Integrated Comparative Genomic Analysis and Phenotypic Profiling of *Pseudomonas aeruginosa* Isolates From Crude Oil

Anming Xu1,2†, Di Wang1†, Yichen Ding3, Yaqian Zheng1,2, Bo Wang1,2, Qing Wei1, Shiwei Wang1, Liang Yang4 and Luyan Z. Ma1,2*

1 State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, 2 University of Chinese Academy of Sciences, Beijing, China, 3 Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Jurong West, Singapore, 4 School of Medicine, Southern University of Science and Technology, Shenzhen, China

*Pseudomonas aeruginosa* is an environmental microorganism that can thrive in diverse ecological niches including plants, animals, water, soil, and crude oil. It also one of the microorganism widely used in tertiary recovery of crude oil and bioremediation. However, the genomic information regarding the mechanisms of survival and adaptation of this bacterium in crude oil is still limited. In this study, three *Pseudomonas* strains (named as IMP66, IMP67, and IMP68) isolated from crude oil were taken for whole-genome sequencing by using a hybridized PacBio and Illumina approach. The phylogeny analysis showed that the three strains were all *P. aeruginosa* species and clustered in clade 1, the group with PAO1 as a representative. Subsequent comparative genomic analysis revealed a high degree of individual genomic plasticity, with a probable alkane degradation genomic island, one type I-F CRISPR-Cas system and several prophages integrated into their genomes. Nine genes encoding alkane hydroxylases (AHs) homologs were found in each strain, which might enable these strains to degrade alkane in crude oil. *P. aeruginosa* can produce rhamnolipids (RLs) biosurfactant to emulsify oil, which enables their survival in crude oil environments. Our previous report showed that IMP67 and IMP68 were high RLs producers, while IMP66 produced little RLs. Genomic analysis suggested that their RLs yield was not likely due to differences at genetic level. We then further analyzed the quorum sensing (QS) signal molecules that regulate RLs synthesis. IMP67 and IMP68 produced more N-acyl-homoserine lactones (AHLs) signal molecules than that of PAO1 and IMP66, which could explain their high RLs yield. This study provides evidence for adaptation of *P. aeruginosa* in crude oil and proposes the potential application of IMP67 and IMP68 in microbial-enhanced oil recovery and bioremediation.

**Keywords:** *Pseudomonas aeruginosa*, crude oil, alkane hydroxylase, rhamnolipids, N-acyl-homoserine lactones
INTRODUCTION

Microorganisms can survive in many harsh environments, such as glaciers, hot springs, and crude oil. In order to survive in these harsh environments, bacteria usually evolve a series of corresponding functional proteins or metabolic systems through adaptive evolution including gene mutation and horizontal gene transfer (HGT) (Ollivier and Magot, 2005). Crude oil is a complex mixture of thousands of compounds that is difficult to be utilized and even toxic for most of bacteria (Xu et al., 2018). Interestingly, bacteria such as Pseudomonads can survive in such harsh environment. Alkanes are saturated hydrocarbons of different carbon-chain length and structure, which are the major components of crude oil. Although they are chemically inert, most of them can be efficiently degraded by several microorganisms (Rojo, 2009; Sun et al., 2018). It has been reported that more than 60 genera of aerobic bacteria and 5 genera of anaerobic bacteria are able to degrade n-alkanes (Prince, 2005).

Alkane hydroxylases (AHs), which catalyze the first step of alkane biodegradation, are the key enzymes in aerobic degradation of alkanes by bacteria. These enzymes hydroxylate alkanes to alcohols, which are further oxidized to fatty acids. Degradation of alkanes by bacteria. These enzymes hydroxylate alkanes to alcohols, which are further oxidized to fatty acids. Alkane hydroxylases (AHs), which catalyze the first step of alkane biodegradation, are the key enzymes in aerobic degradation of alkanes by bacteria. These enzymes hydroxylate alkanes to alcohols, which are further oxidized to fatty acids. Alkane hydroxylases (AHs), which catalyze the first step of alkane biodegradation, are the key enzymes in aerobic degradation of alkanes by bacteria. These enzymes hydroxylate alkanes to alcohols, which are further oxidized to fatty acids.

Among them, P. aeruginosa strains have been isolated from crude oil, including P. aeruginosa IMP66, IMP67, and IMP68, which exhibited significant different phenotypes in RLs and pyocyanin production (Das et al., 2014). In our previous work, we reported the identification of three rhamnolipid-producing Pseudomonas isolates from crude oil (named as IMP66, IMP67, and IMP68), which exhibited significant different phenotypes in RLs and pyocyanin production (Das et al., 2014). The 16s rRNA analysis has suggested that two of them (IMP67 and IMP68) are P. aeruginosa and IMP66 is identified as Pseudomonas sp. Pyr41 (Das et al., 2014). In this study, we performed the comparative genomic analysis for these three Pseudomonas strains to further understand their genetic background. Our analysis indicated that all three strains belong to P. aeruginosa species. Subsequent analysis showed that these three isolates exhibited a high degree of individual genome plasticity along with abundant numbers of alkane hydroxylase encoding genes, conferring survival advantages for P. aeruginosa strains living in the crude oil environment. Moreover, the strains with high yield of RLs produce large amounts of QS signal molecules. This study illustrates the environmental adaptability of P. aeruginosa, and proposes potential applications of these strains in bioremediation as well as in microbial-enhanced oil recovery.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

