Genomic characterization of a polyvalent hydrocarbonoclastic bacterium *Pseudomonas* sp. strain BUN14

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Bioremediation offers a viable alternative for the reduction of contaminants from the environment, particularly petroleum and its recalcitrant derivatives. In this study, the ability of a strain of *Pseudomonas* BUN14 to degrade crude oil, pristane and dioxin compounds, and to produce biosurfactants, was investigated. BUN14 is a halotolerant strain isolated from polluted sediment recovered from the refinery harbor on the Bizerte coast, north Tunisia and capable of producing surfactants. The strain BUN14 was assembled into 22 contigs of 4,898,053 bp with a mean GC content of 62.4%. Whole genome phylogeny and comparative genome analyses showed that strain BUN14 could be affiliated with two validly described *Pseudomonas* Type Strains, *P. kunmingensis* DSM 25974ᵀ and *P. chloritidismutans* AW-1ᵀ. The current study, however, revealed that the two Type Strains are probably conspecific and, given the priority of the latter, we proposed that *P. kunmingensis* DSM 25974 is a heteronym of *P. chloritidismutans* AW-1ᵀ. Using GC-FID analysis, we determined that BUN14 was able to use a range of hydrocarbons (crude oil, pristane, dibenzofuran, dibenzothiophene, naphthalene) as a sole carbon source. Genome analysis of BUN14 revealed the presence of a large repertoire of proteins (154) related to xenobiotic biodegradation and metabolism. Thus, 44 proteins were linked to the pathways for complete degradation of benzoate and naphthalene. The annotation of conserved functional domains led to the detection of putative genes encoding enzymes of the rhamnolipid biosynthesis pathway. Overall, the polyvalent hydrocarbon degradation capacity of BUN14 makes it a promising candidate for application in the bioremediation of polluted saline environments.

Petroleum and dioxins compounds are omnipresent environmental pollutants that threaten the environment and human health due to their toxicological properties and their high resistance to degradation¹². Bioremediation is considered as one of the viable options for remediation of hydrocarbon-polluted environments³⁴. Numerous microorganisms, including bacteria, fungi, archaea and algae, have been investigated for their bioremediation potential¹⁶ with 79 bacterial genera reported to possess the capacity to degrade hydrocarbons². Members of the Gammaproteobacteria class have high hydrocarbon-degrading capacity, and this taxon includes the most obligate hydrocarbonoclastic genera: *Alcanivorax*, *Thalassolituus*, *Oleiphilus*, *Oleispira*, *Cycloclasticus* and *Marinobacter*³⁵⁶. However, other non-obligate hydrocarbonoclastic members of the Gammaproteobacteria, such as the genus *Pseudomonas*, are well known for their hydrocarbon-degrading capacity⁸⁻¹⁰. The genetic basis and enzymatic mechanisms involved in the degradation of oil components, including alkanes and aromatic compounds, by *Pseudomonas* species have been reported in detail⁸⁻¹⁰. The key activating enzymes in the degradation of alkanes are alkane hydroxylases¹⁰. Alkane monoxygenases (AlkB) and cytochrome P450s (CYP153) catalyze the hydroxylation of alkanes to alcohols, which are subsequently oxidized to fatty acids. The fatty acid products are further metabolized by β-oxidation¹⁰. The catabolism of aromatic compounds involves multiple enzymes which convert the aromatic substrates into Krebs cycle intermediates via ortho or meta-cleavage pathways.

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L3-ketoadipate pathway (ortho-cleavage pathway) is the key route for the catabolism of a wide variety of aromatic compounds through the protocatechuate branch (pea genes) and the catechol branch (cat genes).

The phylogeny of the genus *Pseudomonas* is complex, with some 219 validly published and correctly named species. However, the current phylogeny is subject to considerable debate and many of the named species may be considered as conspecific. For example, *Pseudomonas kunmingensis* HL22-2T and *P. chloritidismutans* AW-1T which are closely related to *P. stutzeri* may not deserve separate species status.

To date, the genome sequences of eighteen strains affiliated to *P. kunmingensis* and one affiliated to *P. chloritidismutans* strain are available via the NCBI database. While the genes and pathways associated with n-alkane degradation have been reported for *P. chloritidismutans*, similar information for *Pseudomonas kunmingensis* is not available.

*Pseudomonas* sp. BUN14, isolated from hydrocarbon-polluted sediments from the Bizerte coast refinery harbour, north Tunisia, exhibits the capacity for hydrocarbon degradation. In this study, we combined genomic analysis and degradation experiments to demonstrate that *Pseudomonas* strain BUN14 has potential for application in the bioremediation of hydrocarbon contaminated marine environments.

