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Original Research

Characterization of a Diesel-Degrading *Rhodococcus qingshengii* RHZ01 and Its Genome Analysis

Yujie Huang*#, Zhenjie Wang#, Yali Zhang, Xinjian Zhang, Leilei Wang, Wen Zhang, Qingqing Zhao, Liwen Zheng, Fanyong Song, Jianing Wang

Shandong Provincial Key Laboratory of Applied Microbiology, Ecology Institute of Shandong Academy of Sciences, Qilu University of Technology (Shandong Academy of Sciences), Ji'nan, 250103, China

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Abstract

Bioremediation is a low-cost, eco-friendly technique to reduce and/or eliminate pollutants from environment. Moreover, effective biological degrader for contaminants removal is important during bioremediation process. Here, the strain RHZ01, isolated from petroleum-contaminated soil in Shengli Oilfield, was identified as *Rhodococcus qingshengii* by the morphology, physiology, biochemistry and molecular biology. Strain RHZ01 was able to use diesel as the sole carbon source. The optimal initial pH and temperature for strain RHZ01 growth and diesel degradation were 6-8.5 and 20-30ºC, respectively. Nutrients ($NaNO_3$, NH_4Cl, KNO_3 and Urea) could replace NH_4NO_3. And Mg^{2+} was required for strain RHZ01 growth and diesel degradation. To investigate the diesel degrading mechanisms of this strain, the complete genome was sequenced and annotated. The complete genome consists of one chromosome with a total length 6,506,318 bp and a G+C content of 62.48%. Five putative alkane monooxygenases (AlkB) encoding genes were further analyzed, and their protein sequences were characterized and compared with other published AlkBs in *Rhodococcus* spp.. The physicochemical features of the five AlkBs were analyzed, and these monooxygenase proteins are alkaline, unstable and thermostable. AlkB1, AlkB2 and AlkB3 are hydrophilic protein while the other proteins were hydrophobic. All of these alkane monooxygenases share several conserved regions, such as HYG-motif and three Hist boxes. From the results, it is demonstrated that bacterial strain isolated from petroleum-contaminated could be the potential bioremediation agent for diesel removal.

Keywords: *Rhodococcus qingshengii*, diesel degradation, genome analysis, alkane monooxygenase

#The authors contributed equally to this paper.
*e-mail: huangyj@sdas.org

Introduction

Diesel as the light petroleum product is a complex mixture of hydrocarbons (C atomic number between 9-22) [1-3]. With the high demand for diesel around the world, these hydrocarbon components were released into environment (groundwater, soil and air) through various forms, and as a result, the environment is polluted and human health is endangered [4-7]. There are different techniques used to remove the organic contaminant from the environment, including chemical, physical and biological methods [8]. And as an environment-friendly, and cost-effective technology, bioremediation had attracted the attention of experts from various countries [9, 10]. Bioremediation is a process to remove the pollutants which usually depends on the two techniques, bioaugmentation and biostimulation [11-13].

Bioaugmentation is the main method for bioremediation of diesel pollution, and during the repair process, hydrocarbon-degrading microbes were introduced to the polluted environment to breed and grow [14]. Therefore, potential microbes which can utilize the organic pollutant as carbon source would be useful to remove organic pollutants such as diesel. And it is meaningful to research all kinds of microbes and their ability and characteristics on organic pollutants degradation. Many reports showed that the growth and proliferation of hydrocarbon-degrading microbe, which include bacteria, yeast, fungi, protozoa and algae, are beneficial to the reduction of contaminants [15-19]. Especially, bacteria, such as *Rhodococcus*, *Actinobacter*, *Bacillus*, *Pseudomonas*, *Achromobacter*, *Marinobacter*, *Enterobacter*, play a great role in the biodegradation of hydrocarbon contaminants [20-25]. For instance, the potential of *Bacillus* species for deposited paraffin removal was investigated, and the strains exhibited effective degradation capability of *n*-alkanes between C_{15}-C_{23} and C_{27}-C_{30} [26]. *Acinetobacter pittii* SW-1 was reported that it could utilize long-chain n-alkanes (C_{18}–C_{36}) and the degradation rate of C_{20} was 91.25% [27]. *Rhodococcus* sp. CH91 could utilize a broad range of long-chain n-alkanes from hexadecane to hexatriacontane [28].

