Comparative Genomics of *Pseudomonas stutzeri* Complex: Taxonomic Assignments and Genetic Diversity

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*Pseudomonas stutzeri* is a species complex with extremely broad phenotypic and genotypic diversity. However, very little is known about its diversity, taxonomy and phylogeny at the genomic scale. To address these issues, we systematically and comprehensively defined the taxonomy and nomenclature for this species complex and explored its genetic diversity using hundreds of sequenced genomes. By combining average nucleotide identity (ANI) evaluation and phylogenetic inference approaches, we identified 123 *P. stutzeri* complex genomes covering at least six well-defined species among all sequenced *Pseudomonas* genomes; of these, 25 genomes represented novel members of this species complex. ANI values of ≥∼95% and digital DNA-DNA hybridization (dDDH) values of ≥∼60% in combination with phylogenomic analysis consistently and robustly supported the division of these strains into 27 genomovars (most likely species to some extent), comprising 16 known and 11 unknown genomovars. We revealed that 12 strains had mistaken taxonomic assignments, while 16 strains without species names can be assigned to the species level within the species complex. We observed an open pan-genome of the *P. stutzeri* complex comprising 13,261 gene families, among which approximately 45% gene families do not match any sequence present in the COG database, and a large proportion of accessory genes. The genome contents experienced extensive genetic gain and loss events, which may be one of the major mechanisms driving diversification within this species complex. Surprisingly, we found that the ectoine biosynthesis gene cluster (*ect*) was present in all genomes of *P. stutzeri* species complex strains but distributed at very low frequency (43 out of 9548) in other *Pseudomonas* genomes, suggesting a possible origin of the ancestors of *P. stutzeri* species complex in high-osmolarity environments. Collectively, our study highlights the potential of using whole-genome sequences to re-evaluate the current definition of the *P. stutzeri* complex, shedding new light on its genomic diversity and evolutionary history.

**Keywords:** genome comparison, taxonomy and nomenclature, phylogeny, genetic diversity, pan-genome, *Psuedomonas stutzeri* complex
INTRODUCTION

Pseudomonas stutzeri is a species complex comprising five defined species, P. stutzeri (sensu stricto) (s.s.), P. xanthomarina (Romanenko et al., 2005), P. balearica (Bennasar et al., 1996), P. luteola and P. chloritidismutans (Wolterink et al., 2002), according to the National Center for Biotechnology Information (NCBI) Taxonomy Database. Strains belonging to P. stutzeri complex display extremely broad phenotypic diversity and consequently colonize a large range of ecological niches (Lalucat et al., 2006). Although rarely causing infections, over the past two decades, P. stutzeri complex members have been increasingly recognized as a cause of infection in humans (Scotta et al., 2012; Hequette-Ruz et al., 2018; Halabi et al., 2019). The P. stutzeri complex has received particular attention because of specific physiological properties, such as denitrification (Pena et al., 2012), nitrogen fixation (Lalucat et al., 2006; Yan et al., 2008; Busquets et al., 2013; Paerl et al., 2018), degradation of aromatic compounds (Brunet-Galmes et al., 2012; Busquets et al., 2013; Gomila et al., 2015a; Hirose et al., 2015; Brown et al., 2017), and metal resistance and potential application in bioremediation (Li et al., 2012; Chakraborty et al., 2017; Zheng et al., 2018; Harada et al., 2019). It was shown that one third of P. stutzeri complex members are naturally transformable (Sikorski et al., 2002b; Busquets et al., 2012; Smith et al., 2014), which may be one of the major driving forces causing its high level of intraspecific heterogeneity. Diversity within the species is not limited to physiological traits but is also clearly reflected at the genetic level. To date, the P. stutzeri complex has been divided into at least 21 defined genomovars (Scotta et al., 2013), a provisional taxonomic status for genotypically similar strains within a bacterial species. However, there is lack of defined phenotypic properties for discriminating among different genomovars (Lalucat et al., 2006). Despite focus on its genetic heterogeneity and taxonomy, most investigations of P. stutzeri have been performed on the basis of DNA-DNA hybridization (DDH), 16S rRNA gene similarity, and sequencing of several housekeeping genes (multilocus sequence analysis, MLSA) (Guasp et al., 2000; Scotta et al., 2013; Zhang et al., 2014). With advances in high-throughput sequencing technology, numerous genomes representing this group of strains have been determined. However, a comprehensive, genome-wide analysis of this species complex related to its diversity and taxonomy is lacking.

The DDH, 16S rRNA gene analysis and MLSA are the most frequently used approaches for assigning isolates to species within the P. stutzeri complex. All genomovars of the species complex described so far have been described using these approaches (Rossello et al., 1991; Sikorski et al., 2005; Gomila et al., 2015b). However, each of these techniques has some basic limitations. DDH is the gold standard for prokaryotic species circumscriptions, and construction of the existing prokaryotic classification system was based on the fact that DDH can reveal coherent genomic groups (genospecies) of strains generally sharing DDH values with >70% similarity (Colston et al., 2014). However, laboratory-based DDH measurements are complex and time-consuming. Therefore, the difficulty of obtaining DDH values for a large number of genomes is a major drawback in the bioinformatics era. Similarly, the low variability and conservative nature of 16S rRNA genes gives rise to insufficient resolution for inferring clear taxonomic relationships (Rossi-Tamisier et al., 2015), while putative bias in the selection of genes is a major drawback for MLSA.