IMP66, IMP67, and IMP68 were isolated from the crude oil of Karamay W#8805, Xin-Jiang province, China. Unless otherwise indicated, P. aeruginosa strains were grown in LB without sodium chloride (LBNS) or basal salt medium (BSM, K2HPO4 3.815 g, KH2PO4 0.5 g, (NH4)2HPO4 0.825 g, KNO3 1.2625 g, Na2SO4 0.2 g, CaCl2 0.02 g, FeCl3 0.002 g, MgCl2 0.02 g/l) at 37°C. Culture was supplemented with 300 µg/ml carbenicillin to select and maintain plasmid-carrying P. aeruginosa. A plasmid-borne fusion of the QS-controlled rhlAB promoter sequence (423 bp)
and GFP coding sequence was used to assess the promoter activity of rhlAB in this study. Primers P<sub>rhlAB</sub>-F (5′-ccgagagagcggtcag-3′) and P<sub>rhlAB</sub>-R (5′-ttccacatctaaaaatttctaac-3′) were used to generate an amplicon of the rhlAB promoter by 1-5™ High-Fidelity DNA Polymerase (Tsinke Biological Technology). The PCR product was purified and ligated into pPROBE-AT vector (Miller et al., 2000) through Gibson assembly (Gibson et al., 2010). Recombinant plasmid pTH22 (rhlAB:gsfp) was then transformed into <i>Escherichia coli</i> DH5α and subsequently into <i>P. aeruginosa</i> for determination of the transcription of rhlAB.

**Genome Assembly and Annotation**

Genomic DNA was isolated from an overnight broth culture using the Wizard genomic DNA purification kit (Promega, Madison, WI, United States). A spectrophotometer (NanoDrop 2000 UV-Vis; Thermo Scientific, MA, United States) was used to determine the DNA quantity and ensure the DNA quality was suitable for sequencing. Whole-genome sequencing was performed using the long-read PacBio SMRT sequencing (Pacific Bioscience) platform and the Illumina HiSeq sequencer. Genome sequences were de novo assembled using HGP assembly protocol, which is available with the SMRT Analysis packages and accessed through the SMRT Analysis Portal version 2.1. The pilon v1.5 was then used to correct the assembly (Walker et al., 2014). Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAAP)<sup>1</sup> (Tatusova et al., 2016). The circular genome map of the three strains including all predicted ORFs with COG functional assignments, rRNA, tRNA, G + C content, and GC skew information were generated using CGview (Stothard and Wishart, 2004).

**Phylogenetic Analysis**

The evolutionary position of the three strains relative to other pathovars and species of <i>Pseudomonas</i> was determined by multilocus sequence analysis (MLSA) as described previously (Loper et al., 2012). For phylogenetic annotation, ten conserved housekeeping genes (<i>accA</i>-<i>aroE</i>-<i>dnaE</i>-<i>guaA</i>-<i>gyrB</i>-<i>mutL</i>-<i>ppsA</i>-<i>pyrC</i>-<i>recA</i>-<i>ppsB</i>) from 51 closely related <i>Pseudomonas</i> spp. were retrieved from <i>Pseudomonas</i> Genomic Database (Winsor et al., 2016). Maximum Likelihood analysis was performed for 100 bootstrap replications in RAxML. The final phylogenetic tree was constructed using the iTOLs software (Letunic and Bork, 2016).

In order to detect the strain-level genome content of the three strains, a rapid large-scale prokaryote pan genome analysis by Roary was performed to identify the conserved (core) and non-conserved (accessory) genes (Page et al., 2015), and then created a concatenated core genome for subsequent phylogenetic analysis. 90 closely related <i>Pseudomonas</i> spp. retrieved from <i>Pseudomonas</i> Genome Database (Winsor et al., 2016) were used for the core-genome estimating by Roary bacterial genome analysis pipeline. A cut-off with 90% identity was used and the core genes were defined as those presented in 99% of isolates to generate a multi-FASTA alignment file. These data were then used as the input to the next iteration. RAxML was then run over the final multi-FASTA alignment to provide a high-quality phylogenetic tree in newick format. We used a generalized time reversible (GTR) model with gamma (Y) heterogeneity across nucleotide sites and 100 bootstrap replicates. The final phylogenetic tree was constructed using the iTOLs software (Letunic and Bork, 2016).

**Comparative Genome Analysis**

The MicroScope platform (Vallenet et al., 2016) provided some of the tools used for the comparative genomic analyses, such as the RGP Finder for determination of regions of genomic plasticity (RGP). RGP were defined as DNA segments over 5 kbp possibly related to events of horizontal exchanges, and they were identified using a series of constraints such as G + C deviation, compositional biases as measured by the tools Alien-Hunter (Vernikos and Parkhill, 2006) and SIGI-HMM (Waaack et al., 2006), synteny breaks and proximity to tRNAs. Pan-genome analysis including the genes shared by all these four strains was performed by an “all-against-all” BLAST of the protein sequences of the above genomes using the EDGAR 2.3 software framework (Blom et al., 2016). The genes aligned based on amino acid sequences were used for identifying orthologs. For orthology estimation, EDGAR used a generic orthology threshold calculated from the similarity statistics of the compared genomes, and then generated the venn diagram.