**Results and discussion**

**Isolation, phylogenetic assignment, and characterization of hydrocarbonoclastic bacterial strain BUN14.** Strain BUN14 was isolated on ONR7a mineral medium supplemented with 1% crude oil as the sole carbon source. The comparison of the strain BUN14 16S rRNA gene sequence with those of validly described strains in the EzBioCloud revealed that strain BUN14 showed highest similarity (99.23%) to that of *P. kunmingensis* HL22-2T. Phylogenetic analysis based on the 16S rRNA gene of *Pseudomonas* species placed *P. kunmingensis* HL22-2T as the closest relative of BUN14 (Fig. S1). Growth of strain BUN14 on plates containing TSA (Trypticase soy agar) medium showed that the bacterium formed circular, opaque yellow colonies. Cells were rod-shaped and approximately 0.6 ± 0.1 μm in diameter and 1.8–2.0 μm in length (Fig. S2).

**Optimization of surfactant activities.** When grown on mineral medium ONR7a supplemented with crude oil and vegetable oil as sole carbon sources, BUN14 strain produced biosurfactants, as indicated by the oil spreading, drop collapse and emulsification tests (Fig. S2). Use of the Cetyl Tri Ammonium Bromide (CTAB)-Methylene blue agar method indicated that BUN14 produced anionic biosurfactant (Fig. S2). Optimal conditions for growth and biosurfactant production were assessed using response surface methodology (RSM) experiments. The analysis of variance for the fitted mathematical models shows that the regression sum of squares was statistically significant (P < 0.01) (Table S3). Response surface plots, showing optimal biosurfactant production conditions, are shown in Fig. 1. Statistical analyses of central composite design (CCD) experiments demonstrated that the percentage of oil substrate, NaCl concentration, inoculum size and incubation time all affected the biosurfactant production. Based on desirability function, the optimal BUN14 growth (OD600nm, 0.54), biosurfactant production (OD250nm 2.58) and activities (emulsion index E24, 40.09% and oil-displacement ODA, 20.40 cm²), were obtained after approx. 7 days of cultivation crude oil, 2.50% NaCl concentration inoculum size (Fig. S3). Accordingly, as for previous studies, substrate, NaCl concentration, inoculum size and incubation time significantly affected biosurfactant production (0.01 < p < 0.05).

The basic structure of the isolated biosurfactant was evaluated by Fourier Transform InfraRed (FT-IR) spectrometry and compared to a reference *Pseudomonas aeruginosa* (Fig. 2). The peaks appearing at 3278.56 (Fig. 2a) and 3270.42 cm⁻¹ (Fig. 2b) denoted the presence of −OH stretching (free hydroxyl groups of rhamnose rings) of hydroxyl group. The adsorption peaks at 2924.64 (Fig. 2b) and 3000.0 cm⁻¹ (Fig. 2a) indicated the presence of terminal methyl group of aliphatic stretching bands (CH2, CH3). The absorption peaks at 1097.35 (Fig. 2a) and 1049.1 cm⁻¹ (Fig. 2b) confirmed the presence of C−O−C vibrations (rhamnose ring). The area between 1495.92 and 1150.02 cm⁻¹ (Fig. 2a) represented C−H and OH deformation vibrations typical for carbohydrates, as in the rhamnose units of the biosurfactant. The apparent similarity of the main functional groups determined by FTIR spectrometry of the commercial rhamnolipid (R90) from *Pseudomonas aeruginosa* (AGAE Technologies, Corvalis, OR, USA) and the isolated biomolecule from strain BUN14 (Fig. 2), suggested that the BUN14 biosurfactant product was composed on rhamnose rings with long hydrocarbon chains.

Members of the genus *Pseudomonas* are known for biosynthesis of rhamnolipids with biosurfactant properties.

**Hydrocarbon degradation.** Strain BUN14 could grow on and utilize various hydrocarbons, including pyrene, naphthalene, phenanthrene, carbazole, dibenzofuran, dibenzothiophene, biphenyl, pristane, fluoranthene, crude oil, octadecane and tetradecane as sole carbon and energy sources (Fig. S4a–c). These findings are in agreement with previous reports demonstrating that many *Pseudomonas* species are capable of utilizing hydrocarbons as carbon and energy sources. The kinetics of hydrocarbon biodegradation in liquid media was determined using GC-FID analysis. The total degradation of petroleum TERHCs by strain BUN14 was estimated at 90% after 21 days with rapid degradation of almost all alkanes C12–C36 (Fig. S4d,e). Of the more recalcitrant aliphatic compounds, 22%, 40%, 30% and 14% of pristane, naphthalene, DBT and DBF, respectively, were degraded by strain BUN14 (Fig. S4d) over a period of 21 days. The ability of various bacteria to degrade naphthalene, including *Sphingomonas, Pseudomonas* and *Acidovorax*, has been reported.