Genome sequencing has become a routine practice in microbiology research due to advances in high-throughput sequencing technology and declining costs of sequencing. At the time of writing, there are more than 250,000 prokaryotic genomes that have been released in the NCBI genome database. As a result, new tools are being developed to assess taxonomic relationships and discover novel taxa, primarily digital DNA-DNA hybridization (dDDH) (Chain et al., 2009; Meier-Kolthoff et al., 2013) and average nucleotide identity (ANI) (Richter and Rossello-Mora, 2009). Taxonomists already believe that the reference standard for determining taxonomy will be full genome sequences. Digital DNA-DNA hybridization produces values that compare closely with experimentally derived DDH values (Auch et al., 2010; Meier-Kolthoff et al., 2013). Average nucleotide identity between two genomes has been proposed as a promising standard for defining prokaryotic species and is receiving wide acceptance (Federhen et al., 2016; Ciufo et al., 2018). The most recent proposal recommends using an ANI threshold of 94–96% (Konstantinidis and Tiedje, 2005; Jain et al., 2018), along with support from tetranucleotide frequency correlation coefficient (TETRA) values > 0.99 (Richter and Rossello-Mora, 2009).

An increasing number of P. stutzeri complex genomes have been obtained from a variety of ecosystems (listed in Supplementary Table 1), providing researchers with an opportunity to extensively analyze genetic diversity, assess taxonomic relationships, discover novel taxa and identify genetic determinants associated with specific physiological properties. In this study, by coupling a large complete genome collection with powerful bioinformatics analyses, we documented taxonomic and phylogenetic relationships in this species complex and elaborated on its diversity at the genomic level. Finally, we surveyed the distribution of the ectoine biosynthesis gene cluster (ect) in all Pseudomonas genomes to inquire into a possible origin of the ancestors of the P. stutzeri complex in high-osmolarity habitats. Our results underlined the misidentifications for P. stutzeri complex in taxonomy and nomenclature, and revealed that a substantial proportion of pan-genome has undergone gene loss and gain, essentially shaping the genetic diversity of the P. stutzeri complex.

MATERIALS AND METHODS

Genomic Sequences of Pseudomonas stutzeri Species Complex

Pseudomonas stutzeri has a complex taxonomy encompassing several species. To define P. stutzeri complex genomes, a combination of ANI and MLSA approaches were used to sieve P. stutzeri complex genomes from all sequenced Pseudomonas genomes. All available 9,548 Pseudomonas genomes (as of 6 February 2020) were retrieved from the NCBI genome
database using Aspera. First, 9,548 Pseudomonas genomes were queried against 105 genomes of the P. stutzeri group (Supplementary Table 2) in NCBI for ANI calculation using fastANI version 1.3 (Jain et al., 2018). Then, genomes having ≥80% identity by ANI to reference genomes were retained for curated ANIm calculation using Jspecies version 1.2.1 (Richter and Rossello-Mora, 2009) with default parameters. ANI values were transformed into a “100%-ANI” distance matrix and clustered using MEGAX version 10.0.4 (Kumar et al., 2018) with the neighbor-joining (NJ) algorithm. Simultaneously, ANI values were transformed into a “100%-ANI” distance matrix and clustered using MEGAX version 10.0.4 (Kumar et al., 2018) with the neighbor-joining (NJ) algorithm. Simultaneously, three housekeeping genes (16S rRNA, gyrB and rpoD partial gene sequences) were extracted from the Pseudomonas genomes having ≥80% identity by ANI to reference genomes. Besides, these three genes of 53 well-characterized P. stutzeri complex strains were downloaded from NCBI nucleotide database according to a previous study (Scotta et al., 2013). A NJ phylogenetic tree based on the concatenated analysis of the three genes was generated utilizing MEGAX. Finally, only genomes located in the same branch with the well-characterized (or defined) P. stutzeri complex strains in both ANI and MLSA analyses were considered to be members of the P. stutzeri complex.

Digital DNA-DNA Hybridization

Estimation of dDDH was performed using the Genome-to-Genome Distance Calculator (GGDC) (Chain et al., 2009; Meier-Kolthoff et al., 2013). Accession numbers of P. stutzeri complex genomes were submitted to the GGDC 2.0 Web server, where dDDH calculations were performed. Results of GGDC were used for analysis according to formula 2, which calculates dDDH estimates independent of genome length and is recommended by the authors of GGDC for use with draft genomes (Chain et al., 2009; Meier-Kolthoff et al., 2013).

Determination of Core- and Pan-Genomes

All predicted protein-coding sequences (CDSs) were extracted from each of 123 genomes separately using extract_protein_dir.pl and subjected to identification of homologous clusters using OrthoFinder version 2.3.12 (Emms and Kelly, 2019) with default parameters. This tool is fast, accurate and scalable to numerous genomes compared with OrthoMCL. Results for orthogroups and unassigned genes (strain-specific genes) from OrthoFinder were combined, and the combined data format was modified using format_modification.pl. These data were subsequently used to estimate the core- and pan-genomes for a given number of sequentially added genomes via the PGAP tool v1.2.1 (Zhao et al., 2012). Sample size was set to 20,000 according to recommendations for this software. The core genome was defined as homologous genes shared by all genomes analyzed. The pan-genome was defined as the non-redundant set of all genes found in all genomes analyzed.

In addition, the percentage of homologs shared between pairs of genomes was used to reflect the genetic diversity in their gene content. This was defined as the number of shared homologs divided by the average number of genes in the non-redundant set of all CDSs between two genomes. The number of shared homologs for any pair of genomes and the number of genes in the non-redundant set of all CDSs for each genome were obtained from the results files “Comparative Genomics Statistics Directory” generated by Orthofinder. In total, 15,129 pairwise values were calculated for 123 genomes.