Rhodococcus sp. is a kind of gram-positive bacterium that can be isolated from soil, deep sea and other environments, belonging to *Acitnobacreria*, *Actinobacteria*, *Actinobacteridae*, *Nocardiaceae*. Strains of this genus often contain unique cell wall structure, large genome and circular plasmids, which can secrete a large number of enzymes and use organic compounds as energy and carbon sources [29, 30]. Thus, they can adapt to a variety of substrate environment, and have a wide spectrum of degradation. It has been reported that they could effectively degrade organic pollutants by producing surfactants and changing the structure of cell surface to improve their adaptability to hydrophobic environments [31-33]. In fact, members of *Rhodococcus* genus screened from petroleum-contaminated soils have been shown to degrade hydrocarbon components. During the phenanthrene degradation process of *R. qingshengii* FF, pyrogallol was the predominant accumulated metabolite and 59% of the accumulated metabolites were oxygen-containing PAHs that have only one benzene ring [34]. *Rhodococcus* sp. Y2-2 showed about 80% degradation efficiency both in liquid and soil media by consuming kerosene, gasoline, and diesel as a carbon and energy source at low temperature [35]. *Rhodococcus* sp. LH could degrade diesel oil through terminal or sub-terminal oxidation reactions, and possess the ability to degrade aromatic hydrocarbons [36]. In this study, a *R. qingshengii* strain was isolated and identified from petroleum-contaminated soil on the Shengli Oilfield in China. To further understand its genetic traits for diesel-degradation, the complete genome was sequenced and reported, and genes related alkane degradation were analyzed.

Materials and Methods

Chemical and Growth Media

0# diesel was obtained from Shandong Kexin Oil Products Co., Ltd (Jinan, China). Its density is 827.9 g/cm^3 (20ºC), and the cetane index is 56. The available sulphur content and total pollutant content are 4.0 mg/kg and 4.5 mg/kg, respectively. N-hexane, anhydrous sodium sulfate and other chemicals and solvents were of highest analytical-reagent grade.

Liquid minimal salts medium (MSM) consisted of 1 g NH_4NO_3, 1 g NaCl, 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, per liter, with 0.5 % (v/v) diesel as a sole carbon source. Unless otherwise stated, MSM was adjusted to pH 7.5. The diesel solution is filtered for sterilization and then added to the autoclaved MSM medium. Nutrient Broth medium was used for general bacterial growth.

Isolation of Diesel Degrading Microorganisms

Soil samples used for bacterial enrichment were collected from the Shengli Oilfield (37º28'N, 118º29'E), which is the second-largest oilfield in China and is located in the Yellow River Delta. To select for diesel-degrading microbes, 10 g of soil was placed in a 500 mL Erlenmeyer flask containing 100 mL of MSM supplemented with diesel as the sole carbon source and incubated at 30ºC on a rotary shaker (150 rpm). After 10 days, 5 mL of culture was inoculated to 100 mL fresh MSM-diesel and incubated under the same conditions for another 10 days. After 5 sequential rounds of enrichment, 100 µL of culture was plated onto MSM agar pre-coated with diesel and incubated at 30ºC for 7 days. Bacterial colonies grown on plates with different morphology were tested for their diesel utilizing capabilities and single cell colonies were purified for further analyses.

Identification of Diesel-Degrading Bacteria

Identification of diesel-degrading bacteria was based on morphological, physiological and biochemical characterization according to Bergey's Manual of Systematic Bacteriology. Molecular taxonomy was based on PCR amplification and DNA sequencing of the 16S rRNA gene with the universal primers 7F (5'-CAGAGTTTGATCCTGGCT-3') and 1540R (5'-AGGAGGTGATCCAGCCGCA-3') according to the established protocols. PCR primer synthesis and DNA sequencing were conducted at Sangon Biotech (Shanghai) Co., Ltd, China. The resulting nucleotide sequences were compared to those in GenBank using a BLAST search.