**AHLs Extraction and Quantification**

Extraction and quantification of AHLs were based on a previously described method (Ortori et al., 2011). Briefly, strains were grown in LBNS media for 24 h, 1 ml of bacterial cells were spun down and supernatants were harvested for AHLs extraction. Acidified ethyl acetate was used for AHLs extraction and the extraction was repeated three times. The organic layers were combined and evaporated under vacuumrotary evaporation. Residues were dissolved in 50 μl methanol and subjected to LC-MS/MS analysis using mass spectrometer (QTRAP 6500 System, AB SCIEX). The calibration curve was prepared from a linear dynamic range (from 0.33 to 33.3 μM) of standard 3-oxo-C12-HSL (Sigma-Aldrich, Germany).

**RESULTS**

**Phylogenetic Analysis of Newly Sequenced Strains With the Other <i>Pseudomonas</i> spp.**

IMP66, IMP67, and IMP68 were isolated from the crude oil of Karamay W#8805, Xin-Jiang province, China. The complete genomes of these three strains were sequenced and assembled. A previous report suggested that only IMP67, and IMP68 were <i>P. aeruginosa</i> based on 16S rRNA analysis. It has been shown that the phylogenetic tree based on 16S rRNA lacked resolution at intrageneric level (Anzai et al., 2000). We then used the ten house-keeping genes to further analysis the phylogenetic position of these three strains. A phylogenetic tree was plotted using a maximum likelihood (ML) approach based on multilocus sequence analysis of 54 representative <i>Pseudomonas</i> spp. strains, including 51 strains with complete genome sequence and
these three newly sequenced strains (IMP66, IMP67, and IMP68). E. coli K12 was used as an out-group. Four tightly monophyletic groups could be identified from the phylogenetic tree based on the 10 concatenated housekeeping genes (Figure 1, SC1 to SC3, and SC5). Strains IMP66, IMP67, and IMP68 were all clustered together within the group of P. aeruginosa and were all identified as P. aeruginosa strains (Figure 1).

In order to investigate the evolutionary history of these three newly sequenced strains, we reconstructed the phylogeny of the genus using concatenated core genomes from 90 P. aeruginosa isolates, including representative P. aeruginosa strains with completely genomes (such as PAO1, PA14, and PA7), IMP66, IMAP67, and IMP68, as well as several crude oil-isolated P. aeruginosa strains (Pb18, M8A1, M8A4, M28A1, SJTD-1, DQ8). There were 51,389 SNPs identified in the 820 core genes of the 90 isolates. P. aeruginosa strains were divided into three major clades (Figure 2A), which was consistent with the previous reports (Freschi et al., 2015; Castañeda-Montes et al., 2018). Interestingly, all crude oil isolates were consistently distributed in clade 1, which was represented by strain PAO1 and contained the strains from a wide range of sources, including natural environment and clinical settings (Figure 2A). Although most of the strains from crude oil were randomly distributed within clade 1, strain IMP66, IMP67, and IMP68 were clustered together (Figure 2B), which was consistent with the results of housekeeping gene analysis (Figure 1). Taken together, our data suggested that these three P. aeruginosa strains were closely related in the evolutionary process.

Comparative Genomic Analysis of IMP66, IMP67, IMP68, and PAO1

IMP66, IMP67, and IMP68 were similar in genome size, with a single chromosome of about 6.5 Mb (Table 1 and Figure 3A). The three strains had the same G + C% content of 66.4% (Table 1), which was consistent with other P. aeruginosa strains. The total numbers of coding sequences (CDSs) in the three strains were close to 6100, which were much higher than that of PAO1. We also performed a general comparison of the genomic features including genome size, GC content, and predicted number of CDSs among several crude oil sourced strains and well-studied strain PAO1 (Table 1). The results showed that most of the crude oil isolates harbored bigger genome size than PAO1 strain except SJTD-1. However, SJTD-1 possessed about 200 more CDSs than PAO1 even with similar genome size. These results suggested that genome expansions might confer survival advantages for these isolates living in crude oil.

