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Pangenomic and functional investigations for dormancy and biodegradation features of an organic pollutant-degrading bacterium *Rhodococcus biphenylivorans* TG9

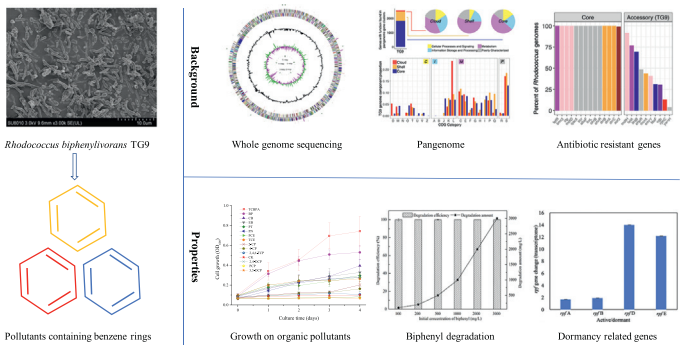
Chungui Yu [a,1], Hui Wang [b,a,1], Ryan Andrew Blaustein [c], Li Guo [a], Qi Ye [a], Yulong Fu [a], Jiahui Fan [a], Xiaomei Su [d,a], Erica Marie Hartmann [c,*], Chaofeng Shen [a,*]

[a] Department of Environmental Engineering, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, Zhejiang, China
[b] College of Eco-Environmental Engineering, Guizhou Minzu University, Guiyang, Guizhou, China
[c] Department of Civil and Environmental Engineering, Northwestern University, Evanston, IL, USA
[d] College of Geography and Environmental Science, Zhejiang Normal University, Jinhua, Zhejiang, China

HIGHLIGHTS

- *Rhodococcus* as a genus showed strong potential of degradation and dormancy propertity.
- Using *R. biphenylivorans* TG9 to study dormancy-to-degradation relationships enables improvement of bioremediation.
- Coupling whole genome sequencing and pangenome analysis of a novel strain revealed key attributes to its genus.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental bacteria contain a wealth of untapped potential in the form of biodegradative genes. Leveraging this potential can often be confounded by a lack of understanding of fundamental survival strategies, like dormancy, for environmental stress. Investigating bacterial dormancy-to-degradation relationships enables improvement of bioremediation. Here, we couple genomic and functional assessment to provide context for key attributes of the organic pollutant-degrading strain *Rhodococcus biphenylivorans* TG9. Whole genome sequencing, pangenome analysis and functional characterization were performed to elucidate important genes and gene products, including antimicrobial resistance, dormancy, and degradation. *Rhodococcus* as a genus has strong potential for degradation and dormancy, which we demonstrate using *R. biphenylivorans* TG9 as a model. We identified four Resuscitation-promoting factor (Rpf) encoding genes in TG9 involved in dormancy and resuscitation. We demonstrate that *R. biphenylivorans* TG9 grows on fourteen typical organic pollutants, and exhibits a robust ability to degrade biphenyl and several congeners of polychlorinated biphenyls. We further induced TG9 into a dormant state and demonstrated pronounced differences in morphology and activity. Together, these results expand our understanding of the genus *Rhodococcus* and the relationship between dormancy and biodegradation in the presence of environmental stressors.

* Corresponding authors.
E-mail addresses: erica.hartmann@northwestern.edu (E.M. Hartmann), ysxzt@zju.edu.cn (C. Shen).
[1] Contributed equally.

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1. Introduction

Dormancy is a strategy used to survive a variety of environmental stresses, including the presence of environmental pollution. As such, dormancy plays an important role in bioremediation, as environmental conditions may induce dormancy, thereby inhibiting biodegradation (Giagnoni et al., 2018; Murugan and Vasudevan, 2018; Ye et al., 2020). Resuscitation may help culture dormant degradative bacteria is helpful that would otherwise evade to culture (Su et al., 2018). Following this line of reasoning, Resuscitation-promoting factor (Rpf) protein produced by *Micrococcus luteus* was added to isolate *Rhodococcus biphenylivorans* TG9 from river sediments contaminated with organic pollutants (Su et al., 2015a). This novel strain has two primary features of interest for research and practical application: its demonstrated ability to transition into and out of the dormant state (Su et al., 2015b), and its potential for biodegradation of a variety of organic pollutants.

Rhodococccus are Gram-positive, aerobic, non-sporulating actinomycetes found ubiquitously throughout the natural environment including soils, surface waters, and the gut contents of arthropods (Goodfellow and Williams, 1983; Danchin, 2002; Alvarez, 2010; Gadd and Sariaslani, 2020; Pátek et al., 2021). Comparative genomic assessment of this genus has demonstrated broad potential for application in biodegradation (Garrido-Sanz et al., 2020). Accordingly, *Rhodococcus* has been used for bioremediation of organic pollutants, which are anthropogenic chemicals with high toxicity and persistence in environment (Larkin et al., 2005; Alvarez, 2010; Henson et al., 2018). While *Rhodococcus* has been applied for bioremediation, the success of that application depends on metabolic activity in situ.

Rhodococcus has been reported to tolerate different stressors, such as physical (osmotic, pH) and chemical (metals, antibiotics) stress and starvation (Cappelletti et al., 2016; de Carvalho et al., 2016; Pátek et al., 2021) through various means. Similar to other environmental bacteria, members of *Rhodococcccus* contain antibiotic resistance genes (ARGs) as a natural result of interspecies communication and competition, as well as to protect themselves against stressors (Czekalski et al., 2014;Berghaus et al., 2015; Huber et al., 2020; Kirkan et al., 2021). Specifically in the presence of antibiotic stress, even strains that were not resistant could survive by entering a dormant state, in which cells remained alive but failed to grow on bacteriological medium (Xu et al., 1982; Su et al., 2015a; Giagnoni et al., 2018). While dormant, bacterial metabolic activity was greatly reduced (Xu et al., 1982; Pinto et al., 2015), leading to decrease in pollutant degradation (Ye et al., 2020). Therefore, understanding the genetic features contributing to these phenotypes is essential for eventual manipulation to promote bioremediation in situ.

Coupling genomic and functional investigations is needed to provide context for key attributes of *R. biphenylivorans* TG9 with respect to the broader genus. These attributes include antimicrobial resistance, dormancy and biodegradation, which may or may not be conserved in the *Rhodococcus* pangenome. Genomic heterogeneity within a bacterial lineage (i.e., the pangenome) is underscored by nucleotide variants in "core" conserved genes as well as presence or absence of "accessory" genes that are dispensable across strains (McInerney et al., 2017). Here, we leveraged 144 high quality genomes of *Rhodococcus* to perform a genus-level pangenome analysis (i.e., define the cumulative set of genes belonging to the organismal group) to determine potential functions that might be characteristic and unique to strain TG9 and closely related strains. Assays further investigating stress response and biodegradation were performed to validate genomic predictions for the strain. Our findings reveal properties and functions, providing much needed context for predicting function in the genus *Rhodococcus*, particularly for elucidating bacterial dormancy-to-degradation relationships.

2. Results

2.1. Whole genome sequencing

We used both Illumina Hiseq4000 and Pacific Biosciences (PacBio) RSII sequencing platforms. A total of 6,532,392 paired-end reads corresponding to 177 folds of genomic coverage were generated on an Illumina Hiseq 4000 platform, and 374,928,577 bp were generated from 46,901 subreads on a PacBio sequencing platform. The complete genome of strain TG9 was composed of one gapless circular chromosome of 5,034,221 bp with 68.0% GC content. Function annotation with a BLAST search against the COG (Clusters of Orthologous Groups of proteins), NR (RefSeq non-redundant proteins), KEGG (Kyoto Encyclopedia of Genes and Genomes) and Swiss-Prot databases indicated that the genome and contained 4882 protein coding genes, 53 tRNA genes, and 12 rRNA genes, with 68.0% GC content. Separate annotation with prokka (Seemann, 2014), which provided the gff files used for downstream pangenome assessment, yielded 4653 total genes, including 61 tRNA genes and 12 rRNA genes.

2.2. Rhodococcus pangenome

Pangenome analysis was conducted to provide genomic and putative functional context to TG9 among the broader *Rhodococcus* genus, especially to determine whether features related to antibiotic resistance, dormancy, and degradation were unique to strain TG9 or core to its genus. The *Rhodococcus* pangenome was constructed from 144 high quality genome assemblies (i.e., each with >99.0% completeness and <1.0% contamination) (Supplementary Table S1). There were approximately 5476 ± 63 genes per genome (mean ± standard error) that comprised a total of 54,865 gene clusters in the pangenome (Fig. 1A and B; Supplementary Fig. S1). There were 37,274 gene clusters in the pangenome annotated as "hypothetical protein" (67.9%), which constituted approximately 46.0% of each individual genome. This large set of genes with unknown function, along with the redundant gene products in the pangenome (i.e., different species encoding genes with the same annotated function), accounted for a largely skewed "cloud" or niche-specific fraction of the pangenome (Fig. 1A).