Phylogenetic Tree Inference

Core genes were obtained using OrthoFinder. Single-copy core genes, which are shared by all genomes and present as only a single copy in each genome, were used to infer phylogenetic relationships among 123 P. stutzeri complex strains plus four outgroup strains (Pseudomonas aeruginosa DSM 50071T, P. azotifigens DSM 17556T, P. mendocina NCTC10897T and P. putida FF4). All nucleotide alignments of core genes were generated using Muscle version 3.8.31 (Edgar, 2004), and core gene sequences were concatenated into a string of nucleotides for each genome using Concat_Seq.pl. The concatenated alignment data were used to infer phylogenies using MEGAX version 10.0.4 with the NJ algorithm, and RAxML version 8.2.11 (Stamatakis, 2014) with the maximum-likelihood (ML) algorithm under the general time-reversible (GTR) + category (CAT) model. To estimate tree reliability, a bootstrap method computing 1,000 bootstrap repetitions was used for both the NJ and ML trees. In addition, GGDC distance (intergenic distance) matrices were used to construct a tree using FastME version 2.1.5 (Lefort et al., 2015). Statistical comparisons of tree topologies were made using the Kendall-Colijn metric implemented through the R package treespace (Jombart et al., 2017). For all pairwise tree comparisons, trees were rooted to four outgroup strains, and a lambda value of 0 (to give weight to tree topology rather than branch lengths) was used along with 100,000 random trees as a background distribution. A z-test was performed comparing the distance observed between the query and the reference to the distances observed between the query and the trees from the background distribution. A threshold of $P < 0.05$ was used to determine that the query tree is more closely related to the reference tree topology than would be expected by chance. All trees were visualized using ITOL (Letunic and Bork, 2019).

Dynamics of Gene Content Analysis

Gain and loss of gene families at each ancestral node were modeled using Count v1.0.4 (Csuros, 2010) using the Pan-genome matrix of 123 genomes and a modified core-gene based ML tree as input files. This tree was generated by removing the outgroup strains of the core-gene based ML tree using iTOL. All
of the model parameters were first optimized by maximizing the likelihood of the data using a gain–loss-duplication model with a Poisson distribution for gene family size at the root. Gamma-distributed rate variation across gene families was assumed, with the shape parameter discretized in four classes; a fixed gain/loss ratio across lineages was also assumed. Rate parameters were optimized after 100 rounds of parameter optimization. Profiles of posterior probabilities of events for each branch of the tree were then estimated. To obtain patterns of gain/loss, these probabilities were transformed into “likely events” using a threshold of 0.5 posterior probability (Brito et al., 2018).

Functional Analysis
We extracted the amino acid sequences of the unique genes and the first genes for each set of homologs in the pan-genome. To characterize the functional classification of pan-genome, these sequences were queried against the Clusters of Orthologous Groups of proteins (COGs, v2021) database (Galperin et al., 2021) using blastP (Boratyn et al., 2013) with E-value of 1e-5 (Zhong et al., 2021). COG_annotation.pl was used to perform the blast analysis and classified the function of the genes according to COG classifications. If a gene was assigned to more than one COG category, each COG category was calculated separately.

Origin of the Cloud Genome
The cloud genome was defined as homologous protein clusters shared by only one or two genomes. To identify potential donor species of the cloud genome, all cloud genes were queried against the NCBI nr database using a blastp search with an E-value of 1e-5. The blastp hits excluded sequences belonging to the 123 strains analyzed in this study.

Detection of Ectoine Biosynthesis Gene Cluster in Pseudomonas ssp.
Ectoine biosynthesis is the common mechanism for balancing osmotic pressure for some bacteria. Biosynthesis of ectoine is catalyzed by ectABC, which encode N-γ-acetyltransferase, L-2,4-diaminobutyric acid transaminase and ectoine synthase, respectively. Some strains hydroxylate ectoine to 5-hydroxyectoine in a stereospecific fashion using the ectoine hydroxylase EctD. ask_ect is a gene encoding aspartokinase that is involved in biosynthesis of the precursor for ectoine biosynthesis, aspartyl-beta-phosphate, and enhances the biosynthesis of ectoine and hydroxyectoine (Stoveken et al., 2011). Several P. stutzeri complex genomes were reported to harbor complete ectoine biosynthesis gene cluster, comprising five genes (ectABCD-ask) (Stoveken et al., 2011; Li et al., 2012). Numerous P. stutzeri complex strains isolated from marine environments (Supplementary Table 1) inspired us to survey whether harboring ectABCD-ask is a common phenomenon in genomes of this species complex strains for surviving in salty environments (Sikorski et al., 2002a; Pena et al., 2012; Paerl et al., 2018; Zheng et al., 2018). Interested_gene_generation.pl

RESULTS AND DISCUSSION
Definition of Pseudomonas stutzeri Complex Genomes
It is well accepted that some genomes are misidentified or could be named more specifically in the NCBI database (Federhen et al., 2016; Ciufo et al., 2018). For this reason, we used two prevalent approaches, ANI analysis and phylogenetic tree reconstruction based on housekeeping genes, to identify P. stutzeri complex genomes among 9,548 Pseudomonas genomes. ANI analysis indicated that 368 of the 9,548 Pseudomonas genomes shared more than 80% identity by ANI with 105 genomes of P. stutzeri complex strains in GenBank. Cluster analysis using the “100%-ANI” distance matrix placed 133 strains in the P. stutzeri complex branch (Supplementary Figure 1). However, P. stutzeri NP_8HO was excluded in the P. stutzeri complex branch. The phylogenetic tree based on the concatenated analysis of the three genes (16S rRNA, ropD and gyrB) is partially incongruent with that of the cluster analysis. The discord is that ten strains (six P. luteola strains, P. lutea LMG 21974, P. zeshuii KACC 15471, and Pseudomonas sp. strains HPB0071 and LTJR-52) were not affiliated with the P. stutzeri complex branch (Supplementary Figure 2). Meanwhile, P. luteola strains had an average GC% of 55.02%, significantly lower than that of P. stutzeri complex (P < 0.01, see below), supporting that P. luteola did not belong to the P. stutzeri complex. Thus, above results demonstrated that 123 genomes should be assigned to the P. stutzeri complex, and they are listed in Supplementary Table 1. Seven genomes (six P. luteola strains and P. stutzeri NP_8HO) did not belong to the P. stutzeri complex. P. stutzeri complex strains had been isolated from different sources, e.g., soil, saline and clinically relevant environments. Overall, the genome size of members of the P. stutzeri complex ranged from 3.674 to 5.319 Mb, with an average value of 4.566 Mb, and their GC contents ranged from 59.6 to 65.18%, with an average value of 63.12%. Strains of this species complex have an average of 4,129 CDs (excluding pseudo-CDSs) (Supplementary Table 1).

