for O-antigen expression (37, 38). It is possible that more OSA-related genes might be present in the P. aeruginosa genomes, which have not been discovered yet. 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 multi-drug 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, genomics-based tools can assist in designing future treatments and containment of outbreaks.
Acknowledgements

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References


476 responsible for the synthesis of the B-band O-antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. Microbiology **145**:3505–3521.


Figure legends

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

**FIG 2** The distribution of the different serogroups (in %) identified via *in silico* serotyping of the *P. aeruginosa* dataset using PAst. The analysis is based on all available *P. aeruginosa* genomes assemblies (n = 1120).

**FIG 3** The distribution of the different serogroups (in %) identified via *in silico* serotyping of CF specific *P. aeruginosa* isolates (n = 529) using PAst.

**FIG 4** Best-hit serotype distribution of the 27 non-typeable isolates as a function of the OSA coverage.
TABLE 1 Serogroup definition in the PAst OSA database.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Reference OSA cluster</th>
<th>Ref. gene</th>
<th>Size (bp)</th>
<th>Serotypes within serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>O1</td>
<td></td>
<td>18.368</td>
<td>O1</td>
</tr>
<tr>
<td>O2</td>
<td>O2</td>
<td>wzy</td>
<td>23.303</td>
<td>O2, O16</td>
</tr>
<tr>
<td>O3</td>
<td>O3</td>
<td></td>
<td>20.210</td>
<td>O3, O15</td>
</tr>
<tr>
<td>O5</td>
<td>O2</td>
<td></td>
<td>23.303</td>
<td>O5, O18, O20</td>
</tr>
<tr>
<td>O4</td>
<td>O4</td>
<td></td>
<td>15.279</td>
<td>O4</td>
</tr>
<tr>
<td>O6</td>
<td>O6</td>
<td></td>
<td>15.649</td>
<td>O6</td>
</tr>
<tr>
<td>O7</td>
<td>O7</td>
<td></td>
<td>19.617</td>
<td>O7, O8</td>
</tr>
<tr>
<td>O9</td>
<td>O9</td>
<td></td>
<td>17.263</td>
<td>O9</td>
</tr>
<tr>
<td>O10</td>
<td>O10</td>
<td></td>
<td>17.635</td>
<td>O10, O19</td>
</tr>
<tr>
<td>O11</td>
<td>O11</td>
<td></td>
<td>13.868</td>
<td>O11, O17</td>
</tr>
<tr>
<td>O12</td>
<td>O12</td>
<td></td>
<td>25.864</td>
<td>O12</td>
</tr>
<tr>
<td>O13</td>
<td>O13</td>
<td></td>
<td>14.316</td>
<td>O13, O14</td>
</tr>
</tbody>
</table>
### TABLE 2 Non-typeable *P. aeruginosa* isolates with %OSA coverage of 80-95% with specification of assemblies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Size (Mb)</th>
<th>Scaffolds</th>
<th>%GC</th>
<th>Best hit</th>
<th>%OSA</th>
<th>wbpM</th>
<th>himD</th>
<th>Gap</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> E2</td>
<td>635.733</td>
<td>196</td>
<td>66.4</td>
<td>O7</td>
<td>83.31</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> IGB83</td>
<td>648.065</td>
<td>249</td>
<td>66.4</td>
<td>O2</td>
<td>84.46</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> VRFP04</td>
<td>681.803</td>
<td>1</td>
<td>66.5</td>
<td>O11</td>
<td>86.96</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>627.851</td>
<td>176</td>
<td>66.1</td>
<td>O6</td>
<td>90.54</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 148</td>
<td>664.374</td>
<td>128</td>
<td>66.1</td>
<td>O11</td>
<td>90.93</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ID4365</td>
<td>677.663</td>
<td>172</td>
<td>66.1</td>
<td>O7</td>
<td>91.74</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>P. aeruginosa</em> C2773C</td>
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<td>200</td>
<td>65.9</td>
<td>O6</td>
<td>93.96</td>
<td>+</td>
<td>+</td>
<td>+</td>
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