Of the 20,591 gene clusters with an annotated product (i.e., not "hypothetical protein"), there were 3812 non-redundant annotations identified from the *Rhodococcus* pangenome. Focusing on these non-redundant annotations, the proportions of cloud (31.6%), shell (35.0%), and core (33.3%) functions (i.e., those present in <15.0%, 15.0–95.0%, or >95.0% of all *Rhodococcus* strains, respectively) were nearly even (Supplementary Fig. S2). There were 2004 ± 9 non-redundant annotated gene products per genome (mean ± standard error) (i.e., excluding "hypothetical protein"), of which approximately 63.0% on average (68.0% for TG9) reflected core functions (Supplementary Fig. S2B). Compared to other members of *Rhodococcus* (the range was 4.86–9.70 MB with a median of 5.93 MB), the 5.03 MB *R. biphenylivorans* TG9 genome was relatively small, ranking as the 6th shortest out of 144 screened high-quality *Rhodococcus* genomes that were 6.04 ± 0.07 MB (mean ± standard error). It contained 4412 protein coding genes assigned to gene clusters in the pangenome matrix (Fig. 1B) and 1849 annotated gene products matching a non-redundant function assigned to gene clusters from the pangenome matrix (i.e., excluding "hypothetical protein;" Supplementary Fig. S2).

The functional components of the *Rhodococcus* pangenome yielded four primary clusters based on analysis of Bray-Curtis dissimilarity (Fig. 1C). Within the cluster that included *R. biphenylivorans* TG9 (Supplementary Table S2), there were two distinct groups. The group including *R. biphenylivorans* TG9 was composed of 14 other *Rhodococcus* strains with a relatively balanced cloud/core pangenome distribution (Fig. 1D), especially compared to the broad *Rhodococcus* pangenome distribution (Fig. 1B). *R. biphenylivorans* TG9 was most similar to *R. pyrinivorans* based on core gene amino acid sequence alignment

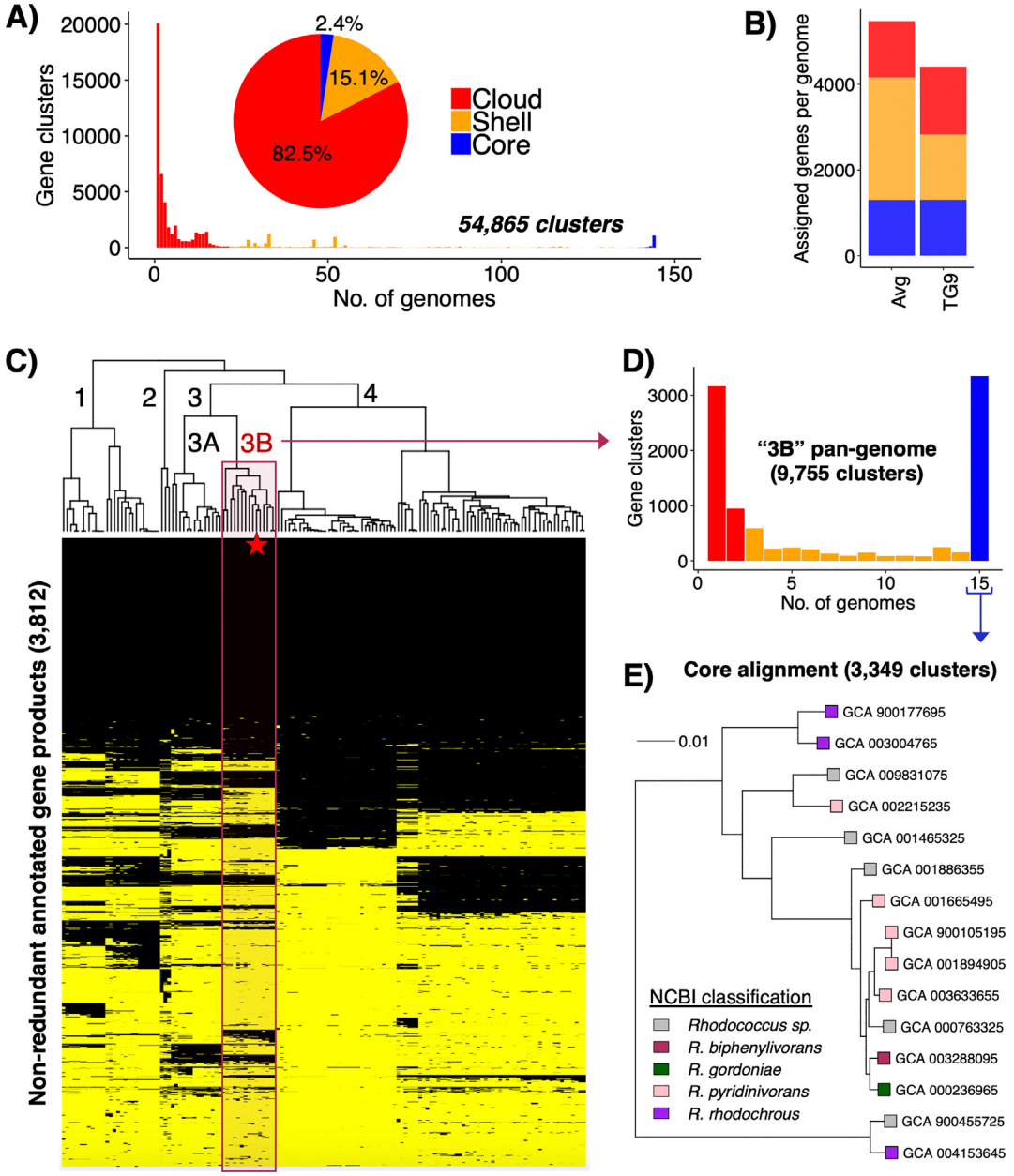

Fig. 1. The *Rhodococcus* pangenome indicates distinct subgroups/clusters.
(A) Histogram distribution of detected genes in the *Rhodococcus* pangenome; pie chart indicates percentage of each fraction (B) total genes per genome assigned to the pangenome gene clusters for *Rhodococcus* (average) and for strain TG9. (C) Presence/absence (black/yellow) of detected functions with non-redundant annotation (i.e., excluding "hypothetical proteins"). The red star indicates strain TG9. (D) Gene distribution for the cluster of *Rhodococcus* strains most functionally similar to TG9 indicates a high proportion of shared genes despite interspecific genetic diversity. (E) Core gene alignment of this cluster demonstrates phylogenetic distinction of *R. biphenylivorans*, suggesting closest relation to *R. pyridinivorans*.

(Fig. 1E), which is consist with our previous results identifying it as a novel strain (Su et al., 2015a).

2.3. COG category

The TG9 genome was categorized with EggNOG to analyze putative function and compare across the *Rhodococcus* genus. From the 4653 prokka-annotated genes within the strain TG9 genome, 2613 had a designated gene product (i.e., were not annotated as "hypothetical protein"). These gene products were screened against the set of non-redundant annotated functions of gene clusters in the pangenome matrix (i.e., the predicted gene function set for all *Rhodococcus*) to elucidate the core, shell, and cloud composition of the TG9 genome (Fig. 2A) with respect to COG functional categories (Fig. 2B). As expected, about half of the core functions in TG9, and really its full genome, reflected bacterial metabolism, primarily energy production (Cog-C), amino acid (Cog-E) and lipid (Cog-I) transport, among other categories (Fig. 2C). The shell functions encoded by TG9; i.e., those common in some but not all *Rhodococcus*, reflected a greater proportion of different types of metabolic pathways, such as secondary metabolite biosynthesis (Cog-Q) and inorganic ion transport (Cog-P) (Fig. 2C). Alternatively, the greater proportion of cloud functions, i.e., those most unique to TG9 and only shared with very few closely related strains if any, reflected processes related to cell division (Cog-D) and DNA replication and repair (Cog-L) (Fig. 2C). Thus, functional attributes that appeared to be more unique to TG9 relative to other *Rhodococcus* included broad processes disproportionately associated with cell signaling and information processing, perhaps reflecting its lifestyle.