Among 123 genomes, we identified 25 novel members of the P. stutzeri complex (Supplementary Table 1), accounting for more than 20% of the total number of P. stutzeri complex genomes. This result provides evidence of mistakes in the taxonomy and nomenclature of the P. stutzeri complex when data were submitted to the NCBI genome database. The novel P. stutzeri complex genomes encompassed all sequenced strains
of four species [Pseudomonas kunmingensis (Xie et al., 2014), P. saudiphocaensis (Azhar et al., 2017), P. songnenensis (Zhang et al., 2015b) and P. zhaodongensis (Zhang et al., 2015a)] and 16 Pseudomonas strains without species assignment. The reason for a large proportion of P. stutzeri complex genomes being deposited at NCBI with incorrect taxonomy and nomenclature most likely stems from their extreme genetic diversity and a high similarity among their 16S rRNA gene sequences. Our results also suggest that it is hard to determine the taxonomy and nomenclature for certain novel P. stutzeri complex strains, which show distant relationships with known P. stutzeri complex genomes. The results presented here suggest that FastANI in combination with phylogenetic analysis is a promising approach for correcting and updating the nomenclature to curtail the promulgation of inaccurate information. FastANI is a fast method for calculating ANI for large numbers of genomes (Jain et al., 2018). We set an ANI value of 80% as the threshold for filtering, which may have caused us to miss some P. stutzeri complex strains if genomes of novel genomovars are not included in the reference genomes. However, it will not happen as ANI values between genomes of different genomovars were greater than 80% (Supplementary Table 3).

**Genome Similarity and Phylogenetic Relationships**

We evaluated the pairwise percentage similarities of 123 genomes using two different bioinformatics approaches, dDDH and ANI. Use of these two approaches has been proposed to overcome the challenges of conventional laboratory-based DDH for evaluating the overall similarity of bacterial genomes (Colston et al., 2014). dDDH and ANI use different algorithms for the calculations (Colston et al., 2014); however, the results obtained here were highly concordant with each other (Figure 1A), and detailed data are shown in Supplementary Table 3. The r² value was 0.986 for the entire data set, demonstrating that results from the two approaches show good congruence and either method can be used for determining overall genome similarities. Usually, two genomes belonging to the same species would have an ANI cutoff of 94–96% (Konstantinidis and Tiedje, 2005; Richter and Rossello-Mora, 2009), which corresponds to the 70% DDH and 70% dDDH. However, for pairs of a few genomes in this study (mainly belonging to gv 7 and gv 18, see below), we found ANI values of around 95% (with TETRA values > 0.99) corresponded to approximately 60% dDDH, noticeably lower than the expected dDDH values. For instance, P. stutzeri DCP-Ps1 and Pseudomonas sp. JL972 have an ANI value of 94.89% and dDDH value of 59.9% [95% confidence interval (CI), 57.1–62.7%] (Supplementary Table 3). This result indicated that ANI values were positively correlated with dDDH values, yet an ANI cut-off of around 95% did not correspond to an absolute dDDH value. A previous study reported that the value of 70% DDH could not be used as absolute boundary, but still a gap between 60 and 70% similarity seemed to embrace clear-cut clusters of organisms; given the large extent of diversity among prokaryotes, the circumscription of each genospecies would, in addition, be dependent on each group being studied (Richter and Rossello-Mora, 2009). Correspondingly, NCBI maintains ANI species cutoff values on a species-specific basis, which span much more (or much less than) the default rule of thumb 96% ANI (Federhen et al., 2016). In addition, the phylogenetic tree based on MLSA analysis confirmed that these genomes belonged to the same genomovar since they clustered with the well-characterized
strains of P. stutzeri complex gv 7 or gv 18 (Supplementary Figure 2). Altogether, for P. stutzeri complex, at the ≈ 95% ANI value (Jain et al., 2018) and ≈ 60% dDDH values cutoff, 123 genomes in our study could be assigned into 27 genomovars (referred to as gv). For each genomovar containing at least three genomes, the range of ANI and dDDH is listed in Table 1.

Based on reference strains of defined genomovars, 82.93% genomes (102 out of 123) belonged to 14 known genomovars. gv 1 was the largest group, comprising 47 genomes, followed by gv 3 (13 genomes), gv 7 (8 genomes), gv 8 (7 genomes), gv 5 (7 genomes), gv 6 (4 genomes), gv 2 (3 genomes), gv 14 (3 genomes), gv 15 (3 genomes), gv 4 (2 genomes) and gv 17 (2 genomes); gv 9, gv 16, and gv 19 contained only one strain, respectively (Lalucat et al., 2006). In addition, according to results of MLSA and ANI analysis (Supplementary Figure 2 and Supplementary Table 3), five genomes were most likely assigned to gv 12 and gv 18. For example, four strains (P. stutzeri 273, Pseudomonas sp. 10B238, Pseudomonas sp. Choline-3u-10 and P. zhaodongensis SST2) were grouped with representative strain P. stutzeri MT-1 of gv 18; they should be members of gv 18. The remaining 11 branches each represented an unknown or novel genomovar (Figure 2). Taken together, these results highlight the extremely high level of genetic heterogeneity within the P. stutzeri complex. It speculation that the study of more genomes will uncover more genomovars (Scotta et al., 2013).