**General features of the draft genome of strain BUN14.** The draft genome sequence of strain BUN14 was assembled into 22 contigs of 4,898,053 bp in size with a G + C content of 62.4%. Comparisons of the BUN14 genome with those of its three closest relatives, *P. kunmingensis* DSM 25974, *P. kunmingensis* CCUG 36651, *P. aeruginosa* and *P. chloritidismutans* AW-1T, which are closely related to *P. stutzeri*, may not deserve separate species status. However, the current phylogeny is subject to considerable debate and many of the named species may be considered as conspecific. For example, *P. kunmingensis* HL22-2T and *P. chloritidismutans* AW-1T which are closely related to *P. stutzeri* may not deserve separate species status.

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and *P. chloritidismutans* AW-1, showed similar genome sizes and G+C contents range of 4.65–5.06 Mb and 62.4–62.6%, respectively (Fig. S5, Table 1).

Annotation of the strain BUN14 genome yielded 4551 protein coding sequences (CDSs) and 55 tRNA genes (Table S1). Evaluation of the predicted proteins using BUSCO showed that 99.8% of gammaproteobacteria conserved single copy orthologs were present in the strain BUN14 draft genome with 0 duplication (Table 1). Functional annotation of the predicted proteome showed that ~87% of the proteins could be assigned to EggNOG orthologous genes (OGs). The overall distribution revealed that the category *metabolism* was overrepresented, comprising ~35% of the OGs compared to cellular processes and signalling, and information storage and processing, which comprise ~23% and 17%, respectively (Table S1).

**Figure 1.** Three-dimensional response surface for the effect of waste frying oil, inoculum size, [NaCl] and incubation time on the response Y1 (a), Y2 (b), Y3 (c) and Y4 (d). The response surface graphs were generated by NEMROD-W statistical software (design NEMROD-W, version 9901 Française, LPRAI-Marseille Inc., France) (https://www.nemrodw.com/fr).
Phylogenomic comparison of strain BUN14 and related *Pseudomonas* strains. To identify appropriate comparison strains, 189 core single copy proteins from 294 *Pseudomonas* Type Strains were used to build a guide tree for subsequent phylogenomic analysis (Fig. 3). Strain BUN14 grouped distinctly with 14 *Pseudomonas* Type Strains, including *P. kunmingensis* HL22-2T. Phylogenetic analysis based on 1,238 single copy proteins shared among the *Pseudomonas* strains that cluster with strain BUN14 and *P. caeni* DSM 24390T, included as an outgroup, showed that strain BUN14, *P. kunmingensis* DSM 25974T, *P. chloritidismutans* AW-1 and *P. kunmingensis* CCUG 36651 formed a subclade, distinct from *P. songnenensis* NEAU-ST5-5T and *P. stutzeri* ATCC 17588T which were included in two separate clades (Fig. S6). Strain BUN14, DSM 25974T, AW-1 and CCUG 36651 shared an average nucleotide identity (ANI) range of 96.66–97.18% (Fig. S7). By contrast, the four strains shared ANI ranges of 86.74–86.88% with *P. stutzeri* ATCC 17588T. Furthermore, AW-1 and DSM 25974T shared in silico DNA-DNA hybridization (iDDH) of 71.9% (CI 68.9–74.8%) while strain BUN14 shared iDDH
of 72.2% (CI 69.2–75.1%) and 70.3% (CI 67.3–73.2%) with AW-1 and DSM 25974T, respectively (Fig. S8). Two important species discriminatory genomic metrics, ANI and $i$ DDH, provided further support for the clustering of these strains. Interestingly, the two type strains (DSM 25974T and AW-1), strains BUN14 and CCUG 36651 share ANI and $i$ DDH values above 96 and 70%, respectively, suggesting that they belong to the same species30–33. The ANI range of 86.74–86.88% between the four strains and \textit{P. stutzeri} ATCC 17588T and the distinct clustering of the latter in the presented phylogeny provide strong evidence for the separation of the above

Table 1. Genomic comparison with related \textit{Pseudomonas} strains obtained from the NCBI database.