Microbial Diesel-Degradation

Diesel biodegradation was quantified by monitoring decreasing concentration of the diesel in liquid culture over time. Strain RHZ01 was grown in NB broth at 30ºC on a rotary shaker (150 rpm) for 16 h, cells were collected by centrifugation (4000 g for 10 min), washed twice and re-suspended to an OD600 = 0.8 (Lambda Bio Spectrophotometer, Perkin Elmer, USA) in sterile 0.9% NaCl solution. The cell suspension (approx. 1×108 cell/mL) was used to inoculate (2% v/v) 100 mL flasks of MSM-diesel and incubated at 30ºC on a rotary shaker (150 rpm). The cultures were taken out at different time points to detect the cell concentrations and the diesel residues. Non-inoculated control was set and each treatment was replicated 3 times.

Detection and Analysis of Diesel Utilization

N-hexane was added to the flasks containing cultures with a volume ratio of 1:1. The flasks were subjected to oscillation extraction in a shaker (25ºC, 150 rpm) for 30 min, and then liquid-liquid extraction using a separating funnel. All procedures were repeated 3 times. During each extraction, the organic phase was collected in a 250 mL flask with anhydrous sodium sulfate to absorb water, then allowed to rest overnight and removed by filtration. The organic phase was evaporated with a rotatory evaporator and finally quantified to 5 mL with n-hexane. The diesel concentration was determined with gas chromatography-flame ionization detection (GC-FID) equipped with an Agilent HP-5 capillary column (30 mm × 0.32 mm × 0.25 μm), and the analytical procedures were as follows: carrier gas (N2) flow rate of 1.5 mL/min, air flow rate of 300 mL/min, H2 flow rate of 30 mL/min, makeup gas flow rate of 28 mL/min, and injection volume of 1 μL. The oven temperature program was as follows: start at 40ºC, hold for 2 min, increase to 350ºC at 10ºC/min, and hold for 10 min. The inlet temperature and the detector temperature were both 300ºC.

Effect of Nitrogen Source, Cations, pH and Temperature on Diesel Utilization by Strain RHZ01

Four experiments were established to determine the individual effects of nitrogen source, cations, pH and temperature on diesel degradation by RHZ01. Unless otherwise indicated, the strain RHZ01 was inoculated into 50 mL of NB liquid medium and then cultured at 30ºC and 150 rpm. After incubation for 16 h, the bacteria were collected through centrifugation at 5000 r/m for 10 min and washed two times and diluted as the inoculum with sterile 0.9% NaCl solution. To examine the effects of different source of nitrogen on diesel degradation, 0.1% urea, 0.1% NH_4Cl, 0.1% KNO_3, 0.1% $NaNO_3$ were respectively added to MSM-diesel in the absence of NH_4NO_3. Similarly, substitutions of $MgSO_4$ in MSM-diesel with equimolar amounts of $MnSO_4$, $ZnSO_4$, $Fe_2(SO4)_3$, $CaCl_2$ or $CuSO_4$ were used to determine the effects of alternative cations on diesel degradation. To determine the influence of temperature on diesel degradation, the medium was cultured at 20ºC, 25ºC, 30ºC, 35ºC and 40ºC. And the medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 to investigate the effect of pH on diesel degradation.