To infer the phylogenetic relationship for P. stutzeri complex strains, we built both ML (Figure 2) and NJ (Supplementary Figure 3) phylogenetic trees based on concatenated single-copy core genes, using four closely related strains as an outgroup. These two trees showed accordance with each other in strain grouping (P < 0.05) by Kendall-Colijn test with λ = 0 implemented in R package Treespace (Jombart et al., 2017). Their topology was also similar with that of the phylogenetic tree reconstructed using the GGDC distance matrix (Kendall-Colijn test, P < 0.05) (Supplementary Figure 4). Overall, P. stutzeri complex strains could be divided into two major clades (Figure 2). Clade I comprised 96 genomes covering 15 genomovars. Most of the known genomovars were located in this clade, e.g., gv 1 and gv 5. These two genomovars were most closely related to each other. Interestingly, blastp analysis using the nifH gene (encoding nitrogenase Fe protein) as a query indicated that all 16 strains possessed the potential ability to fix nitrogen. These strains all belonged to Clade 1, mainly belonging to gv1 (Supplementary Table 4). Clade II had 27 genomes covering 12 genomovars; more than half of the unknown genomovars belonged to this clade. In good accordance with the results of ANI and dDDH, the phylogenetic tree based on core genes divided all genomes of the P. stutzeri complex into 27 branches (marked with different colors in Figure 2), and members of the same genomovar clustered together on the same branch (Figure 2). Hence, we conclude that the core-gene-based tree reflected the actual affiliations among strains of the P. stutzeri complex. Our results also robustly support that 25 newly identified genomes belong to P. stutzeri complex, since these strains were mixed well with known P. stutzeri complex strains in the tree, with most of them located in known genomovars (Figure 2 and Supplementary Figure 2), e.g., P. kunmingensis strains. By contrast, in previous studies, phylogenetic analyses of the 16S rRNA gene and four housekeeping genes (ITS1, gyrB, rpoB and rpoD) distinctly placed P. stutzeri complex and P. balearica on different phylogenetic branches (Bennasar et al., 1996; García-Valdés et al., 2010). It can be concluded that the phylogenetic tree based on the whole-genome sequences gives much better resolution than a phylogeny based on several housekeeping genes.

### TABLE 1 | The range of ANI and dDDH values within each genomovar.

| Genomovar* | ANIm Minimum value (%) | ANIm Maximum value (%) | dDDH Minimum value (%) | dDDH Maximum value (%) |
|------------|------------------------|------------------------|------------------------|------------------------|
| gv 1       | 97.31                  | 99.96                  | 73.4                   | 100                    |
| gv 2       | 97.67                  | 97.74                  | 77.9                   | 78.9                   |
| gv 3       | 96.51                  | 99.92                  | 69.6                   | 98.8                   |
| gv 5       | 96.54                  | 98.38                  | 68.6                   | 86.1                   |
| gv 6       | 98.45                  | 98.62                  | 84.6                   | 87.6                   |
| gv 7       | 94.89                  | 98.51                  | 59.9                   | 87.6                   |
| gv 8       | 96.91                  | 98.95                  | 72.4                   | 91.5                   |
| gv 14      | 96.65                  | 98.68                  | 70.7                   | 88.6                   |
| gv 15      | 97.25                  | 97.47                  | 75.5                   | 77.5                   |
| gv 18      | 95.57                  | 99.56                  | 62.7                   | 97.7                   |
| un 1       | 98.22                  | 98.35                  | 83.5                   | 85                     |

*Each genomovar containing at least three genomes is involved in statistical analysis.
species level. In total, there were 16 strains without species names, distributed in nine genomovars, including eight known genomovars. *P. stutzeri* CCUG 16156 is the first sequenced representative of gv 2 and a model organism for denitrification studies (Pena et al., 2012). *Pseudomonas* sp. TTTU2014-105ASC and strain CCUG 16156 had an ANIm value of 97.7% and a dDDH value of 77.9%, demonstrating that strain TTTU2014-105ASC should belong to gv 2. The core-gene phylogeny placed *Pseudomonas* sp. R2A2 and *Pseudomonas* sp. THAF7b within gv 5 group. ANI values between these two strains and the gv 5 strain *P. stutzeri* DNSP21 were > 96.5%, while the dDDH values were > 68.8%. Taken together, the data strongly support assigning strains R2A2 and THAF7b to gv 5. Three strains (*Pseudomonas* sp. HMP271, JL972 and Chol1) had ANI values of ≥ 95.9% and dDDH values of ≥ 65.8% to strain DSM 50238, the reference strain of gv 7. Four strains, *Pseudomonas* sp. GL14, s199, TTTU2014-096BSC and TTTU2014-066ASC clustered with *P. stutzeri* DSM 10701, which is the reference strain of gv 8 and considered as a model organism for natural transformation. These strains shared ANIm and dDDH values with DSM 10701.
of ≥ 97.6% and ≥ 78.7%, respectively. *Pseudomonas* sp. 9Ag belongs to gv 15, since it had an ANI value of 97.3% and a dDDH value of 75.9% to *P. stutzeri* 4C29, the reference strain of gv 15. *Pseudomonas* sp. BAY1663 was assigned to gv 17, as it had an ANIm value of 98.04% and a dDDH value of 82.2% with the gv 17 strain *P. stutzeri* 24a75 (Lalucat et al., 2006). According to MSLA, ANI and dDDH analyses, *Pseudomonas* sp. 10B238 and Choline-3u-10 belonged to gv 18, and *Pseudomonas* sp. IC_126 affiliated to gv 12. *Pseudomonas* sp. SCT and *P. stutzeri* RCH2 grouped together in unknown genomovar un 2.