We then analyzed the group of shared and unique genes between IMP66, IMP67, IMP68, and PAO1. All the shared and unique genes were determined and visualized by EDGAR platform. The four bacteria shared a conserved core genome comprising of 5,376 CDSs. PAO1 possessed 168 distinct singletons, which was the largest number of singletons among these four strains. Interestingly, there were 592 unique genes shared by IMP66, IMP67, and IMP68 (Figure 3B). The largest portion (46 genes) of the 592 unique
FIGURE 2 | Phylogeny of P. aeruginosa population structure. Unrooted (A) and circular (B) maximum likelihood trees of three newly sequenced crude oil isolates combined with 87 representative P. aeruginosa isolates from diverse niches are generated from multiple-sequence alignment with 51,389 SNPs from 820 universally conserved (core) genes. The phylogenetic trees are constructed using RAxML with GTR + $\gamma$ nucleotide substitution model and 100 bootstrap replicates. Strains are divided into three major clades. Clade 2 is contracted for visualization purpose. A framed miniature of the true appearance of this tree is presented. Representative crude oil sourced P. aeruginosa strains are highlighted in yellow.
| Genome features | PAO1 | IMP66 | IMP67 | IMP68 | Pb18  | M8A1  | M8A4  | M28A1 | SJTD-1 |
|----------------|------|-------|-------|-------|-------|-------|-------|-------|--------|
| Size (bp)      | 6,264,404 | 6,486,336 | 6,500,677 | 6,481,180 | 6,401,521 | 6,368,297 | 6,349,902 | 6,499,441 | 6,243,825 |
| CDS (total)    | 5697  | 6077  | 6096  | 6070  | 5,823  | 5,803  | 5,766  | 5,913  | 5,808  |
| CDS (coding)   | 5572  | 5996  | 6007  | 5991  | 5,684  | 5,687  | 5,671  | 5,772  | 5,761  |
| G + C (%)      | 66.6  | 66.4  | 66.4  | 66.4  | 66.2   | 66.4   | 66.4   | 66.1   | 66.5   |

*The features identified for each category were retrieved from NCBI after submission and annotation of genome data.

### Figure 3

(A) Circular chromosome map of *P. aeruginosa* IMP66, IMP67, and IMP68. Pan-genome analysis of three crude-oil sourced *P. aeruginosa* isolates and PAO1. (B) The unique and shared genes among *P. aeruginosa* IMP66, IMP67, IMP68 and PAO1 are performed using the EDGAR software platform based on the orthology analysis. The results are visualized in four degrees. Degree one represents the distinct singletons harbored by each single strain; degree two represents the genes shared by every two strains; degree three represents the genes shared by every three strains; degree four is the number of core genes shared by all the four strains. (C) COG annotation of all 592 exclusive CDSs shared by these three *P. aeruginosa* isolates.

Genes belonged to mobilomes (prophages and transposons, category X) according to the COG annotation. Other major unique genes were in category K (transcription), category L (replication, recombination, and repair), and category M (cell wall/membrane/envelope biogenesis), respectively. A small portion of genes were related to energy production and conversion, amino acid transport, and metabolism, etc (Figure 3C). The mobilome genes are commonly associated with HGT as they often carry transposons, phage fragments, and IS elements. These results suggested that HGT might commonly occur in these three strains. To further investigate HGT events in these three strains, the MicroScope platform...
was used to compare the genomes of IMP66, IMP67, IMP68 with PAO1. We selected IMP66 genome as the representative reference (the same results were obtained when using IMP67 or IMP68) of these three strains against the query genome of strain PAO1. A total of 23 conserved RGPs were identified in IMP66 (Figure 4A and Table 2). Most of these regions contained the sequences related to transposable elements and were flanked by genes coding for tRNAs or phage-structures (Table 2), indicating the possible horizontal gene acquisition. Although most of these regions encoded proteins of unknown functions, some RGPs with important genomic information were identified. RGP9 carried an insertion sequence at its 5’-end (IS3 family, DBX28_13665) and harbored CDSs with predicted functions that belonging to type I-F CRISPR-Cas system. RGP1 is a 48.9 kb genomic element with 56 CDSs including the genes for IS3 family transposases and a possible alkane degradation cluster with a long-chain alkane hydroxylase (almA, DBX28_14785). A noteworthy plasticity region with an aromatic ring-hydroxylating dioxygenase (DBX28_20755) was found in RGP11 (Table 2), which might provide competitive advantages for these three strains living in crude-oil areas. A large number of RGPs detected in IMP66, IMP67, and IMP68 indicated a complex exchange pattern of genetic material segments that occurred during the chromosome reshaping process throughout the evolutionary history.

### Analysis of Genes Involved in Alkane Degradation

Alkane hydroxylases (AHs) catalyzes the first step of alkane degradation and is involved in an AH system that includes electron transport protein rubredoxin and rubredoxin reductase. In the present study, whole-genome screening was used for identification of alkB, P450, almA, and ladA from IMP66, IMP67, and IMP68 through blasting against the reported AHs. We observed that the three strains carried up to 9 AH-encoding genes including two alkB homologous genes, three P450 homologous genes, two almA homologous genes and two ladA homologous genes (Figure 4B). As the “persistent genome” is generally considered to be more stable replicons, it is not surprising that most of the alkane hydroxylase genes were located in the “persistent genome”. Interestingly, two alkane hydroxylases were found in the “shell genome” (an almA gene in RGP10, and a cytochrome P450 gene in RGP14) (Figure 4B and Table 2). RGP10 was later identified to be a possible alkane degradation cluster that