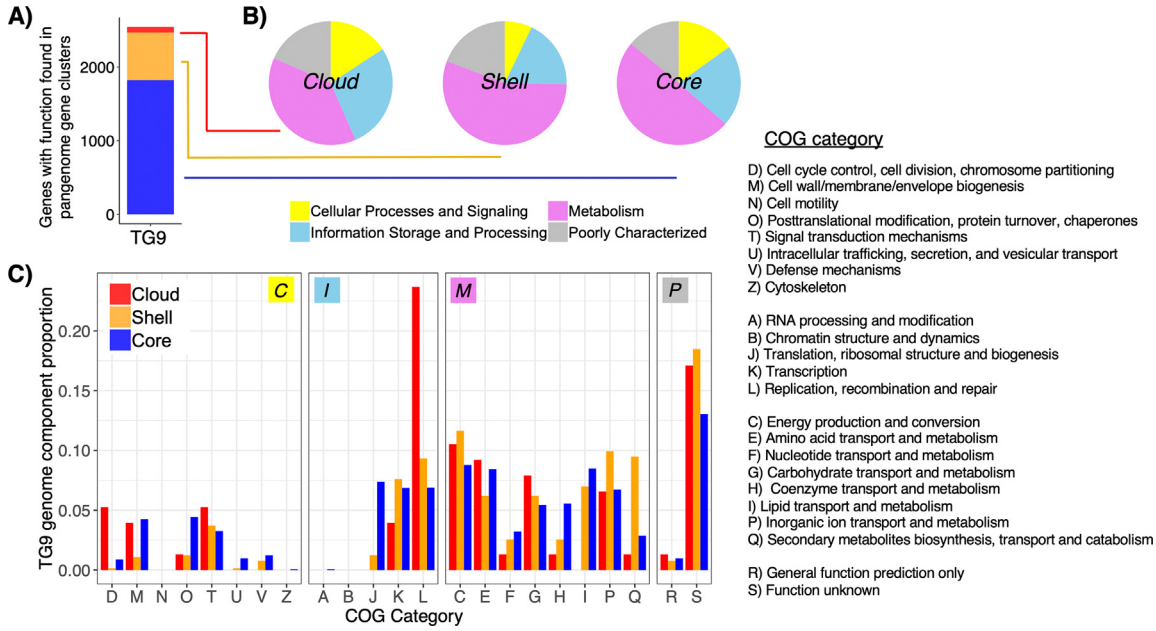

Fig. 2. COG functional categories comprising the *R. biphenylivorans* TG9 genome based on EggNOG analysis.
(A) Total genes within the TG9 genome with an annotated function matching that of the gene clusters in the pangenome ($n = 2545$ genes; i.e., excluding the 2040 genes annotated as "hypothetical protein" and 68 genes with product name not matching an annotation from the pangenome matrix). Colors correspond to frequency of the predicted function across the pangenome matrix (i.e., cloud, shell, or core functions). (B) Percentage of COG categories assigned to the respective fractions of the TG9 genome displayed in panel A. (C) Proportion of COG subcategories that make up the respective TG9 genome components displayed in panel A. Panels are separated by broader COG category.

2.4. Resistance to antimicrobials and other chemical stressors

The ability to resist stressors is critical for survival in situ as well as for successful bioremediation. Antimicrobial resistance genes play an important role in mediating stress from chemical sources. To understand the resistance profile of both the genus and the strain TG9, we examined the distribution of antimicrobial resistance genes and corroborated their function in TG9.

The results showed that *Rhodococcus* has characteristic resistance to a variety of clinical antimicrobials, non-antibiotic compounds, and other chemical stressors (e.g., heavy metals). Nearly all members of the genus were predicted to be resistant to tetracycline (*tetA*, *tetR*), arsenic (*acr3*), and antitumor drugs doxorubicin/daunorubicin (*drrA*, *drrB*, *drrC*), and contain several multidrug efflux pumps (i.e., encoded in core fraction of the pangenome) (Fig. 3; Supplementary Table S4). In addition, several genes are broad spectrum and confer resistance for not only antibiotics but also other antimicrobials and heavy metals (Fig. 3). Analysis of the TG9 genome assembly with Comprehensive Antimicrobial Resistance Databases (CARD) also predicted a potentially broad ARGs profile. There was 1 "strict hit" and 225 "loose hits." The strict hit predicted RbpA (i.e., rifamycin resistance) via a protein homolog model with 89.2% identity to the matching region. Loose hits with >50.0% identity included ARGs that may confer resistance to fosfomycin (*abaF*, *lfrA*, *murA*), rifamycin (*epfA*, *rphB*), and tetracycline (*tetA*, *tetV*) antibiotics, as well as additional mechanisms for multidrug efflux (*mtrA*, *soxR*).

Predicted antimicrobial resistance of *R. biphenylivorans* TG9 was validated with minimum inhibitory concentration (MIC) assays (Supplementary Table S5). Based on MICs and breakpoint assessment of other model Gram-positive strains and *Rhodococcus equi* (also known as *Rhodococcus hoagie*), TG9 appears to be resistant to aminoglycosides

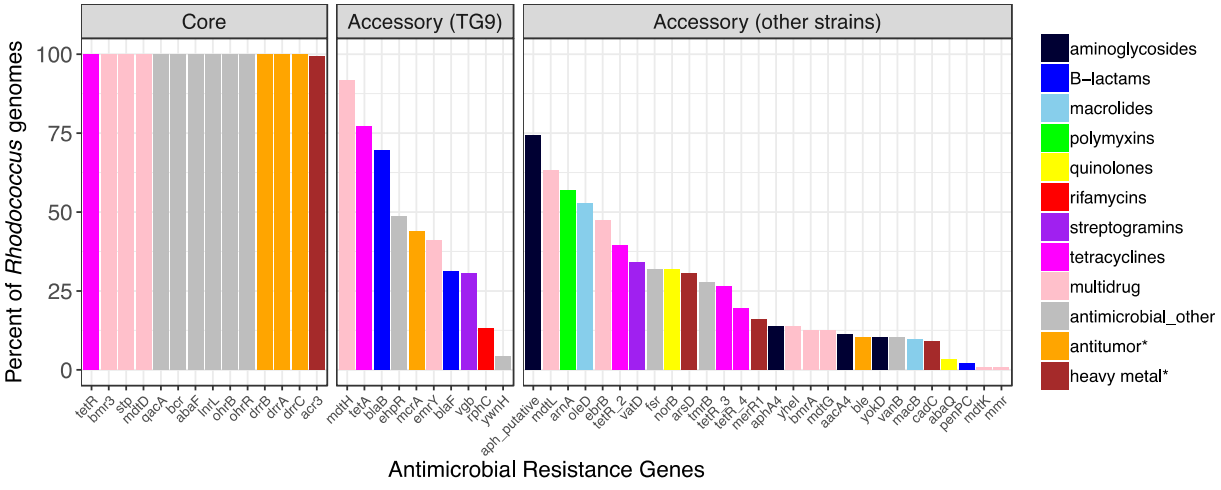

Fig. 3. Antibiotic resistance genes (ARGs) detected in the *Rhodococcus* pangenome. Core and accessory genes are those present in greater or less than 95.0% of all strains, respectively. Accessory ARGs are distinguished by presence or absence in the *R. biphenylivorans* TG9 genome. Colors correspond to drug class. The "*" indicates genes conferring resistance to non-antibiotic compounds, though these genes have implications for antimicrobial resistance.

(i.e., resistance to gentamicin and streptomycin, though not kanamycin), rifampin, tetracycline, chloramphenicol, and polymyxin (note that Gram-positive bacteria are generally not susceptible to polymyxin) (Supplementary Table S5). Notably, *R. biphenylivorans* TG9 also appeared tolerant, though not resistant, to β-lactams such as ampicillin and amoxicillin. Overall, the observed phenotype was generally consistent with our genome-based prediction.

2.5. Prediction of dormancy-related genes

Dormancy is an effective survival strategy and has important implications for isolation of new strains. *R. biphenylivorans* TG9 was originally collected from sediment, and then was recovered from a dormant state. Some dormant bacteria can be resuscitated by Rpf, which might play an important role in the transition out of dormancy. Rpf-encoding genes are widely distributed in *Rhodococcus*. For example, our pangenome assessment identified a gene cluster annotated as *rpfB* to be present in 82.7% of all *Rhodococcus* genomes. By further blasting the TG9 whole genome sequencing against database of COG, NR, KEGG and Swiss-Prot, we found *rpfA*, *rpfB*, *rpfD*, and *rpfE* genes as well. In order to determine the similarity between the *rpf* gene of *M. luteus* and that of TG9, the sequence of all *rpf* genes were aligned using BLAST (National Center for Biotechnology Information). The results showed that four *rpf* genes of TG9 had 46.1%–56.4% identity with the *rpf* gene from *M. luteus* (Supplementary Table S3). Moreover, we also blasted each *rpf* gene of TG9 to find its closest homologs and found that they had high identity (97.9%–99.7%) with other *Rhodococcus rpf* genes (Supplementary Table S3). We further searched these sequences for conserved structural domains using CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The four Rpfs of TG9 and that of *M. luteus* all had specific hits (a high confidence level of domain-specific threshold score) to transglycosylase (has peptidoglycan hydrolytic activity, similar to RPF) or RPF (core lysozyme-like domain of resuscitation-promoting factor proteins). In addition, RpfB of TG9 had a G5 domain (involved in metabolism of bacterial cell walls), and *M. luteus* Rpf had a specific hit to LysM (involved in bacterial peptidoglycan binding and cell wall degradation). Thus, while the Rpf proteins of strain TG9 and *M. luteus* are similar on many levels, there are also key distinctions that may have interesting applications for studying the role of exogenous or endogenous Rpf in resuscitation.