In addition, our results revealed mistakes in the nomenclature of 12 strains, especially seven *P. xanthomarina* strains, which were assigned into three different genomovars. Group un 5 represented the actual *P. xanthomarina* species, as the type strain *P. xanthomarina* DSM 18231T was located on this branch. *P. xanthomarina* UASWS0955 and *P. stutzeri* DSM 10701 had an ANIm value of 97.2% and a dDDH value of 74.7%. Therefore, *P. xanthomarina* UASWS0955 should be re-assigned as *P. stutzeri* UASWS0955. Four *P. xanthomarina* strains, S11, PX_371, PX_177 and PX_067, belong to gv 3, as they had minimum ANIm of 96.8% and dDDH of 71.1% with *P. stutzeri* CCUG 29243, the reference strain of gv 3. Surprisingly, a number of taxonomic controversies existed within the gv 3 group. This group contained two newly described species, *P. kunmingensis* [type strain *P. kunmingensis* DSM 25974T (Xie et al., 2014)] and *P. chloritidismutans* [type strain *P. chloritidismutans* AW-1T (Wolterink et al., 2002)]; however, the ANI and dDDH for any pair of genomes in this group exceeded the specified threshold for species delineation. This result was in accordance with previous reports for *P. chloritidismutans* (Wolterink et al., 2002; Cladera et al., 2006; Scotta et al., 2013; Mehboob et al., 2016). *Pseudomonas chloritidismutans* has been identified as a novel species because strain AW-1 can dissipate perchlorate or chlorate, which was not found in *P. stutzeri* DSM 50227, the reference strain of gv 3. However, this unique feature is most likely a result of lateral gene transfer in the environment; additionally, dissimilation of perchlorate or chlorate is also found in strains belonging to gv 1, gv 3 and gv 5 (Cladera et al., 2006). One crucial reason for describing *P. kunmingensis* as a novel species was that *P. kunmingensis* DSM 25974T has a DDH value of 38.92% (± 3.44%, standard deviation of three replicates) with *P. stutzeri* ATCC 17588T (= CGMCC 1.1803T), the reference strain of gv 1 (Xie et al., 2014). In fact, both *P. kunmingensis* DSM 25974T and BUM14 have dDDH values of > 70% and ANI values of > 95% with the strains of the gv 3 branch. Therefore, we suggest that a mistake occurred in the nomenclature for *P. chloritidismutans* and *P. kunmingensis*, and representatives of these two taxa should belong to the same species. Each pair of genomes among *P. stutzeri* DW2-1, *P. songnenensis* L103 and *P. songnenensis* NEAU-ST5-5T had an ANIm value > 98% and dDDH > 83%. Hence, *P. stutzeri* DW2-1 should be renamed *P. songnenensis* DW2-1. In the gv 6 group, *P. stutzeri* YC-YH1 and three *P. balearica* strains (LS401, st101 and DSM_6083T) grouped together, supporting assignment of *P. stutzeri* YC-YH1 to *P. balearica*. Our results showed that *P. zhaodongensis* SST2 and *P. zhaodongensis* NEAU-ST5-21T belong to different genomovar branches, as the shared ANI and dDDH values between them greatly below the species cutoff value. This demonstrated mistaken taxonomic nomenclature for *P. zhaodongensis* SST2. Our results could

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**FIGURE 3** | Homologous gene cluster based pan-genome. (A) Composition of the *P. stutzeri* pan-genome. (B) Relative frequency of softcore, shell and cloud pan-genomes for individual genomes; on the X-axis, each vertical bar represented one genome. (C) Simulations of the increase of pan-genome size and decrease of core-genome size. Genomes were sampled as 20,000 random combinations of each given number of genomes. Solid black lines indicate pan- and core-genome curves fitted using points from all random combinations according to the models implemented in PGAP software.
assign some groups to valid species. For example, un 11 contained two strains, the type strain \textit{P. saudiphocaensis} 20\_BN\textsuperscript{T} and \textit{P. saudiphocaensis} PS\_399, suggesting that un 11 should represent \textit{P. saudiphocaensis}.

Collectively, our results conclude that either of the ANI and dDDH indexes enables robust determination of genomic taxonomy for prokaryotic species. However, ANI is much appropriate for re-evaluating the taxonomy of \textit{P. stutzeri} complex, since this species complex has ANI genomovar cut-off for set at approximately 95%, but dDDH genomovar cutoff value greatly below 70% (Meier-Kolthoff et al., 2013; Colston et al., 2014). Furthermore, dDDH can only be calculated through a Web-based interface that requires manually uploading genome sequences, while ANI can be easily analyzed through many standalone tools, such as Jspecies (Richter and Rossello-Mora, 2009), OrthoANI (Lee et al., 2016) and OrthoANIu (Yoon et al., 2017). In addition, NCBI developed a protocol for using ANI genome neighboring statistics in conjunction with reference genomes from type and proxytype to improve taxonomic assignments (Federhen et al., 2016; Ciufo et al., 2018). Consequently, we recommend ANI as it is more time-effective when it comes to large comparisons (Richter and Rossello-Mora, 2009; Colston et al., 2014).

**Pan- and Core-Genome Within \textit{Pseudomonas stutzeri} Complex Genomes**

Based on protein sequence similarity analysis using OrthoFinder, the 123 \textit{P. stutzeri} complex genomes possessed a pan-genome size of 13,261 homologous protein clusters. We categorized clusters in the pan-genome according to their frequency of presence (Koonin and Wolf, 2008): 1,104 strict core gene clusters shared by all genomes, 2,476 softcore gene clusters shared by more than 95% genomes (118–123 genomes), 5,663 shell gene clusters shared by 2–95% genomes (3–117 genomes), and 5,122 cloud gene clusters present in only one or two genomes (Figure 3A). This classification system can overcome some of the technical uncertainty, particularly for the softcore, where strict core genes could be placed if they were missed from draft genomes (Gordon et al., 2017). The strict core genes and softcore genes constituted only 8.33% and 18.67% of the pan-genome, respectively. These analyses may lead to better understanding of the minimal set of genes for \textit{P. stutzeri} complex. The relatively small size of the core genome reflects the high genetic diversity of \textit{P. stutzeri} complex. Of the pan-genome clusters, 81.3% belonged to accessory genes (shell and cloud), which may be vital for this species in occupying diverse habitats and ecological niches. The high fraction of shell genome (42.7% overall) is key for understanding genome dynamics of \textit{P. stutzeri} complex because it reflects how genes present at intermediate frequencies drive adaptation of species. Furthermore, a previous study showed that proportion of shell genes is independent of genome size (Gautreau et al., 2020). Relative proportions of the three gene categories per genome were relatively constant within \textit{P. stutzeri} complex. On average, each individual genome was composed of 62.80% ± 3.32% (mean and standard deviation) softcore pan-genes, 35.83 ± 3.01% shell genes and 1.37 ± 1.20% cloud genes (Figure 3B). Several genomes had more than twice (and even four times, e.g., \textit{P. stutzeri} strain B1SMN1 and \textit{P. stutzeri} 19) the average proportion of cloud genes. It appeared to be partly explained by a large genome size for these genomes, driven by large gene gain and small gene loss events. For example, of 123 genomes, \textit{P. stutzeri} strain B1SMN1 has the largest genome size, encompassing many more...
genes, and recently experienced 859 gene gain and 77 gene loss events (see below).