| Strains                  | Accession         | Assembly                  | # contigs | Size (bp) | G+C (%) | # proteins | # tRNA | *completeness (%) |
|--------------------------|-------------------|---------------------------|-----------|-----------|---------|------------|-------|-------------------|
| \textit{Pseudomonas kunmingensis} DSM 25974T | GCF_900114065.1 | IMG-taxon 2663763606     | 33        | 4,654,543 | 62.6    | 4341       | 55    | 99.6              |
| \textit{Pseudomonas chloritidismutans} AW-1 | GCF_000495915.1 | PseChl                    | 77        | 5,056,349 | 62.5    | 4719       | 85    | 96.9              |
| \textit{Pseudomonas kunmingensis} CCUG 36651 | GCF_002890855.1 | ASM289085v1               | 65        | 4,810,086 | 62.4    | 4441       | 55    | 99.8              |
| \textit{Pseudomonas kunmingensis} BUN14 | GCF_002922251.1 | ASM29222v1                | 22        | 4,898,053 | 62.4    | 4552       | 55    | 99.8              |
| \textit{Pseudomonas songnenensis} NEAU-ST5-5T | GCF_003696315.1 | ASM369631v1               | 25        | 4,321,177 | 63.2    | 4021       | 58    | 100               |
| \textit{Pseudomonas stutzeri} ATCC 17588T | GCF_000219605.1 | ASM21960v1                | 1         | 4,547,930 | 63.9    | 4191       | 65    | 99.6              |
| \textit{Pseudomonas balnearia} DSM 6083T | GCF_000818015.1 | ASM81801v1               | 1         | 4,283,460 | 64.7    | 4060       | 64    | 99.7              |
| \textit{Pseudomonas saudiphocaensis} 20_BN | GCF_000756775.1 | FRJH647478_assembly_1     | 1         | 3,673,759 | 61.2    | 3414       | 54    | 99.8              |
| \textit{Pseudomonas zhaodongensis} NEAU-ST5-21 | GCF_003696365.1 | ASM369636v1               | 27        | 4,668,208 | 59.6    | 4293       | 58    | 100               |
| \textit{Pseudomonas xanthomarina} DSM 18231T | GCF_900129835.1 | IMG-taxon 2687453778     | 17        | 4,308,853 | 60.3    | 3968       | 54    | 99.5              |
| \textit{Pseudomonas azotifigens} DSM 17556T | GCF_000425625.1 | ASM42562v1               | 59        | 5,017,423 | 66.7    | 4520       | 55    | 99.8              |
| \textit{Pseudomonas flexilis} ATCC 29606T | GCF_000802425.1 | ASM80242v1               | 49        | 3,762,694 | 65.8    | 3503       | 62    | 99.4              |
| \textit{Pseudomonas oryzae} KCTC 32247T | GCF_900104805.1 | IMG-taxon 2667527434     | 1         | 4,642,193 | 67.4    | 4130       | 87    | 99.5              |
| \textit{Pseudomonas linyingensis} LMG 25967T | GCF_900109175.1 | IMG-taxon 2663762776     | 43        | 4,721,122 | 66.3    | 4308       | 62    | 100               |
| \textit{Pseudomonas sagittaria} JCM 18195T | GCF_900115715.1 | IMG-taxon 2663762798     | 44        | 4,607,575 | 66.7    | 4164       | 68    | 100               |
| \textit{Pseudomonas fluvialis} ASS-1T | GCF_002234375.1 | ASM223437v1              | 69        | 3,291,143 | 62.6    | 3049       | 56    | 99.3              |
| \textit{Pseudomonas caeni} DSM 24390T | GCF_00421765.1 | ASM42176v1               | 24        | 3,022,325 | 48.3    | 2781       | 43    | 99.1              |

Figure 3. Phylogenomic analysis of strain BUN14 and 16 closest \textit{Pseudomonas} type strains. The maximum likelihood (ML) tree was inferred from the concatenated protein alignment (376,464 amino acids) of 1238 single copy proteins. The phylogeny was generated using IQ-TREE version 1.6.7 based on the LG + F + R5 model with the bootstrap option-obb 1000.
four from the P. stutzeri group. Mehboob et al.\textsuperscript{17} reported similar ANI values to the current data for AW-1 and strains P. stutzeri except P. stutzeri CCUG 29243 which share ANI value of 97% with AW-1. However, our work included only the type strain of P. stutzeri. \textit{P. chloritidismutans} AW-1. The latter was first described as a novel species in 2002 based on DDH, physiological and biochemical data\textsuperscript{14} and later proposed as conspecific with \textit{P. stutzeri}, specifically \textit{P. stutzeri} genovar 3, based on multigenic phylogeny and by disputing the validity of the phenotypic features that separate the two taxa\textsuperscript{34}. Subsequent studies\textsuperscript{17} and this work clearly demonstrate that \textit{P. chloritidismutans} AW-1 and \textit{P. stutzeri} ATCC 17588\textsuperscript{T} are distinct species\textsuperscript{34}. \textit{P. kunmingensis} DSM 25974\textsuperscript{T} was proposed as a distinct species based on a combination of 16S rRNA gene analysis, phenotypic characterisation and DDH values\textsuperscript{13}. However, the analysis omitted \textit{P. chloritidismutans} AW-1 from the comparison. For instance, the low 16S rRNA gene similarity value reported between DSM 25974\textsuperscript{T} and closely related taxa was only possible without AW-1. The full-length 16S rRNA genes of AW-1 and DSM 25974\textsuperscript{T} (data not shown), extracted from their respective genomes, share a similarity of 99.74%. Moreover, the phylogenetic and the genomic metrics presented here indicate that DSM 25974\textsuperscript{T} and AW-1 share ANI and DDH values that are below the threshold for species separation\textsuperscript{36–38}. Considering the priority of \textit{P. chloritidismutans} AW-1\textsuperscript{T} and the multiple lines of phylogenetic and genomic evidence, we suggest that \textit{P. kunmingensis} DSM 25974\textsuperscript{T} is a heterotypic synonym of \textit{P. chloritidismutans} AW-1\textsuperscript{T}. Similarly, the strains BUN14 and CCUG 36651, and perhaps CCUG 29243\textsuperscript{17}, may be more appropriately affiliated with \textit{P. chloritidismutans}.