2.6. Biodegradation capabilities of TG9 and other Rhodococcus

The potential for organic pollutant degradation in strain TG9 was predicted based on genomic analysis. Except for genes related to furfural degradation, the genes involved in the other 20 KEGG pathways for xenobiotics biodegradation and metabolism were annotated, including benzoate, aminobenzoate, fluorobenzoate, chloroalkane and chloroalkene, chlorocyclohexane and chlorobenzene, toluene, xylene, nitrotoluene, ethylbenzene, styrene, atrazine, caprolactam, bisphenol, dioxin, naphthalene, polycyclic aromatic hydrocarbons (PAHs) and steroid degradation, together with metabolism of xenobiotics by cytochrome P450, drug metabolism - cytochrome P450, and drug metabolism - other enzymes. In particular, genes related to biphenyl and polychlorinated biphenyl (PCB) degradation were widely annotated in the TG9 genome (Supplementary Fig. S3). Key genes responsible for steroid degradation and the related xylene degradation pathway were also found in the genome of strain TG9 (Supplementary Fig. S4). In addition, most of the pollutants predicted to be degraded by TG9 contained one or more benzene rings (e.g., PCBs, PAHs). Such organic pollutants are highly toxic and often difficult to degrade. Much like *R. biphenylivorans* TG9, other members of *Rhodococcus* encode broad pollutant degradation potential as well. Many *Rhodococcus* have been reported to have a *bph* gene cluster, and some *bph* genes were similar to those of the TG9 (Fig. 4E). More broadly, there were 142 genes encoding degradation pathways in the TG9 genome, of which 43 (30.3%) were assigned as "core" functions of *Rhodococcus* genomes. Core genes of TG9 included those involved in pathways such as degradation of atrazine, benzoate, chloroalkane/chloroalkene, naphthalene, and xylene. Accessory TG9 degradation genes that were conserved in some, but not all *Rhodococcus* included even more pathways for benzoate, benzene, and xylene, among others. Thus, TG9 may be unique in its genus with respect to its predicted abilities for degradation of organic pollutants.

Accordingly, some of the pollutants were chosen as the sole carbon source to do a growth test to confirm degradative function. The results showed that TG9 was able to grow in media containing only phenanthrene (PT), ethylbenzene (EB), tetrachlorobisphenol A (TCBPA), chlorobenzene (CB), biphenyl (BP), phenol (PN), 2-chlorophenol (2-CP), 2,4,6-trichlorophenol (2,4,6-TCP), 4-chlorophenol (4-CP) and several PCB congeners including 2-chlorobiphenyl (2-CB), 3-chlorobiphenyl (3-CB), 2,4′-dichlorobiphenyl (2,4′-DCB), 3,3′-dichlorobiphenyl (3,3′-DCB) or 3,4-dichlorobiphenyl (3,4-DCB) as the sole carbon source (Table 1, Supplementary Fig. S5). To further confirm the degradation ability of TG9, degradative capacity was evaluated under exposure to varying concentrations of biphenyl and PCBs. Across a range of increasing biphenyl concentrations from 100 to 3000 mg L^{-1}, biphenyl was efficiently removed and concurrent cell growth in mineral medium indicated that strain TG9 could utilize biphenyl as the sole carbon source (Fig. 4AB). From 20 to 100 h, while the amount of biphenyl decreased, the volume of benzoic acid increased (Fig. 4C), which indicated that biphenyl was degraded into benzoic acid as an intermediate metabolite. Given longer time, benzoic acid would continue to degrade and finally enter into the tricarboxylic acid (TCA) cycle. Furthermore, strain TG9 exhibited significant degradation of chlorobiphenyl, dichlorobiphenyl, trichlorobiphenyl, with degradation efficiency ranging from 99.9% to 33.1% (Fig. 4D).

The mechanism encoded by the *bph* gene cluster underscores the biphenyl and PCB degradation through strain TG9, which seems to have a unique *bph* cluster (*bph* A1A2A3A4DC1H1J1C2J1I1H2H3H4BI2J2H5) (Fig. 4E). The pathway for biphenyl and PCB degradation contains an upper and lower pathway. BphA (biphenyl 2,3-dioxygenase, the first step, deciding substrate degradation range) and BphC (biphenyl-2,3-diol 1,2-dioxygenase, the third step, influencing degradation efficiency) have been identified as two of the most important proteins involved in the degradation of biphenyl and PCB (Pieper and Seeger, 2008). Biphenyl and 2,3-dihydroxybiphenyl are the substrates of the BphA and BphC, respectively. Thus we further tested the transformation of biphenyl and 2,3-dihydroxybiphenyl. The results showed that when using biphenyl and PCBs (2-CB, 3,4-DCB) as the sole carbon source, the transformation of biphenyl and 2,3-dihydroxybiphenyl were both significant higher than in the control (without substrate) and 2,4′,5-TrCB (which could not be used as a carbon source) (Fig. 5). The results suggest that strain TG9 has the ability to metabolize biphenyl and contains a complete, functional biphenyl catabolic pathway.

2.7. Induction, morphology and transcription analysis of dormant TG9 cell

To investigate the characteristics of dormancy, TG9 cells were induced into the dormant state using low temperature and oligotrophic culture. Laser scanning confocal microscope (LSCM) images showed that the dormant cells were alive, but the number of culturable cells was lower than 0.1 colony forming units mL^{-1} (Supplementary Fig. S5). The result of scanning electron microscopy showed that active cells were rod-shaped and most of them were arranged in chains, while the dormant cells became shorter, or globose, and the average cell size was reduced (Supplementary Fig. S6). Analysis of previously generated transcriptomic data suggested that expression of *rpf* genes was higher in active than dormant cells, and these results were confirmed with reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR; Fig. 6). Thus, *rpf* genes might be an indicator of bacterial dormancy.