Modeling of pan-genome size by iterative random sampling of genomes showed that members of *P. stutzeri* displayed an open pan-genome. The gene accumulation curve for an increasing number of genomes fitted the power law, \( y = ax^{0.316} + b \) (95% confidence interval for \( a = 2630.59 \pm 1.21 \), and \( b = 1166.33 \pm 5.14 \), R-square of curves fitting = 0.9998) (Figure 3C). Approximately 31 to 644 new genes would be added to each new genome, resulting in an open pan-genome whose size shows a tendency to increase with the addition of new genomes. An open pan-genome is likely to be typical in species that colonize multiple environments and have multiple ways of exchanging genetic material. It is largely responsible for the ability of *P. stutzeri* complex to adapt to different ecological niches. The gene occurrence plot showed a closed core-genome with a finite number of core genes for *P. stutzeri* complex, which fitted well with the exponential law, \( y = ae^{-0.03x} + b \) (95% confidence interval for \( a = 1980.84 \pm 7.00 \), and \( b = 1147.46 \pm 2.31 \), R-square of curves fitting = 0.9582) (Figure 3C).

We characterized the functions of pan-genome genes using COG functional categories (Galperin et al., 2019). The core proteomes (strict core and softcore) carried a large proportion of genes involved in J (Translation, ribosomal structure and biogenesis), E (Amino acid transport and metabolism), and M (Cell wall/membrane/envelope biogenesis) (Fisher’s exact test, \( p < 0.001 \), Supplementary Figure 5). The accessory proteomes (shell and cloud) had more than twice the number of genes associated with categories X (Mobilome: prophages, transposons) and V (Defense mechanisms) compared with their respective core proteomes (Fisher’s exact test, \( p < 0.001 \)). These two functional categories reflect the dynamics of *P. stutzeri* complex genomes (Richter and Rossello-Mora, 2009; Galperin et al., 2019). Functional analysis of the pan-genome showed that approximately 45% of pan-genome genes (5907/13261) do not match any sequence present in the COG database for *P. stutzeri* complex. These poorly characterized genes mainly belonged to the cloud genes (3719 out of 5122) and shell genes (1974 out of 5663). The proportion of poorly characterized genes is larger than that in *P. aeruginosa* (Freschi et al., 2019). These results highlighted that our knowledge of the functions of *P. stutzeri* complex genes is still far from being complete, and more investigations need to be performed for this species complex.

### Size Variation of Gene Families

We used Count software with a birth-death likelihood model to delineate the dynamics of genome content across the genealogy of *P. stutzeri* complex. Results revealed substantial gene gain and loss events occurred in *P. stutzeri* complex genomes (Figure 4). Most of the gene gain events occurred in genes belonging to accessory clusters (shell and cloud genes). In contrast, nearly all gene loss events occurred in genes belonging to shell and softcore categories (Supplementary Table 5). This demonstrates that shell genes are the result of complex histories characterized by frequent events of gain and losses (Brito et al., 2018). Compared with internal phylogenetic nodes, most gene gain and loss events occurred at terminal nodes. This result is fully compatible with previous findings indicating that genes are gained and lost at higher rates on the tips of phylogenetic trees (Hao and Golding, 2006). Overall, gain of gene families dominates the evolution of *P. stutzeri* complex, occurring at more than three times the rate of loss. This suggested that gene gains contribute more in driving the divergence of genome content and pan-genome expansion. High rates of gene gain appear to coincide with the ability for natural transformation in many *P. stutzeri* complex strains. Nine strains had suffered from gene gain/loss events affecting more than 16% gene content: *P. stutzeri* B1SMN1, *P. stutzeri* 19, *P. stutzeri* KOS6, *P. chloritidismutans* AW-1, *P. xanthomarina* PX_177, *Pseudomonas* sp. BAY1663, *P. stutzeri* YC-YH1, *P. stutzeri* TS44 and *P. stutzeri* PM101005. Bacterial genomes can be highly variable, with loss and gain of genes greatly affecting the evolution of bacterial populations. A previous study using mathematical modeling showed that extensive gene gain and loss within the accessory genome pushes prokaryotes beyond the homologous recombination barrier and accelerates genome sequence divergence, resulting in speciation (Iranzo et al., 2019). We conclude that gene gain and loss is the central factor driving the genetic diversity of *P. stutzeri* complex and the subsequent adaptation of this species complex to diverse ecological niches (Rodríguez-Valera et al., 2016).