### Table 2 | Regions of Genomic Plasticity (RGPs) identified in the chromosome of IMP66, IMP67, and IMP68.

| Region | Coordinates | Length (kbp) | No. of CDSs | G + C% | Features | Best hit (% coverage, % identity)* |
|--------|-------------|-------------|-------------|--------|----------|----------------------------------|
| RGP1   | 290261–302956 | 12966       | 22          | 57.9   | tRNA     | P. aeruginosa T63266 (100%, 100%) |
| RGP2   | 631096–652201 | 21106       | 35          | 57.8   | tRNA, transposase, integrase       | P. aeruginosa PAE14, 119 (44%, 94.1%) |
| RGP3   | 1730348–1738782 | 8435      | 15          | 56     | tRNA     | P. aeruginosa T63266 (100%, 99.9%) |
| RGP4   | 1975456–1982437 | 6982      | 14          | 49.9   | unknown function                    | P. aeruginosa PK (100%, 99.9%) |
| RGP5   | 2339727–2346716 | 6990      | 22          | 65.5   | tRNA     | P. aeruginosa T63266 (100%, 100%) |
| RGP6   | 2366073–2406282 | 40210     | 64          | 63.5   | integrase, phage                    | P. aeruginosa F63912 (88%, 97.4%) |
| RGP7   | 2461950–2475724 | 13775     | 18          | 57.3   | tRNA, transposase                   | P. aeruginosa T63266 (100%, 100%) |
| RGP8   | 2798834–2807221 | 8388      | 28          | 49.5   | Transposase                          | P. aeruginosa T63266 (100%, 99.9%) |
| RGP9   | 2858781–2870683 | 11923     | 20          | 62.5   | transposase, type I-F CRISPR        | P. aeruginosa PABL017 (100%, 94.6%) |
| RGP10  | 3122707–3171699 | 48993     | 56          | 61.9   | alkane hydroxylase                  | P. aeruginosa T63266 (100%, 100%) |
| RGP11  | 4391909–4404142 | 12234     | 21          | 61.8   | Aromatic ring-hydroxylating dioxygenase subunit alpha, acetyltransferase | P. aeruginosa T63266 (100%, 100%) |
| RGP12  | 5104668–5141335 | 36668     | 65          | 64.5   | transposase, phage                  | P. aeruginosa JD024 (90%, 97.3%) |
| RGP13  | 5178764–5291282 | 112519    | 148         | 60.8   | tRNA, transposase, type II          | P. aeruginosa B136-33 (69%, 97.4%) |
| RGP14  | 6456744–6463635 | 6892      | 15          | 60     | cytochrome P450, isopenicillin N    | P. aeruginosa T63266 (100%, 100%) |
| SIGIVOM1 | 280000–283595 | 3586       | 11          | 60.4   | transposase                          | P. aeruginosa T63266 (100%, 100%) |
| SIGIVOM2 | 731236–769656 | 38421     | 62          | 58.9   | tRNA                                | P. aeruginosa SJTD-1 (100%, 99.9%) |
| SIGIVOM3 | 1287864–1292500 | 4637   | 13          | 58.2   | unknown function                    | P. aeruginosa M8A1 (100%, 99.7%) |
| SIGIVOM4 | 1869178–1874512 | 5335    | 16          | 47.3   | diguanylate phosphodiesterase       | P. aeruginosa T63266 (100%, 100%) |
| SIGIVOM5 | 3479225–3484687 | 5483    | 14          | 45.5   | unknown function                    | P. aeruginosa T63266 (100%, 100%) |
| SIGIVOM6 | 4501618–4515000 | 13383   | 23          | 59.6   | tRNA, type I fimbrial protein       | P. aeruginosa M8A1 (100%, 99.8%) |
| SIGIVOM7 | 4522500–4525211 | 2712     | 13          | 63.1   | unknown function                    | P. aeruginosa T63266 (100%, 100%) |
| SIGIVOM8 | 4669092–4686046 | 19655    | 39          | 57.9   | integrase, phage                    | P. aeruginosa T63266 (77%, 100%) |
| SIGIVOM9 | 5590764–5596000 | 4237     | 19          | 57.1   | phage                               | P. aeruginosa UCBPP-PA14 (67%, 97.8%) |

*BLASTN performed against the “nr” database at the NCBI website.
FIGURE 4 | Whole genome comparison of IMP66, IMP67, IMP68, and PAO1. (A) Analysis of regions of genomic plasticity among IMP66, IMP67 and IMP68 (IMP66 was used as a representative of the three strains). The innermost ring represents the G + C skew of IMP66, followed by its G + C content. Light green, dodger blue and light blue rings that follow depict BLASTN comparisons between the genome of IMP66 and those of strains PAO1, IMP67, and IMP68, respectively. The red ring represents the core genome of the four strains. Then the two black rings are the CDSs of IMP66. The outermost interspaced ring (in dark green) represents the localization of the predicted regions of genomic plasticity (RGP) in the IMP66 genome, and the labels of each region follow the ones in Table 2. (B) Regions with different colors represent potentially horizontally transferred genes (HGT) which are gathered in genomic regions (Region of Genomic Plasticity, RGP). All potential alkane hydroxylases were marked at the chromosome with their corresponding colors.

contained the genes coding for alkane hydroxylase (almA, DBX28_14785), SDR family oxidoreductase (DBX28_14685, DBX28_14840), phytanoyl-CoA dioxygenase (DBX28_14740, DBX28_14775), NAD(P)/FAD-dependent oxidoreductase (DBX28_14785, DBX28_14815), and NAD-dependent alcohol dehydrogenase (DBX28_14795). Almost all the genes in RGP10 were related to metabolism and energy production (Table 3). These results suggested that these three strains might have the ability to degrade and utilize a broad-spectrum of alkanes for growth.