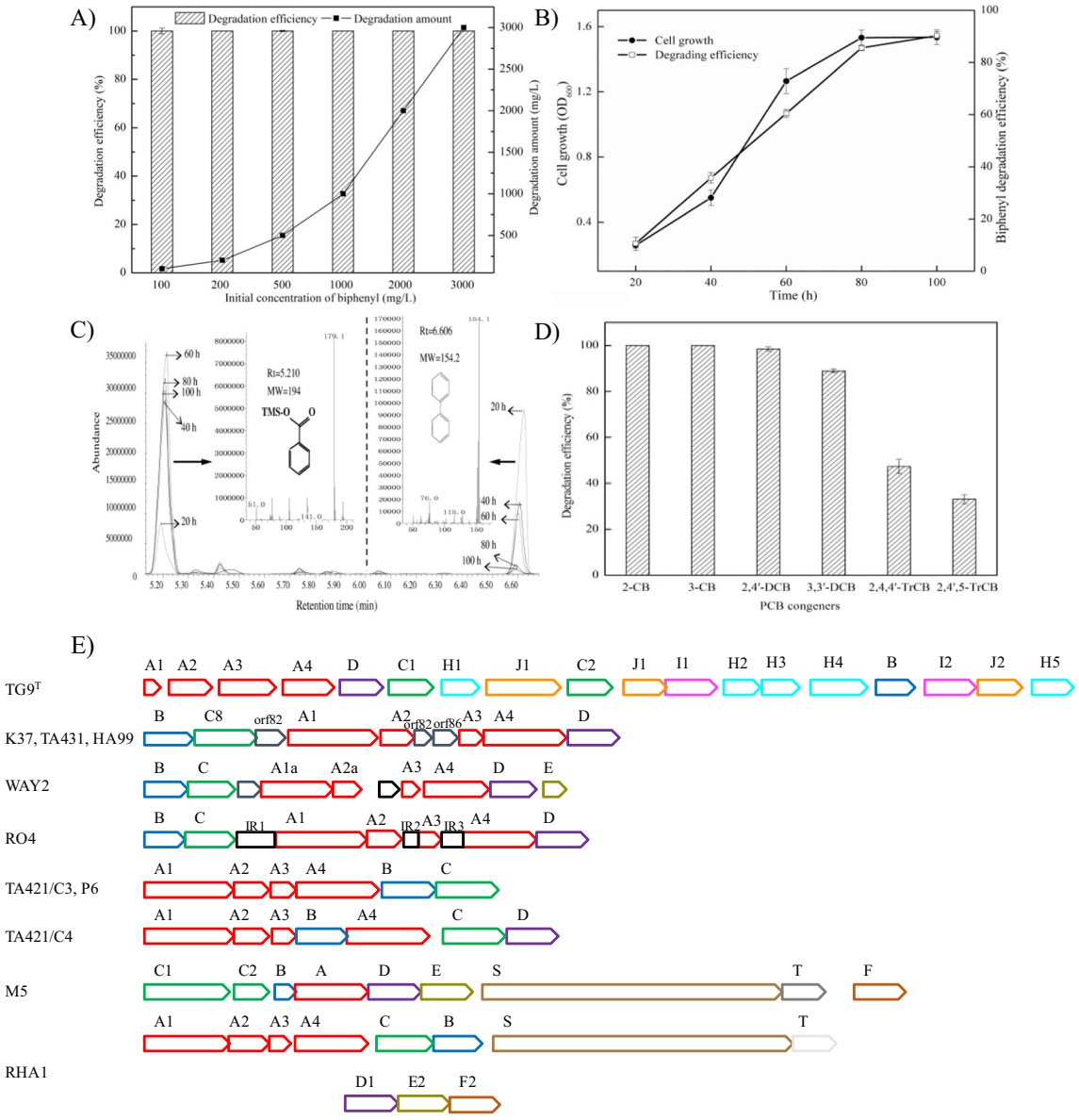

Fig. 4. Degradation ability of *R. biphenylivorans* TG9 and Organization of *bph* gene clusters in various *Rhodococcus* strains.
Degradation of various concentrations of biphenyl by strain TG9 (A). Time course of biphenyl degradation and cell growth of strain *R. biphenylivorans* TG9 in minimal media (B), and the variation trends of biphenyl and benzoate (C). Degradation of PCB congeners by strain TG9 in 72 h (D). Organization of *bph* gene clusters in various *Rhodococcus* strains (E).
For panel E, different colors indicate different kind of *bph* genes, named as marked. The most close homologs to each *bph* gene of TG9 has been showed in Supplementary Table S6.

Overall, coupling the pangenome analysis with experiment validation demonstrated that the strain TG9 appeared to have unique implications for exploring dormancy and degradation of pollutants.

3. Discussion

We sequenced the genome of *R. biphenylivorans* TG9 and revealed diverse predicted functions, including the ability to enter and leave dormancy, antibiotic resistance to survive hazards and organic pollutant degradation. Pangenome analysis revealed that some of these traits were shared among many members of the genus *Rhodococcus*. A genus-level pangenome analysis is inherently limited due to genomic dissimilarity across species arising from mutations and gene gain/loss events that occur with divergence of lineages. However, it is useful for high level predictions to provide functional context for specific isolates with respect to broader bacterial taxa. The genomic analysis suggested that genus of *Rhodococcus* has strong potential for degradation and dormancy, which we further now validated using culture-based and molecular biology methods. By focusing on functional annotations of the gene clusters in the *Rhodococcus* pangenome, *R. biphenylivorans* TG9 appears to be a good candidate for the study of the relationship between degradation and dormancy.

Degradation is one of the most important functions and applications for both the genus *Rhodococcus* and strain TG9. Our pangenome analysis showed a broad potential for members of *Rhodococcus* to degrade organic pollutants (mainly those containing benzene ring), which was consistent with previous findings (Garrido-Sanz et al., 2020). Strain TG9 encoded genes involved in 20 out of 21 pathways for xenobiotic biodegradation and metabolism. The result of growth tests showed that strain TG9 could grow on fourteen organic pollutants. Interestingly, TG9 increased cell density on medium containing TCBPA. Aerobic degradation of TCBPA has rarely been reported, and only Yuan et al. demonstrated TCBPA degradation in sediment containing *Bacillus megaterium* and *Pseudomonas putida* (Yuan et al., 2010). TCBPA degradation thus merits further study.

Table 1
Growth of *R. biphenylivorans* TG9 using different substrates as sole carbon source.

Full name	Short name	As sole carbon source
Phenanthrene	PT	Yes
Ehylbenzene	EB	Yes
Tetrachlorobisphenol A	TCBPA	Yes
Chlorobenzene	CB	Yes
Biphenyl	BP	Yes
2-Chlorobiphenyl	2-CB	Yes
3-Chlorobiphenyl	3-CB	Yes
2,4′-Dichlorobiphenyl	2,4′-DCB	Yes
3,3′-Dichlorobiphenyl	3,3′-DCB	Yes
3,4-Dichlorobiphenyl	3,4-DCB	Yes
Phenol	PN	Yes
2-Chlorophenol	2-CP	Yes
2,4,6-Trichlorophenol	2,4,6-TCP	Yes
4-Chlorophenol	4-CP	Yes
2,4,4′-Trichlorobiphenyl	2,4,4′-TrCB	NO
2,4′,5-Trichlorobiphenyl	2,4′,5-TrCB	NO
3,5-Dichlorophenol	3,5-DCP	NO
2,4-Dichlorophenol	2,4-DCP	NO
Pentachlorophenol	PCP	NO
Negative control	CK	NO

The control group was the same as other treatments without adding the pollutants as sole carbon source.

Strain TG9 also showed a strong ability to degrade biphenyl and thus likely PCBs. Biphenyl is a useful substrate to screen for degrader and compare the degradation potential of different bacteria for PCBs. Our experiments indicated that strain TG9 could grow and fully degrade of 3000 mg L^{-1} biphenyl in 96 h, as well as utilizing it as the sole carbon source and metabolizing it to benzoic acid. During biodegradation of biphenyl, benzoic acid easily accumulates (Potrawfke et al., 1998; Egorova et al., 2010; Egorova et al., 2020). It has been shown that benzoic acid could suppress the enzyme activity of Bph and inhibit the growth of degraders (Camara et al., 2004). However, benzoic acid could be further degraded by strain TG9, which explaining its strong degrading ability. Similarly, other *Rhodococcus* have been reported to use biphenyl as their sole carbon source. *Rhodococcus erythropolis* T902.1 could degrade 53.0% of 500 mg L^{-1} biphenyl in 18 d (Wannoussa et al., 2015). *Rhodococcus* sp. R04 was reported to degrade about 90% of 154 mg L^{-1} of biphenyl in 30 h (Yang et al., 2007). Thus, *R. biphenylivorans* TG9 has a relatively strong ability to degrade biphenyl. Nevertheless, it should be noted that comparing biphenyl degradation across studies may have inherent biases due to different experimental systems and parameters such as biomass, substrate concentration, and growth conditions.

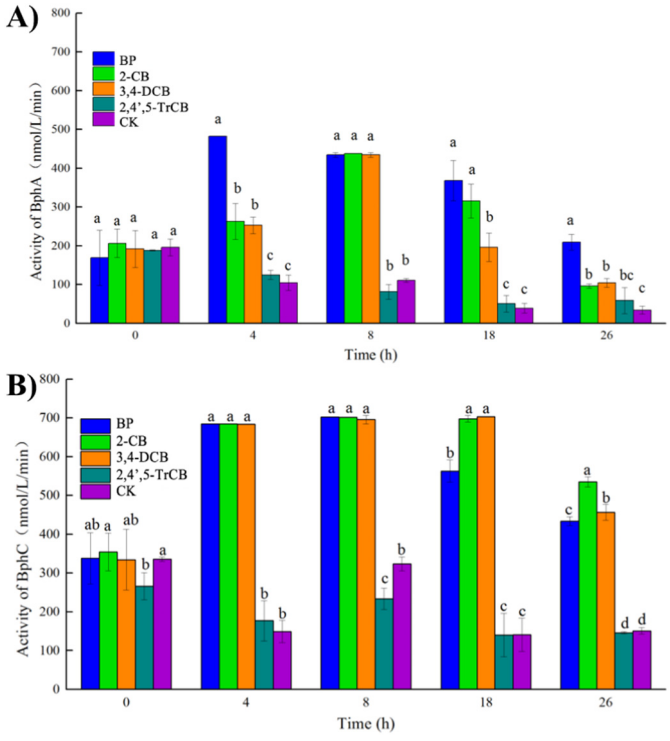

Fig. 5. Changes of BphA and BphC activity of *R. biphenylivorans* TG9 under different substrate conditions
The figure marked with different letters means experimental and control group was significantly different $P < 0.05$, marked with same letter means experimental and control group was insignificant $P > 0.05$.