Genomic determinant of hydrocarbon degradation in strain BUN14 and comparisons with closely related \textit{Pseudomonas} spp. To identify the proteins potentially implicated in hydrocarbon degradation, the proteome of strain BUN14 was annotated using BlastKOALA\textsuperscript{37}. Of the 4551 proteins, 2564 (56.34%) could be assigned to 2062 KEGG Orthologues (KOs) including 154 linked to xenobiotic biodegradation and metabolism (Fig. S9). Of the 154 proteins, 44 have been associated with the pathways for complete degradation of benzoate and naphthalene (Table S2). Gene product prediction using PGEnESB revealed that 39 of these proteins are encoded in four genomic loci, comprising five putative operons, while the genes of five proteins appear to be transcribed as independent transcription units.

The strain BUN14 genome contained six genes (\textit{dmpP}, \textit{dmpO}, \textit{dmpN}, \textit{dmpM}, \textit{dmpL} and \textit{dmpK}) encoding enzymes that catalyse the conversion of benzene to catechol (KEGG module: M00548; Fig. 4). The genome also contained the genes \textit{benA-xylX}, \textit{benB-xylY}, \textit{benC-xylZ} and \textit{benD-xylL}, which encode the enzymes for degradation of benzoate to catechol (KEGG module: M00551) (Fig. 4). Two distinct genomic loci contained \textit{bphH}, \textit{bphI}, \textit{bphJ}, \textit{dmpB}, \textit{dmpC}, \textit{dmpD}, \textit{dmpH}, \textit{mphE}, \textit{mphF} and \textit{praC}, encoding enzymes for the complete pathway for degradation of catechol (KEGG module: M00569, meta-cleavage) to yield Acetyl-CoA (Fig. 4), and the \textit{catB}, \textit{catC}, \textit{catA}, and \textit{pcaD} genes that encode enzymes for conversion of catechol (KEGG module: M00568; ortho-cleavage) to 3-oxoadipate (Fig. 4). The complete degradation pathway of benzoates and
benzoate derivatives, yielding acetyl-CoA and pyruvate, has been identified in other Pseudomonas species such as Pseudomonas putida, Pseudomonas aeruginosa and Pseudomonas alcaliphila.

Although partial degradation of dioxin was detected in culture experiments and the analysis of genome showed only some of genes of the dioxin degradation pathway; praC, dmpH bphI, bphH, bphJ, mhpE and mhpF (Table S2), associated with the conversion of 2-hydroxymuconate to acetyl-CoA. However, dxnA/dbfA, dbfB and dxnB which encode enzymes involved in the conversion of dibenzo-p-dioxin to 2-hydroxymuconate have not been identified. Genomic analysis of Pseudomonas putida strain B6-2 revealed genes for the complete biphenyl degradation pathway (represented by the gene cluster bphA-D and pbhH-K)\textsuperscript{40}. Like strain BUN14, the genome sequence of Sphingomonas wittichii contains only putative genes that code for enzymes in the initial part of the dioxin degradative pathway\textsuperscript{41}.