Difference in RLs Production Among the Three Strains is Not at Genomic Level

Our previous study described that three strains produced different amounts of RLs. IMP67 and IMP68 produced much larger amounts of RLs than IMP66 (Das et al., 2014). The pan-genome analysis found that IMP67 and IMP68 possessed several unique genes compared to IMP66 (Figure 3B). These unique genes belong to type II secretion system, lipopeptide, non-ribosomal peptide synthetase, and hypothetical protein, which were not reported to be directly relevant to the RLs biosynthesis. Moreover, the average nucleotide identity (ANI) value between IMP66 and IMP68 is 99.99%. These results implied that the reason for the different yield of RLs of these strains is unlikely at genomic level.

To find the reason for the difference in RLs production among three strains, we then detected the QS signal molecules (C4-HSL and C12-HSL) by LC-MS/MS quantification, which positively regulate the expression of rhlAB, the two key RLs synthesis genes. After 24 h incubation, IMP67 and IMP68 produced significantly higher (3–10-folds) level of the two QS signal molecules (C4-HSL and C12-HSL) than that of PAO1, yet IMP66 produced trace amount of C4-HSL as well as C12-HSL, which might explain the reason for the less RLs production in IMP66 (Figure 5A).

In addition, we used the rhlAB:gfpl fluorescence-based reporter plasmid pTH22 to track the intracellular rhlAB transcription level. GFP fluorescence intensity was normalized by the whole cell protein concentration. IMP67 and IMP68 presented higher rhlAB expression than that of IMP66 and PAO1 (Figure 5B), which is correlated with the RLs and QS signal molecule production. Moreover, rhlAB:gfpl intensity was significantly improved when IMP67 and IMP68 were grown in the nutrient broth (NB) medium (Figure 5B), which is a known RLs-promoting medium, suggesting that rhlAB transcription is related to the nutrient in the growth medium.

Rhamnolipids can enhance the bioavailability of crude oil, and therefore are crucial for microbial consortium living in the crude oil environment. To investigate whether these three strains possess the ability to produce RLs when grown with crude oil, especially n-alkanes, as the sole carbon source. We used again the rhlAB:gfpl to determine the transcription of rhlAB when they cultured with alkane (tetracosane, C24; eicosane, C20) as the sole carbon source. The three strains displayed noticeable growth indicating by total protein concentration, and rhlAB transcription after 72 h incubation in the two types
TABLE 3 | Detail information of the probable alkane degradation cluster in RGP10.