Microbial degradation of PCBs evolved from biphenyl metabolism, and both biphenyl and PCB degradation rely on the *bph* pathway (Kumamaru et al., 1998; Egorova et al., 2020; Gorbunova et al., 2021; Ines et al., 2021). The reported bph gene clusters of *Rhodococcus* were classed into four categories, including *bph*BCA1A2A3A4D (Taguchi et al., 2007; Yang et al., 2007; Garrido-Sanz et al., 2018; Garrido-Sanz et al., 2020b), *bph*A1A2A3A4BC (Asturias et al., 1995; McKay et al., 1997; Taguchi et al., 2007), *bph*C1C2BAD1E2STF2 (Labbe et al., 1997), and *bph*A1A2A3A4CBSTD1E2F2 (Fukuda et al., 1998; Taguchi et al., 2007; Yang et al., 2007; Patrauchan et al., 2008; Pieper and Seeger, 2008; Araki et al., 2011; Li et al., 2012). The *bph* gene cluster of strain TG9 (*bph*A1A2A3A4DC1H1J1C2J1I1H2H3H4BI2J2H5) was very different from the other four *bph* gene clusters of *Rhodococcus* in the composition and order of genes (Fig. 4E), despite the fact that TG9's *bph* genes were individually homologous with those of other *Rhodococcus* (Supplementary Table S6). The particular structure of the TG9 *bph* gene cluster was different from those of other genera, including *Burkholderia xenovorans* LB400 (Denef et al., 2005), *Pseudomonas putida* KF715 (Hayase et al., 1990), *Bacillus* sp. JF8 (Mukerjee-Dhar et al., 2005) and *Sphingobium yanoikuyae* B1 (Kim and Zylstra, 1999). Strain TG9 might have a whole different *bph* gene cluster, and interestingly the downstream pathway (*bph*IJH) has multicomponent genes (*bph*I1I2J1J2J3H1H2H3H4H5). Moreover, except for strain M5, all the other typical *Rhodococcus* have several *bph*A genes. Indeed, TG9 has four genes involved in *bph*A. BphA, responsible for initializing aerobic degradation, are usually multicomponent enzymes containing a catalytic iron-sulfur protein and a ferredoxin-NADH-reductase (Butler and Mason, 1997). Only strain M5 has a single *bph*A gene; however, this genome may be incomplete. The upper pathway (*bph*ABCD) is crucial for successful degradation. Biphenyl or PCBs are transformed to *cis*-2,3-dihydro-2,3-dihydroxybiphenyl compound by BphA, and the product is then converted to 2,3-dihydroxy-biphenyl through BphB (*cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase). 2,3-dihydroxy-biphenyl is degraded into 2-hydroxy-6-oxo-6(2-dydroxyphenyl)-hexa-2,4-dieneoate by BphC, which is in turn catalyzed by BphD (2,6-dioxo-6-phenylhexa-3-enoate hydrolase) to yield 2-hydroxypenta-2,4-dienoate and benzoate. Lower pathways mainly degrade these two products via BphH (2-keto-4-pentenoate hydratase), BphI (4-hydroxy 2-oxovalerate aldolase) and BphJ (acetaldehyde dehydrogenase) to acetyl-CoA, which can then enter the TCA cycle (Pieper and Seeger, 2008). Strain TG9 potentially has a novel *bph* gene cluster that enables high degradation efficiency of biphenyl or PCBs. Thus, *R. biphenylivorans* appears to be an effective candidate for application in degrading PCBs or other compound with a similar structure to biphenyl.

As many organic pollutant-contaminated environments contain multiple chemical stressors, such as extreme pH, metals, antibiotics and so on, the ability to tolerate these stressors and survive in situ is critical for successful bioremediation. Many antimicrobial resistance genes may play a role in survival under these conditions. Based on our analysis, *Rhodococcus* might be resistant to stressors such as tetracyclines and rifampin, other antimicrobials, and heavy metals. Genome-based prediction and experimental validation indicated that TG9 is resistant to some aminoglycosides, chloramphenicol, and polymyxin. Strain TG9

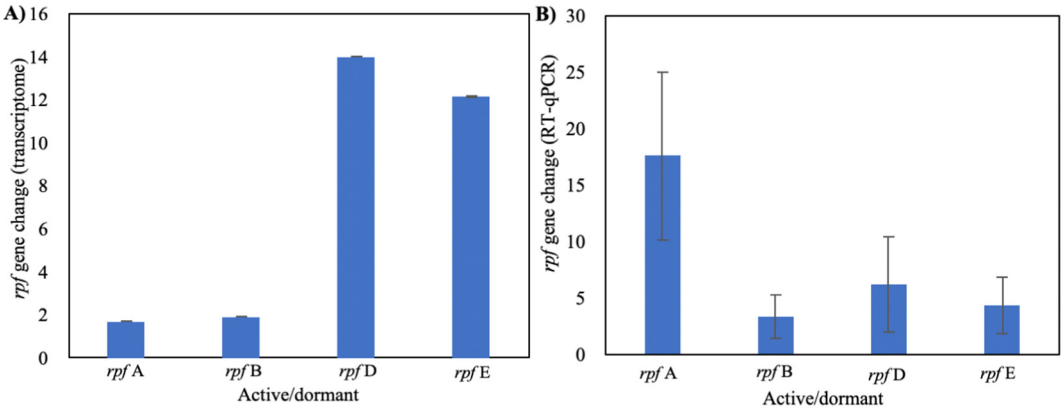

Fig. 6. The expression of *rpf* genes in active and dormant *R. biphenylivorans* TG9 using transcriptome (A) and RT-qPCR (B) analysis.

was isolated from Taizhou city where electronic waste recycling has been conducted for more than 30 years (Su et al., 2015a). The local environmental conditions explain its strong ability to degrade various organic pollutants and tolerate heavy metals or metalloids. In addition, the presence of antibiotics released by surrounding people and animals might induce and enhance resistance towards various clinical antimicrobials. ARGs commonly exist naturally in environmental bacteria, and may be enriched in bacteria found in environments contaminated with compounds like antibiotics, heavy metals and organic pollutants (Zhu et al., 2013; Sun et al., 2015; Younessi et al., 2019). Prior research has indicated that members of the genus *Rhodococcus* can tolerate many stressors, including osmotic pressure, extreme pH, metals, antibiotics and others (Cappelletti et al., 2016; de Carvalho et al., 2016; Pátek et al., 2021). Also, the coexistence contaminants such as metals, organic pollutants and other stressors could increase the expression of ARGs (Stepanauskas et al., 2006), which would enhance the tolerance of TG9 strain to antibiotics in environment.

Despite the presence of resistance genes, added biodegraders might become inactive or enter into dormancy in situ due to the presence of physical/chemical stressors, which poses a great challenge for bioremediation (Giagnoni et al., 2018). Dormancy in particular represents an advantage to the bacteria, as it may help them survive under stress, but a detriment to the success of in situ bioremediation. Transcription analysis of the active and dormant TG9 indicated that *rpf* genes, which are related to the dormancy and resuscitation, were all down-regulated in dormant cells (Fig. 6). Adding Rpf protein to resuscitate dormant degraders could improve the degradation efficiency of biphenyl or PCBs (Ye et al., 2020). Rpf has been reported to re-activate dormant bacteria from environmental samples for degrader isolation (Su et al., 2019). Strain TG9 itself was resuscitated and isolated by adding external Rpf from *M. luteus* (Su et al., 2015a). Moreover, it has four *rpf* genes, which are different from the *rpf* of *M. luteus*. The Rpfs from both TG9 and *M. luteus* were predicted to have core lysozyme-like domain of Rpf proteins but also key structural differences. The application of exogenous Rpf, and the range of organisms that respond to specific Rpf activities, merits further study.