When tracing the origin of cloud genes, Blastp analysis querying the NCBI non-redundant database identified best hits

### TABLE 2 | Taxonomic characterization of best blastp hits for cloud genes,

| Taxonomic status   | Number |
|--------------------|--------|
| Archaea            | 1      |
| Eukaryota          | 7      |
| Viruses            | 11     |
| Unclassification   | 42     |
| Bacteria           | 88     |
| Proteobacteria     | 4,076  |
| γ-proteobacteria   | 3,666  |
| Pseudomonadales    | 3,215  |
| Pseudomonadaceae   | 3,166  |
| Pseudomonas        | 3,095  |

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for 4225 out of 5122 genes (Table 2). The taxonomy of the best hits indicated that 96.47% of cloud genes had a close homolog within the phylum Proteobacteria, 73.25% within the genus Pseudomonas. These results showed that most genes acquired were obtained from closely related species through horizontal gene transfer (HGT). We also identified 88 genes obtained...
from distant taxa. These genes have closest homologs in other phyla, mainly Firmicutes (18 genes) and Bacteroidetes/Chlorobi group (10 genes) (Table 2). These genes were most likely obtained through HGT, allowing their hosts to exploit new habitats (Moulana et al., 2020). In addition, we found no significant blastp hits for 897 genes in the NCBI non-redundant database, representing 17.51% of the cloud genome. This result is compatible with the observation that a large proportion of cloud genes could not be assigned to any known COG functional category. No significant blastp hits for many cloud genes could be explained that as with the large amount of genomic data available today, many genes are uncharacterized.

Genotypic Diversity Within Pseudomonas stutzeri Complex Genomes

We used the fraction of homologs shared between pairs of genomes to reflect genetic diversity. As shown in Figure 1B. We found that the fraction of homologs correlates (Spearman’s 𝜌 = 0.77, 𝑃 = 2.2e-16) with the evolutionary distance measured by ANI (Konstantinidis and Tiedje, 2005), which is consistent with results reported previously based on other bacterial groups (Konstantinidis et al., 2006, 2009; Jain et al., 2018). To a large extent, the genotypic similarity between two genomes may be predicted from their evolutionary distance. However, a larger correlation between percentage of homologs and ANI was observed for pairs of genomes from different genomovars (Spearman’s 𝜌 = 0.63, 𝑃 = 2.2e-16) than those within the same genomovar (Spearman’s 𝜌 = 0.22, 𝑃 = 1.858e-15). Meanwhile, overall, pairs of genomes within the same genomovar had significantly higher fractions of homologs than those within different genomovars (𝑃 < 0.01). Correspondingly, strains within the same and different genomovars differed in 0.04–19.22% and 6.42–34.17% of their total genes, respectively, revealing extensive genetic diversity within P. stutzeri complex. The fraction of homologs between and within genomovars partially overlapped. For inter-genomovar genomes, some pairs of genomes with less than 95% identity by ANI showed relatively large percentages of homologs, e.g., > 90%. This may be due to close affiliations among these strains, as most of these genomes clustered together in Clade II and had short branch lengths compared with other genomes. Some pairs of genomes within the same genomovar had a relatively low percentage of homologs, mainly due to the high frequency of gene gain and loss events occurring in these genomes, such as P. stutzeri BISMN1 and P. stutzeri 19 in gv1, and P. chloritidismutans AW-1 and P. xanthomarina PX_177 in gv3. For instance, P. stutzeri BISMN1 obtained 859 genes and lost 77 genes, accounting for 17.25% and 1.55% of its total predicted protein-coding genes, respectively (Figure 4). Extensive gene gain and loss events occurring in P. stutzeri complex genomes produced inconsistencies in the relationship between the fraction of homologs and evolutionary relatedness.

We speculate that the high genetic diversity reflects the many and different ecological niches potentially occupied by members of P. stutzeri complex, and that the genomic groups that have been described so far are evolving in a speciation process that may give rise to 27 novel species from a genomic point of view.

Distribution of the Ectoine Biosynthesis Gene Cluster in Pseudomonas stutzeri Complex

Blastp analysis showed that out of 9,548 Pseudomonas genomes, 553 genomes carried ectC genes. However, most of these genomes contained an orphan ectC, and only 165 genomes possessed the entire ectoine biosynthesis gene cluster, accounting for 1.73% of the total Pseudomonas genomes used in this study (data not shown). It was noted that ectA or ask_ect gene was missing from some genomes out of 165 genomes. A surprising finding was that all 123 P. stutzeri genomes contained the ectoine biosynthesis gene cluster, except for six genomes lacking the ectA gene, possibly resulting from microevolution as these genomes were located in the same branch (Figure 5). This result indicated ect cluster was mainly distributed in P. stutzeri complex, compared with other Pseudomonas species. According with above result, many P. stutzeri complex strains can grow in high concentrations of NaCl, such as 7.02% (w/v) NaCl for P. stutzeri TS44 (Li et al., 2012), 8% (w/v) NaCl for P. xanthomarina (Romanenko et al., 2005), and 8.5% (w/v) NaCl for P. borealis (Bennasar et al., 1996). The ect genes and their flanking genes displayed high levels of similarity and conserved synteny among P. stutzeri complex strains. In addition, we observed good coherence between the phylogeny and organization of ect genes and corresponding flanking genes (Figure 5). Compared with P. aeruginosa DSM 50071, ect cluster seems to be obtained by insertion at the site near to zwf gene, encoding glucose-6-phosphate dehydrogenase (Ma et al., 1998). We suspect that obtaining the ect cluster was an early evolutionary adaptation for the ancestor of P. stutzeri complex, after it split from other members of the Pseudomonas genus. Taken together, our results provided strong evidences that the ancestor of P. stutzeri complex originated from a high-salt habitat.

CONCLUSION

Deep bioinformatics is a powerful tool for clarifying the taxonomic status and nomenclature of P. stutzeri complex. We revealed that a large proportion of P. stutzeri complex genomes (33 out of 123 genomes) are currently misidentified, including 25 genomes submitted as other Pseudomonas in the NCBI genome database that actually belong to P. stutzeri complex strains, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name. All 123 sequenced genomes could be ascribed to 27 genomovars, and that 8 out of 98 genomes have the incorrect species name.
genomes and their evolutionary distance. We also revealed that all P. stutzeri complex genomes harbor an ectoine gene cluster, possibly supporting a high-salt origin for this species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

XL conceived and designed the study and performed the bioinformatic analyses. XL, ZY, WL, and GZ analyzed all the data. XL, ZW, and HY wrote the original draft of the manuscript. XL and ZY supervised the bioinformatic analysis work and reviewed and edited the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.755874/full#supplementary-material

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