Figure 5. Heat map showing the distribution of orthologous proteins of strain BUN14 and closely related species, associated benzoate, dioxin and naphthalene degradation pathways. The heat map was based on the presence/absence matrix of orthologous proteins determined using Orthofinder. Heat map was produced using Clustvis version 1 (https://biit.cs.ut.ee/clustvis/).
The strain BUN14 proteome included the nine proteins (nahD, nahE, nahC, nahF, nahB, nahAd, nahAc, nahAb and nahAa) that catalyse the complete pathway for the degradation of naphthalene (KEGG module: M00534) to salicylate (Fig. 5). Comparisons of the strain BUN14 genome with those of 15 related Pseudomonas species, including its closest relatives, *P. kunmingensis* DSM 25974T, CCUG 36651 and *P. chloritidismutans* AW-1, showed that only BUN14 and *P. balearica* DSM 6083T harboured the complete pathway for naphthalene degradation (Fig. 6). Apart from DSM 18231T, which has an orthologue of nahD, all the compared strains lacked the orthologues of nahD and nahE. However, DSM 18231T lacked other genes associated with the naphthalene degradation pathway, and the other Pseudomonas genomes lacked between two and all nine genes of the complete pathway. The catechol degradation pathway has been shown to be the major catabolic route by which many bacteria biodegrade naphthalene and a repertoire of genes linked to the complete naphthalene degradation pathway has been reported in many taxa, including *Pseudomonas, Paraburkholderia, Alcaligenes* and *Rhodococcus*.

The BUN14 genome encoded a membrane bound acyl-CoA desaturase (WP_104098302.1; FADS-like; alkB like), rubredoxin-2 (WP_003283319.1) and rubredoxin-NAD(+) reductase (WP_104098084.1), which are likely to determine its ability to degrade n-alkane. Orthologues of these three proteins were also found in the proteomes of nine of the compared genomes including the closest relatives of BUN14, *P. kunmingensis* DSM 25974T, CCUG 36651 and *P. chloritidismutans* AW-1 (Fig. S10). Unlike *P. chloritidismutans*, no alkane 1-monoxygenase (AlkB; WP_023446487.1) genes were identified in the strain BUN14 genome. Most AlkB proteins have been shown to carry membrane fatty acid desaturase (FADSs), the actions of which depend on rubredoxin and rubredoxin reductase, encoded on a distinct genomic region. Both rubredoxin and rubredoxin reductase homologues were identified in the BUN14 genome, suggesting that either an alternative, and unknown, enzyme catalyses the initial activation of alkanes, or that the sequence homology of the BUN14 putative AlkB gene was too low to be detected by Blast analysis.
Given the identification of putative rhamnolipids in the extracted biosurfactant fraction of BUN14 cultures, the proteome was scanned for proteins potentially involved in the synthesis of this group of compounds. Blastp searches against 3-(3-hydroxydecanoyloxy) decanoate synthase RhlA (Q51559), rhamnosyl transferase RhlB (Q51560) and Rhamnosyltransferase 2 RhlC (Q9IK5S) of P. aeruginosa did not yield orthologues of these proteins. The BUN14 proteome was subsequently annotated for glycosyltransferase (GT1 and GT2) signatures using dbCAN2 and for conserved domains using the NCBI conserved domains database search tool. This combined strategy revealed four proteins (Table S3) that showed similar domain architecture to P. aeruginosa RhlA, RhlB and RhlC. WP_104098192.1 (296 aa) which shared only 13.6% similarity with Q51559 (295 aa) of P. aeruginosa, harboured conserved domains similar to Q51559 and was identified as a specific hit for the alpha/beta hydrolase superfamily. Only one BUN14 protein, WP_104098902.1 (310 aa), was predicted to be a member of the glycosyltransferase family GT1. This protein shared 22.1% similarity with Q51560 (325 aa) of P. aeruginosa and the domain architecture of both proteins was similar. Seven BUN14 proteins were predicted to be members of the glycosyltransferase family GT2. Of these, two hypothetical proteins, WP_042926909.1 (291 aa) and WP_003300529.1 (357 aa) were predicted to harbour functional domains similar to those of Q9IK5S (325 aa) of P. aeruginosa. However, WP_042926909.1 and WP_003300529.1 share only 20.5 and 22% similarity, respectively, with Q9IK5S. Therefore, WP_104098192.1 and WP_104098902.1 could potentially carry the molecular functions of the RhlA and RhlB proteins, respectively, while WP_042926909.1 and WP_003300529.1 are potential RhlC functional homologues. Indeed, the genetic characterization of rhamnolipid biosynthesis pathways is hindered by high levels of sequence diversity in the key genes, as seen in the low levels or absence of homology in the rhlA-C genes/proteins in P. aeruginosa, Burkholderia and related organisms.