We recognize that our approach was subject to several key limitations. Performing a genus-level pangenome analysis was limited by the necessity to use relatively low sequence similarity threshold (i.e. 70.0%) to maintain paralogs in gene clusters and overcome potential challenges for grouping genes across different species. This may yield different gene clusters encoding redundant gene products. Thus, we assigned core and accessory functions to TG9 based on annotated product names prior to EGGmapper analysis, which may have provided a limited or misleading scope of overall function (e.g., protein domains and related function may drastically differ from annotated description). Future research is needed to benchmark the ability to make coarse genome-level pangenomic comparisons, perhaps using additional pangenome-building tools such as Anvi'o (Eren et al., 2015) or Panaroo (Tonkin-Hill et al., 2020). Moreover, classification of the functional potential of TG9 and other *Rhodococcus* was limited by the large abundance of genes with unknown function, i.e., on average, 46.0% of the genes within each *Rhodococcus* genome ($n = 144$ genomes) were annotated as "hypothetical protein." It is common for newly sequenced bacterial genome assemblies to have 30.0–50.0% proportion of hypothetical proteins (Stevens, 2005; Kotaru et al., 2013; Marklevitz and Harris, 2017; Uddin et al., 2019). More than 30% of the XDR-*Mycobacterium tuberculosis* genome is annotated as hypothetical proteins (Uddin et al., 2019). Even antibiotic-resistant *Staphylococcus aureus*, which is widely studied because it was a major public concern, has about 50% of its genome annotated as hypothetical proteins (Marklevitz and Harris, 2017). This proportion for environmentally sourced organisms is even higher because they are under-characterized relative to human-associated organisms and particularly pathogens. Genetic, bioinformatic and biochemical experiments have been used to elucidate the function annotation of these hypothetical proteins (Eisenstein et al., 2000; Younessi et al., 2019). As a result, various transporters, binding and regulatory proteins, and enzymes with biotechnological potential have been found (Yang et al., 2019; Gazi et al., 2020). Future studies focused on functional characterizations of TG9 would be beneficial to further explore these hypothetical proteins and elucidate mechanisms associated with biodegradation of organic pollutants and survival in harsh environments.

4. Experimental procedures

4.1. Genome sequencing, assembly and annotation

Whole genome sequencing of *R. biphenylivorans* TG9 was conducted to reveal its properties and functions. DNA was extracted using the Omega BioTek kit (Norcross, GA, USA) following the instruction. Whole genome sequencing was performed using the Illumina Hiseq4000 and Pacific Biosciences (PacBio) RSII sequencing platforms. The Illumina pair-end sequencing library was prepared with a TruSeq DNA sample prep kit and the Pacbio library was prepared with a SMRTbell® Express Template Preparation Kit 2.0 (Pacific Biosciences, USA) for 20 kb insert size. The sequence data were assembled de novo using the hierarchical genome-assembly process (HGAP) protocol RS-HGAP Assembly 3 in SMRT analysis version 2.3.0 (http://www.pacb.com/products-andservices/analytical-software/smrt-analysis/) (Chin et al., 2013). The genes in the TG9 genome were identified using Glimmer (version 3.02, parameter, -o*-g*-t*-l linear, http://ccb.jhu.edu/software/glimmer/index.shtml). Function annotation was performed based on BLAST search (-e 1e-5 -F F -a 4 -m 8) against the COG (Galperin et al., 2015), NR (Pruitt et al., 2007), KEGG (Kanehisa et al., 2016) and Swiss-Prot (Bateman et al., 2015) databases.

The complete genome data of *R. biphenylivorans* TG9 has been deposited at GenBank under the accession numbers CP022208. This strain TG9 is available from the China General Microbiological Culture Collection Center (CGMCC 1.12975^T), Korean Collection for Type Cultures (KCTC 29673^T) and Marine Culture Collection of China (MCCC 1K00286^T).

4.2. Pangenome analysis

To understand genes and functions (especially those related to antibiotic resistance, dormancy, and degradation) of *R. biphenylivorans* TG9 relative to other species within the *Rhodococcus* genus, a pangenome analysis was performed. All available genome assemblies for the *Rhodococcus* genus were obtained from NCBI GenBank (n = 398; www.ncbi.nlm.nih.gov/assembly accessed on 02/24/2020) and assembly quality was evaluated with CheckM v1.0.7 (Parks et al., 2015). Genomes with >99.0% completeness and <1.0% contamination (n = 144) were used to construct the *Rhodococcus* pangenome (Supplementary Table S1). The high-quality genomes were annotated with prokka v1.12 with flag "–genus *Rhodococcus*" (Seemann, 2014). The output.gff files were processed with Roary v3.12.0 (Page et al., 2015) using flags for minimum blastp identity of 70.0% (-i 70; set relatively low since comparing across different species) and to not split gene clusters containing paralogs (-s; to avoid inflating the number of clusters of core genes) to build the pangenome matrix. The matrix was screened for redundant annotations to different gene clusters to determine presence/absence of unique predicted functions. Gene clusters and predicted gene products were grouped into categories of "cloud," "shell," and "core" corresponding to presence in <15.0%, 15.0 to 95.0%, and >95.0% of all genomes analyzed, respectively. While the collection of gene families present in every member of a particular organismal group are "core" (McInerney et al., 2017), a 95.0% conservation rate was selected (i.e., almost all genomes) as there may have been inherent biases or batch effects in sequence data across studies accessed in NCBI (Snipen and Liland, 2015; Blaustein et al., 2019). The cutoffs assigned to the fractions of accessory genes (i.e., cloud and shell genes) were selected based on observed distributions for the rates for conserved genes/gene products. All bioinformatics scripts and generated data that may be used to reproduce our analyses are available at https://github.com/hartmann-lab/Rhodococcus_pangenome."

All pangenome-related data visualizations were performed in R v3.2.1. To infer phylogenetic similarity of *R. biphenylivorans* TG9 to other species within the *Rhodococcus* genus, the subset of genomes that clustered around strain TG9 (n = 15 genomes) based on Bray-Curtis distance for presence/absence of gene products were reprocessed with Roary. The generated core gene amino acid sequence alignments for the subset were processed with FastTree v2.1.10 using the Jones-Taylor-Thornton model and CAT approximation (Price et al., 2010). The Newick trees were processed with Phangorn v2.4.0 (Schliep, 2011) for midpoint rooting and plotted with Ape v5.1 (Paradis and Schliep, 2019).

4.3. COG functional category

To further know the functional categories of the stain and the genus, focusing on the *R. biphenylivorans* TG9 genome, genes with an annotated function (i.e., not including "hypothetical proteins") were assigned "core," "shell," or "cloud" categories based on the frequency of particular function presence across all *Rhodococcus* (which was the same as pangenome analysis, i.e., >95.0%, 15.0–95.0%, or <15.0% across strains, respectively). Each fraction of the TG9 genome (i.e., core, shell, cloud), as well as the full genome, were processed with BlastKOALA and EggNOG to elucidate KEGG pathways and COG categories, respectively.

4.4. Prediction of dormant genes, antimicrobial susceptibility and the potential of degradation ability in both the strain TG9 and the genus *Rhodococcus*

Whole genome sequencing and pangenome assessment also enabled comparison of key aspects of *R. biphenylivorans* TG9 (e.g., dormant genes, antimicrobial resistance properties, biodegradation capabilities) relative to members of the broader *Rhodococcus* genus with regard environmental implications. The *rpf* genes and potential genes with degradation properties of the strain TG9 and *Rhodococcus* genus were characterized by searching the keywords of in both whole genome sequencing as well as pangenome analysis. The essential genes of dormancy, *rpf* genes, their sequence were also blasted against the NCBI database. While the important gene cluster of degradation, *bph* gene cluster, their sequences were searched and blasted based on publications and databses. ARGs within the *R. biphenylivorans* TG9 genome and *Rhodococcus* pangenome were identified by screening the respective gene product lists for associated keywords (i.e., "resistance," "aminoglycoside," "*cillin," "lactam," "macrolide," "*mycin," "polymyxin," "rifampin," "quinolone," "streptogramin," and "tetracycline"). For any positive hits, UniProtKB (http://www.uniprot.org) was used to confirm antibiotic resistance as the biological process (i.e., ensure that it was not antibiotic biosynthesis). ARGs that were core to *Rhodococcus* and those that were accessory (i.e., present in <95.0% of all genomes) with presence or absence in TG9 were identified. The TG9 genome assembly was further processed with the CARD, using the RGI online (https://card.mcmaster.ca/analyze/rgi) to characterize its ARG profile.

4.5. Validation of antimicrobial susceptibility

To test the antimicrobial susceptibility of *R. biphenylivorans* TG9 predicted by the pan-genome analysis, minimum inhibitory concentration (MIC) test of antimicrobial substances was conducted using a serial broth dilution method in 96-well microtiter plate method by Wiegand with some modification (Wiegand et al., 2008). Briefly, bacteria suspension with a a final density equivalent to $5 * 10^5$ cfu mL^{-1} was inoculated into a Mueller-Hinton broth in the presence of different concentrations of antibiotic (0.01, 0.1, 1, 10, 200, 500, 1000 mg L^{-1}). Visible growth was assessed after incubation for a period of time (16–20 h) and the lowest concentration to prevent growth was known as the MIC value.