| Label       | Begin       | End         | Type   | Product                                           | GC Region | SIGI | IVOM |
|-------------|-------------|-------------|--------|--------------------------------------------------|-----------|------|------|
| DBX28_14665 | 3119086     | 3120522     | CDS    | undecaprenyl-phosphate glucose phosphotransferase |           |      |      |
| DBX28_14670 | 3120517     | 3120845     | CDS    | Glycosyltransferase                              | −2SD      |      |      |
| DBX28_14675 | 3121054     | 3121725     | CDS    | hypothetical protein                             |           |      |      |
| DBX28_14680 | 3121772     | 3122491     | CDS    | MOSC domain-containing protein                   | +1SD      |      |      |
| DBX28_14685 | 3122707     | 3123447     | CDS    | SDR family oxidoreductase                        |           |      |      |
| DBX28_14690 | 3123497     | 3123859     | CDS    | hypothetical protein                             |           |      |      |
| DBX28_14695 | 3124233     | 3125303     | CDS    | Hydrolyase                                       |           |      |      |
| DBX28_14700 | 3125694     | 312635     | CDS    | hypothetical protein                             | −2SD      |      |      |
| DBX28_14705 | 3126780     | 3127468     | CDS    | IS3 family transposase                           |           |      |      |
| DBX28AM_2945| 3130845     | 3130875     | CDS    | protein of unknown function                      | −2SD      |      |      |
| DBX28_14710 | 3130845     | 313091     | CDS    | hypothetical protein                             | −2SD      |      |      |
| DBX28_14715 | 3131276     | 3131785     | CDS    | hypothetical protein                             | −1SD      |      |      |
| DBX28_14720 | 3132014     | 3132588     | CDS    | hypothetical protein                             | −2SD      |      |      |
| DBX28AM_2951| 3133431     | 3133694     | CDS    | IS3 family transposase                           | −2SD      |      |      |
| DBX28AM_2952| 3133851     | 3133934     | CDS    | protein of unknown function                      | −2SD      |      |      |
| DBX28_14730 | 3134510     | 3135052     | CDS    | hypothetical protein                             | −2SD      |      |      |
| DBX28_14735 | 3135075     | 3136274     | CDS    | hypothetical protein                             |           |      |      |
| DBX28_14740 | 3136668     | 3137434     | CDS    | phytanoyl-CoA dioxygenase family protein         | −1SD      |      |      |
| DBX28_14745 | 3137519     | 3138481     | CDS    | glycosyl hydrolyase                              |           |      |      |
| DBX28_14750 | 3138492     | 3140894     | CDS    | RND transporter                                  |           |      |      |
| DBX28_14755 | 3140903     | 3142489     | CDS    | DUF1302 domain-containing protein                |           |      |      |
| DBX28_14760 | 3142573     | 3143997     | CDS    | DUF1329 domain-containing protein                |           |      |      |
| DBX28AM_2960| 3144149     | 3144388     | CDS    | protein of unknown function                      | −2SD      |      |      |
| DBX28_14765 | 3144552     | 3145199     | CDS    | hypothetical protein                             | −2SD      |      |      |
| DBX28_14770 | 3145310     | 3146877     | CDS    | FAD-binding oxidoreductase                       |           |      |      |
| DBX28_14775 | 3146739     | 3147626     | CDS    | phytanoyl-CoA dioxygenase family protein         | −1SD      |      |      |
| DBX28_14780 | 3148075     | 3148856     | CDS    | TelR/AcrR family transcriptional regulator       | −1SD      |      |      |
| DBX28_14785 | 3149040     | 3150566     | CDS    | NAD(P)/FAD-dependent oxidoreductase, almA like   | −1SD      |      |      |
| DBX28_14790 | 3150563     | 3151438     | CDS    | alpha/beta hydrolase                             |           |      |      |
| DBX28_14795 | 3151669     | 3152826     | CDS    | NAD--dependent alcohol dehydrogenase             |           |      |      |
| DBX28_14800 | 3152839     | 3154170     | CDS    | APC family permease                              |           |      |      |
| DBX28_14805 | 3154249     | 3155673     | CDS    | gamma-aminobutyraldehyde dehydrogenase          |           |      |      |
| DBX28_14810 | 3155719     | 3156972     | CDS    | aspartate aminotransferase family protein        |           |      |      |
| DBX28_14815 | 3157060     | 3157980     | CDS    | NAD(P)-dependent oxidoreductase                  |           |      |      |
| DBX28_14820 | 3158054     | 3158206     | CDS    | amino acid permease                              | −1SD      |      |      |
| DBX28_14825 | 3158284     | 3159942     | CDS    | cation acetate symporter                         |           |      |      |
| DBX28_14830 | 3159939     | 3160262     | CDS    | DUF485 domain-containing protein                 |           |      |      |
| DBX28_14835 | 3160319     | 3161968     | CDS    | acyl-CoA synthetase                              |           |      |      |
| DBX28_14840 | 3162052     | 3162819     | CDS    | SDR family NAD(P)-dependent oxidoreductase       | +1SD      |      |      |
| DBX28_14845 | 3162819     | 3163991     | CDS    | acyl-CoA dehydrogenase                           |           |      |      |
| DBX28_14850 | 3164042     | 3164398     | CDS    | acyl-CoA dehydrogenase                           |           |      |      |
| DBX28_14855 | 3164395     | 3165450     | CDS    | phosphotransferase family protein               |           |      |      |
| DBX28_14860 | 3165647     | 3167599     | CDS    | sigma-54-dependent Fis family transcriptional regulator |           |      |      |
| DBX28_14865 | 3167665     | 3168168     | CDS    | transcriptional regulator                        | −1SD      |      |      |
| DBX28_14870 | 3168227     | 3170530     | CDS    | MCE family protein                               |           |      |      |
| DBX28_14875 | 3170523     | 3171143     | CDS    | paraquat-inducible protein A                     |           |      |      |
| DBX28AM_2983| 3171472     | 3171699     | CDS    | conserved protein of unknown function            | −1SD      |      |      |
| DBX28_14880 | 3171792     | 3173375     | CDS    | aldehyde dehydrogenase [NADP(+) ]                | +1SD      |      |      |
| DBX28_14885 | 3173054     | 3174499     | CDS    | FAH family protein                               |           |      |      |
| DBX28_14890 | 3174527     | 3175702     | CDS    | L-rhamnionate dehydratase                        |           |      |      |
| DBX28_14895 | 3175733     | 3177055     | CDS    | MFS transporter                                  |           |      |      |
of n-alkanes, while they exhibited similar growth rate and grewed better in C20 than C24 (Figures 6A,B). Altogether, our data indicated that high yield of RLs in IMP67 and IMP68 was due to the transcriptional regulation, not at genomic level.

**DISCUSSION**

The environmental microorganism *P. aeruginosa* possesses great ecological flexibility, which enables it to thrive in diverse ecological niches. Under different environmental settings, *P. aeruginosa* may use different strategies to survive, such as forming biofilms in high-temperature oilfields (Orphan et al., 2000). It was widely believed that crude oil is a harsh habitat for microbes because of its high toxicity and hydrophobicity. However, growing evidence has revealed the presence of living microbes in crude oil (Yoshida et al., 2005). It has been reported that microbial communities in the crude oil phase were dominated by *Pseudomonas*, accounting for 96.84–98.87% of the total OTUs (Yamane et al., 2008; Cai et al., 2015), which exhibited significant survival advantages over other genera.