Conclusion
This study has demonstrated the versatility of Pseudomonas strain BUN14 in the degradation of a wide range of polyaromatic and aliphatic hydrocarbons. The genome of strain BUN14 was fully sequenced to identify the genetic determinants of its functional capacity, including the ability to utilize various hydrocarbons and to produce biosurfactants. The complete degradation pathways of some hydrocarbons, such as benzoate and naphthalene, were identified in the BUN14 genome, while the absence of some key enzyme genes suggested that the genome may harbor genes encoding novel functionalities, or functional homologues with very low sequence homology. On the basis of whole genome phylogeny and other genomic metrics (ANI and iDDH), the current study demonstrates that P. chloritidismutans AW-1T is the closest relative of strain BUN14. Furthermore, P. chloritidismutans AW-1T and P. kunmingensis DSM 25974 are conspecific and given that the former has been widely described prior to P. kunmingensis DSM 259, we suggest that DSM 25974 strain should be considered as a heteronym of P. chloritidismutans AW-1T.

Materials and methods

Strain isolation and carbon source utilization. Strain BUN14 was isolated by enrichment culture in mineral salts medium (ONR7a) supplemented with 1% crude oil as a sole carbon source, after incubation for 21 days at 30 °C with shaking (150 × g). ONR7a contained (per litre of distilled or deionized water) 22.79 g of NaCl, 11.18 g of MgCl2·6H2O, 3.98 g of Na2SO4, 1.46 g of CaCl2·2H2O, 1.3 g of TAPSO {3-[N-tris(hydroxymethyl) methylamino]-2-hydroxypropanesulfonic acid}, 0.72 g of KCl, 0.27 g of NH4Cl, 89 mg of Na2HPO4·7H2O, 83 mg of NaBr, 31 mg of NaHCO3, 27 mg of H2BO3, 24 mg of SrCl2·6H2O, 2.6 mg of NaF, and 2.0 mg of FeCl3·4H2O. 1% of crude oil was used as only energy and carbon source. To determine the phylogenetic affiliation of the strain, the 16S rRNA gene was amplified, sequenced, and queried using the 16S rRNA identification module in the EzBioCloud database were aligned using Mafft and the domain architecture of both proteins was similar. Seven BUN14 proteins were predicted to be members of the glycosyltransferase family GT2. Of these, two hypothetical proteins, WP_042926909.1 (291 aa) and WP_003300529.1 (357 aa) were predicted to harbour functional domains similar to those of Q9IK5S (325 aa) of P. aeruginosa. However, WP_042926909.1 and WP_003300529.1 share only 20.5 and 22% similarity, respectively, with Q9IK5S. Therefore, WP_104098192.1 and WP_104098902.1 could potentially carry the molecular functions of the RhlA and RhlB proteins, respectively, while WP_042926909.1 and WP_003300529.1 are potential RhlC functional homologues. Indeed, the genetic characterization of rhamnolipid biosynthesis pathways is hindered by high levels of sequence diversity in the key genes, as seen in the low levels or absence of homology in the rhlA-C genes/proteins in P. aeruginosa, Burkholderia and related organisms.

Optimization of surfactant activities. Strain BUN14 was screened for biosurfactant production using the Cetyl Trimethyl Ammonium Bromide agar plate assay (CTAB), the drop collapse test, emulsion index (E18) and the oil-displacement test (ODA). For the optimization of surfactant activities, response surface methodology (RSM) using central composite design (CCD) was performed (Table S4). A CCD composed of 29 experiments have been planned (Table S5). The effects of various independent variables (substrate (waste frying oil) concentration (X1), NaCl concentration (X2), inoculum size (X3) and incubation time (X4)) on the biosurfactant production yield (Response Y) were evaluated at three levels (Table S6). The specific codes for each independent variable and range are given in Table S6. The relationship between these variables and the biosurfactant production yield was defined by the following model:
Y = b0 + b1X1 + b2X2 + b3X3 + b4X4 + b11X1² + b22X2² + b33X3² + b44X4² + b12X1X2 + b13X1X3 + b23X2X3 + b14X1X4 + b24X2X4 + b34X3X4 where Y are the response (emulsion index \(E_{44}\)) and oil displacement activity (ODA); \(X_j\) is the variables of studied factors and \(b_0\), \(b_1\), \(b_3\), and \(b_4\) model coefficients.

The five replicates at the center point were carried out in order to estimate the pure error variance. The significance of the fitted model was tested by the means of the analysis of variance (ANOVA). The relationship between the response and the experimental variables was illustrated graphically by plotting the response surfaces. The NemrodW software was used for experimental design and statistical analysis\(^{56}\). The optimum conditions for maximum biosurfactant production was determined using the desirability functions\(^{57}\).