4.6. Growth test under organic pollutants

The organic pollutants were added to seed medium as sole carbon source of the strain TG9 to test its potential of degradation. To prepare seed medium, TG9 was inoculated into Luria-Bertani broth and cultured in 30 °C, 180 r min^{-1} for 24 h. Cells were collected by centrifuging at 8000 r min^{-1} for 10 min, washed twice by phosphate buffer (pH = 7.4, 0.05 mol L^{-1}), and finally suspended in mineral medium (OD_{600} ≈ 0.06– 0.08) to prepare seed medium for further experiments. The mineral medium contained the following ingredients per liter deionized water: 1 g KH_2PO_4, 3 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4$, 0.02 g $FeSO_4 \cdot 7H_2O$, 1 g NaCl, 3 g $(NH_4)_2SO_4$, 0.01 g $CaCl_2$, and 2 mL trace elements solution (g/L: 0.004 g MoO_3, 0.028 g $ZnSO_4 \cdot 5H_2O$, 0.02 g $CuSO_4 \cdot 5H_2O$, 0.004 g H_3BO_3, 0.004 g $MnSO_4 \cdot 5H_2O$, and 0.004 g $CoCl_2 \cdot 6H_2O$), pH = 7.3 (Su et al., 2015b; Ye et al., 2020).

Different concentrations of pollutants, including 5 mg L^{-1} PN, EB, TCBPA, CB, BP; 10 mg L^{-1} PCBs (2-CB, 3-CB, 2,4′-DCB, 3,3′-DCB, 3,4-DCB, 2,4,4′-TrCB, 2,4′,5-TrCB), 100 mg L^{-1} 3,5-DCP (3,5-dichlorophenol), 2,4-DCP (2,4-dichlorophenol), 2,4,6-TCP, 4-CP, PCP (Pentachlorophenol), PN, and BP; 250 mg L^{-1} 2-CP dissolved in *n*-hexane were added into wells in 96 well plates, respectively, and *n*-hexane evaporated to dry. Then 200 μL of prepared seed medium of TG9 was inoculated (initial OD_{600} = 0.06 or 0.08) before culturing at 30 °C, 180 r min^{-1} and growth monitoring using multifunctional enzyme

marker. The well which showed increase of cell density was validated through agar plate to approve the growth of TG9. The negative controls were those without adding the pollutants as carbon source. Since the seed medium lack carbon source, the cell growth showed the strain could use the organic pollutant as sole carbon source.

4.7. Validation the degradation ability of biphenyl and PCBs of strain TG9

Biphenyl and PCBs were chosen as representatives of organic pollutants to confirm the strain's potential of organic pollutants biodegradation. Seed medium contained no carbon source of TG9 was prepared as mentioned before.

4.7.1. Tolerance and degradation of biphenyl

Tolerance of biphenyl was conducted at nominal concentrations of 100, 200, 500, 1000, 2000, and 3000 mg L^{-1} media for strain TG9 over 96 h. Different concentrations of biphenyl dissolved in *n*-hexane were added into a 50 mL flask; after the solvent *n*-hexane completely dried, the biphenyl was adsorbed to the bottom of the flask. Then 20 mL of TG9 suspended in mineral medium (as described in Section 4.6) was added before culturing at 30 °C, 180 r min^{-1}. We further evaluated the degradation of 1500 mg L^{-1} biphenyl by monitoring the growth, degradation efficiency and metabolic pathway at 20 h intervals over the course of 100 h. 5 mL of prepared seed medium of TG9 was added into a 50 mL glass tube contained 1500 mg L^{-1} biphenyl before culturing at 30 °C, 180 r min^{-1}. The growth of strain TG9 was monitored by measuring the OD_{600} at the time of extraction.

To assess biphenyl degradation (classically transformed via BphA (Lajoie et al., 1994; Gomez-Gil et al., 2007; Yang et al., 2007)), mineral medium (5 mL) and biphenyl (15.6 mg L^{-1}) were added to a glass tube, closed tightly and incubated at 30 °C for 60 min. HCl (100 μL, 3 mol L^{-1}) was added to lyse the cells and terminate the reaction. The residual amount of biphenyl in the system was determined. The assay to assess transformation of 2,3-dihydroxybiphenyl (classically intermediate in biphenyl degradation, performed by BphC), was similar to that for biphenyl, except that the cells were lysed by ultrasonication in an ice bath (120 W, 12 min, the working time was 4 s, and the interval time was 6 s), centrifuged (4 °C, 13000 rpm min^{-1}, 30 min), and 2,3-dihydroxybiphenyl (14.9 mg L^{-1}) was used as substrate and detected. In both experiments, the glass bottle cap was covered with a layer of aluminum foil and closed tightly to inhibit biphenyl loss due to volatilization.

4.7.2. Degradation of PCBs

We further evaluated the potential for *R. biphenylivorans* TG9 to degrade PCBs. Similarly, 5 mL of prepared seed medium was added into a 50 mL glass tube contained different initial concentrations of 2-CB, 3-CB, 2,4′-DCB, 3,3′-DCB, 2,4,4′-trichlorobiphenyl (2,4,4′-TrCB), 2,4′,5-trichlorobiphenyl (2,4′,5-TrCB), which was 5.57, 6.41, 7.18, 7.93, 7.43, 9.36 mg L^{-1}, respectively. The caps were capped tightly. Tests of different congeners of PCB were conducted for 72 h at 30 °C, 180 r min^{-1} before extracting for concentration determination.

4.7.3. Extraction and determination concentration of biphenyl and PCB

For both experiments of biphenyl and PCB, the controls were done the same as the samples, except replacing the active TG9 solution with sterilized TG9 culture. The negative controls were done to verify that the degradation of the treatment group comes from the active strain TG9 rather than other factors such as the volatility of pollutants or the experiment system. The degrading efficiency was expressed as the difference between the reduction rate of the experimental group and the control group. For extraction and determination, samples were cultured for the predetermined time before the container was removed out of the shaker. The analysis was conducted as described in (Shen et al., 2008). The pH of the sample was adjusted with 3 mol L^{-1} hydrochloric acid, after which 3 mL of ethyl acetate was added, and the mixture was shaken vertically for 10 min, and let stand for 2 h to allow for phase separation. The whole procedure was then repeated, and the resulting organic phase extracts were combined. Water was removed using anhydrous sodium sulfate and gently blowing with nitrogen to dryness, after which 100 μL *n*-hexane and derivatization reagent (BSTFA + TMCS 99: 1) each were added, and the mixture was kept at 60 °C for 15 min. Finally, *n*-hexane was added to a volume of 1 mL before analysis using GC–MS. The parameters for GC–MS analysis were as follows. The column (30 m HP-5) and electron bombardment (70 eV) were chosen, ion source temperature (230 °C), injection temperature (290 °C) and quadrupole temperature (150 °C) were set. Full scan mode (50– 550 amu, the flow rate of helium is l mL min^{-1}) was conducted. The temperature was programmed as follows: the initial temperature was 80 °C for 1 min, increased to 140 °C at 25 °C min^{-1}, then 8 °C min^{-1} to reach 180 °C, and finally raised to 280 °C at 15 °C min^{-1}. The injection volume was 2 μL.

4.8. Inducement of dormant TG9, morphology and transcription analysis of active and dormant TG9

To find the difference of degradation and dormancy capabilities in active and dormant TG9, we induced TG9 into the dormant state using low temperature and oligotrophic culture. Briefly, seed medium of TG9 as prepared before was put in 4 °C refrigerator and regularly determined the viable and culturable cell. The viable cell was stained using LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes Inc., USA) following the instruction before counted through laser scanning confocal microscope (LSCM, Carl Zeiss, Germany). The culturable cell was determined using spread plate method.

The morphological analysis of active and dormant TG9 was conducted using scanning electron microscopy (SEM). Briefly, the active and dormant TG9 were fixed with glutaraldehyde, filtered, dehydrated and coated, before being observed under SEM.

A transcription analysis of the active and dormant TG9 was performed to find the different regulation of dormancy related genes (mainly *rpf* genes in the current study). The previous transcriptomic (Su et al., 2015b) has been reanalyzed using the whole genome sequencing data of TG9 as the reference database rather than the reference genome (https://www.ncbi.nlm.nih.gov/search/all/?term=NC_023150.1). The *rpf* genes of active and dormant TG9 were validated using reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). The RNA extraction, primers design, and the procedure of the RT-qPCR were the same as (Ye et al., 2020). The amplification efficiencies of all standard curves (90% to 110%, $R^2 > 0.99$) met the expected standards.

CRediT authorship contribution statement

CS and EMH supervised the study. CY designed the research. HW provided the resources. RAB and CY analyzed the data. CY, HW, and RAB wrote the paper. CY, HW, LG, QY, and XS performed the experiments. JF and YF helped design and modify the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151141.

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