In this study, we reported that three *P. aeruginosa* strains isolated from crude oil, IMP66, IMP67, and IMP68, exhibited excellent capabilities in n-alkane utilization and RLs production, which are two important factors for *P. aeruginosa* strains to maintain sustainable growth in the crude oil environment. Our work provides a better understanding of the adaptability of *P. aeruginosa* in crude oil.

Alkanes are the main components of petroleum hydrocarbons, and therefore, alkane degradation ability is critical for microorganisms to survive in petroleum-contaminated environments. Alkane hydroxylases are the key enzymes for aerobic degradation bacteria. Previous reports have identified AHs in several *P. aeruginosa* strains cultured from petroleum-contaminated environments (Zhang et al., 2011; Liu et al., 2012; Das et al., 2015). Compared to these *P. aeruginosa* strains, IMP66, IMP67, and IMP68 possess more AHs related genes, especially the coding genes for long-chain alkane monooxygenase, *almA*, and *ladA*. The coexistence of multiple alkane hydroxylases including *alkB*, *P450*, *almA*, and *ladA* in one bacterium is quite common in *Marinobacter* strains (Sun et al., 2018), *Dietziasp.*
DQ12-45-1b (Nie et al., 2011), Amycolicicoccus subflavus DQ53-9A1T(AEF42720) (Nie et al., 2013), Acinetobacter spp. ADP1 (Barbe et al., 2004), and was also observed in many Alcanivorax isolates (Wang et al., 2010; Wang and Shao, 2012). However, nine AH genes identified in one single chromosome is rare in P. aeruginosa species. These alkane hydroxylases were demonstrated to be complementary and cover expanded substrate ranges, thereby enhancing the adaptive ability of P. aeruginosa in crude oil.

Gene duplication and horizontal gene transfer are common evolutionary processes that generate novel genes or functions for rapid adaptation (Kondrashov et al., 2002; Gogarten and Townsend, 2005). In this study, alkB, P450, almA and ladA paralogs with multiple copies were found in IMP66, IMP67, and IMP68. Moreover, a P450 and an almA gene were found integrated into the chromosome presumably through HGT (Figure 3B). These paralogs may exhibit different functions. For example, alkW1 and alkW2 are two paralogous genes encoding AlkB rubredoxin fusion proteins, which has been reported originally in Dietzia sp. DQ12-45-1b. It was shown that AlkW1 could hydroxylate n-alkanes ranging from C14 to C32, whereas AlkW2 was not expressed in this condition (Nie et al., 2011). Results from the whole genome comparison and pan-genome analysis have indicated that many flanking mobile genetic elements exist in the genomes of IMP66, IMP67, and IMP68. For instance, a 50 kb-long alkane metabolism-related genomic island (RGP10) containing an almA-like (50% identity) alkane monoxygenase along with several IS3 transposases is found in these three strains (Tables 2, 3), suggesting that horizontal gene transfer may occur during their life cycles.

Pseudomonas aeruginosa is previously demonstrated to use QS to regulate the synthesis of RLs, which are critical for P. aeruginosa to emulsify hydrocarbons and make them easier for uptake and assimilation. However, RLs also act as costly extracellular public goods. When the population growth was limited by restricted nutritional conditions, the regulation of QS-controlled public-good related genes may exhibit a metabolic prudent manner (Xavier et al., 2011). This could be a reasonable explanation for the fact that IMP67 and IMP68 expressed huge amount of QS signal molecules (Figure 5A), yet produced just a little higher amount of RLs than PAO1 (Das et al., 2014). Another plausible explanation could be that both RLs and exopolysaccharides share the common sugar precursors catalyzed by AlgC (Olvera et al., 1999; Wang et al., 2014). It could be a tactic for P. aeruginosa to synthesize proper amount of RLs even with excess AHLs, and balance the synthesis of RLs and exopolysaccharides.

In conclusion, our results have showed that the alkane degradation ability and the superior AHL synthesis ability are two promoting factors for P. aeruginosa adaption in crude oil environment, which may also confer these three new isolates potential engineering performance in bioremediation of crude-oil pollution as well as microbial-enhanced oil recovery. Moreover, the native type I-F CRISPR-Cas system possessed by IMP66, IMP67, and IMP68 could be further established as a highly efficient, in situ genome-editing technique to modify the strains for future applications in such as industrial RLs production.

DATA AVAILABILITY STATEMENT

The assembled annotated complete genomes used in this study are available at the National Center for Biotechnology Information (NCBI), under the accession code CP028959, CP028848 and CP028849. The other sequenced genomes are abtained from Pseudomonas database (www.pseudomonas.com).

AUTHOR CONTRIBUTIONS

LM and SW acquired the funding. DW and LM designed the experiments. AX, YD, YZ, BW, and QW carried out the work and made contributions to acquisition of the data. AX wrote the original draft. LM, DW, LY and SW revised and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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