The extraction of the biosurfactant was performed using liquid–liquid extraction\(^{20}\). The supernatants of BUN14 cultures were collected after centrifugation (12,000 rpm/20 min at 4 °C) adjusted to pH 2.0 with 6 N HCl and left overnight at 4 °C. The precipitates surfactant was collected by centrifugation at 12,000 rpm for 30 min at 4 °C. For additional purification, the crude biosurfactant was extracted at three successive washes with a mixture of the chloroform–methanol (2:1, v/v) and concentrated using rotary evaporation at 40 °C. For additional purification, the crude biosurfactant was extracted at three successive washes with a mixture of the chloroform–methanol (2:1, v/v) and concentrated using rotary evaporation at 40 °C. Functional group

Hydrocarbon degradation. For hydrocarbon degradation analysis, strain BUN14 was cultured in ONR7a liquid mineral medium supplemented with 5 g L\(^{-1}\) Na-acetate for 72 h in shaking (150 × g) at 30 ± 1 °C. Bacterial cells in logarithmic phase were collected by centrifugation (10 min, 14,000g), washed twice in phosphate buffered saline (PBS 1×; 140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O and 1.5 mM KH₂PO₄), resuspended and inoculated (−10⁶ cells mL⁻¹) measured by the DAPI count method) into 50 ml ONR7a liquid mineral medium supplemented with sterile Arabian Light Crude Oil (1%, v/v), pristane (1% v/v) and 50 ppm (final concentration) of naphthalene, DBT and DBF (Sigma Aldrich, Milano—Italy). Cultures containing the same amount of hydrocarbon but without inoculation were used as abiotic controls. Cultures were incubated at 30 ± 1 °C for 21 days with shaking. Total Extracted and Resolved Hydrocarbons and their derivatives (TERHCs) were extracted from cultures with dichloromethane (Sigma-Aldrich, Milan; 10% v/v) following the 3550C EPA (Environmental Protection Agency) procedure as previously reported\(^{59,60}\). Degradation rates were quantified by a Master GC DANI Instruments GC-FID (Development Analytical Instruments DANI Instruments S.p.A., Milan, Italy), equipped with SSL injector and FID detection. The extent of biodegradation was expressed as the percentage of hydrocarbon degraded compared to the abiotic control\(^{60}\).

BUN14 genome sequencing, assembly, annotation and analysis. DNA extraction was performed on mid-log phase cells by sodium dodecyl sulfate (SDS)-protease K treatment with an additional equal volume of chloroform/isooamyl alcohol (2:1 v/v). Purified genomic DNA was sequenced on an Illumina MiSeq platform (MRDNA, Clearwater, Tx, USA). The 11,335,376 paired reads were filtered according to read quality, and reads below a mean quality, score of 23 were removed using prinseq-lite software. The reads were assembled using SPAdes\(^{61}\). The genome of strain BUN14 was structurally annotated using PROKKA\(^{62}\) and FGENESB (http://www.softberry.com/). The CGView Server\(^{63}\) was used for circular representation of multiple genomes. The draft genome of strain BUN14 was used as the reference genome and was compared with genomes of \(P\). kunmingensis DSM 25974, \(P\). kummingensis CCUG 36651 and \(P\). chloritidismutans AW-1. Functional annotation was accomplished using a combination of RAST\(^{63}\), BlastKOALA\(^{37}\), KEGG\(^{64,65}\) and EggNOG\(^{66}\). Orthologous relationships among the predicted proteomes of strain BUN14 and close relatives were determined using orthofinder\(^{37}\). Single copy orthologs were aligned using mafft\(^{50}\), trimmed using Gblocks v0.91b\(^{51}\) and maximum likelihood phylogeny was constructed using iq-tree\(^{46}\). Orthologous average nucleotide identity (OrthoANI) and in silico DNA-DNA hybridization (iDDH) were determined using OAT\(^{38}\) and GGDC\(^{38}\), respectively. Comparisons of genomic regions among related \(P\). chloritidismutans \(AW-1\) species were accomplished using SimpleSynteny\(^{38}\) and a heat map was generated using ClustVis\(^{17}\).

Nucleotide sequence accession number. The genome sequence has been deposited in the Bioproject and Biosample Genomes online database under PRJNA420855 and SAMN08122818 accession numbers, respectively. Whole Genome Shotgun sequence data has been deposited at DDBJ/ENA/GenBank under the accession number PISM00000000.

Data availability The genome sequence has been deposited in the Bioproject and Biosample Genomes online database under PRJNA420855 and SAMN08122818 accession numbers, respectively. Whole Genome Shotgun sequence data has been deposited at DDBJ/ENA/GenBank under the accession number PISM00000000.
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Author contributions

M.M. and H.A. performed experiments and wrote the manuscript. M.M., H.A., H.C., M.N. and S.C. assisted with data processing and analyses and contributed to writing the manuscript; D.A.C., A.S.M., Y.S. and A.C. contributed to review and editing the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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