Genome Sequencing and Comparative Genomics Analysis

Genome was sequenced using a combination of PacBio RS II Single Molecular Real Time (SMRT) and Illumina sequencing platforms. The Illumina data was used to evaluate the complexity of the genome. The data generated from PacBio and Illumina platform were used for bioinformatics analysis. The complete genome sequence was assembled using both the PacBio reads and Illumina reads. All of the analyses were performed using the free online platform of Majorbio Cloud Platform (www.majorbio.com) from Shanghai Majorbio Bio-pharm Technology Co.,Ltd. Glimmer 3.02, tRNA-scan-SE 2.0 and Barrnap 0.8 were used for CDS, tRNA and rRNA prediction, separately. The predicted CDSs were annotated from NR, Swiss-Prot, Pfam, GO, COG and KEGG database using sequence alignment tools such as BLAST, Diamond and HMMER. Based on 16S rRNA gene analysis result, *Rhodococcus qingshengii* JCM 15477, representative *Rhodococcus* strains with the most similarity or relativity with strain RHZ01 were selected, and the data on the genomes of the *Rhodococcus* strains were obtained from NCBI. Digital DNA-DNA hybridization (dDDH) values were calculated using GGDC 3.0 [37], a state-of-the-art *in silico* method for genome-to-genome comparison.

Analysis of Alkane Monooxygenase

According to the genomic information, the amino acid sequences of alkane monooxygenases from RHZ01 were compared with other monooxygenases database

from *Rhodococcus* spp. downloaded from NCBI (www.ncbi.nlm.nih.gov). Primary sequence analysis including sequence composition and physiochemical properties was analyzed by Expasy protparam online tool (https://web.expasy.org/protparam/). The conserved motifs were analyzed by MEME (https://meme-suite.org/) and were compared by running clustalw locally by MEGA software. PSIPRED was used to predict secondary structure (http://bioinf.cs.ucl.ac.uk/psipreD). And the 3-D model of the monooxygenases was achieved by SWISS-Model workspace (https://swissmodel.expasy.org/).

Statistical Analysis

Biodegradation of diesel by strain RHZ01 was assessed by comparing differences in diesel concentration between treatments, each consisting of 3 replicates. All data were analyzed by analyses of variance (ANOVA), using SPSS 16.0 statistical software (SPSS Inc., USA). Pairwise comparisons of means were used to compute Fisher's least significant difference values (LSD, P = 0.05).

Results and Discussion

Isolation and Identification of the Diesel-Degrading Strain Rhz01

Enrichment cultures established from petroleum-contaminated soils were plated onto NB agar to select for putative microbes that degrade diesel. Bacterial representing different colony morphologies were purified and confirmed to have diesel degrading function. Strain RHZ01, quantitatively assessed as the most effective diesel degrader, was selected for further study using MSM medium with diesel as the sole carbon source. The strain grew well in liquid NB media and reached its maximum growth rate after 8 h of shaking culture (Fig. 1a). And it could also grow in MSM media containing 0.5% (v/v) diesel as the sole carbon source at 30ºC, and according to the growth curve in MSM with diesel as carbon, the strain reached its maximum valve on the eighth day and then grew slowly (Fig. 1b). 85.02 % of diesel in media was removed, which suggests its strong diesel removal ability under the culture conditions. And the growth of strain RHZ01 was positively correlated with the removal of diesel (Fig. 1b).

Strain RHZ01 was a gram-positive, non-motile, rod-shaped bacterium with cells 0.8-1.5 μm long and 0.4-0.6 μm wide observed by scanning electron microscope (HITACHI SUB8011, Japan) (Fig. 2a). The colony surface on NB plates displayed smooth, and the single colony was round, light orange with regular margin after 36 h at 30ºC (Fig. 2b). The strain RHZ01 showed positive urease, acetoin and catalase production, but negative for oxidase, starch hydrolysis and nitrate reductase in physiological and biochemical tests. It was able to use mannose, citrate, maltose, sodium benzoate as the sole carbon sources and asparagine and acetylamine as sole sources of carbon and nitrogen.

16S rRNA gene fragment was amplified from strain RHZ01 and sequenced. And a phylogenetic tree was constructed based on its 16S rRNA sequence (GenBank accession number: MZ960909). The strain RHZ01 showed 100% identical to that *Rhodococcus qingshengii* JCM 15477^T (LRRJ01000016) and the corresponding phylogenetic tree analysis supported a strong relationship between RHZ01 and members of *Rhodococcus* (Fig. 3). Therefore, according to the morphological characteristics, the physiological and biochemical properties and 16S rRNA sequence analysis, it could be concluded that the isolate RHZ01 is a number of *Rhodococcus qingshengii*.

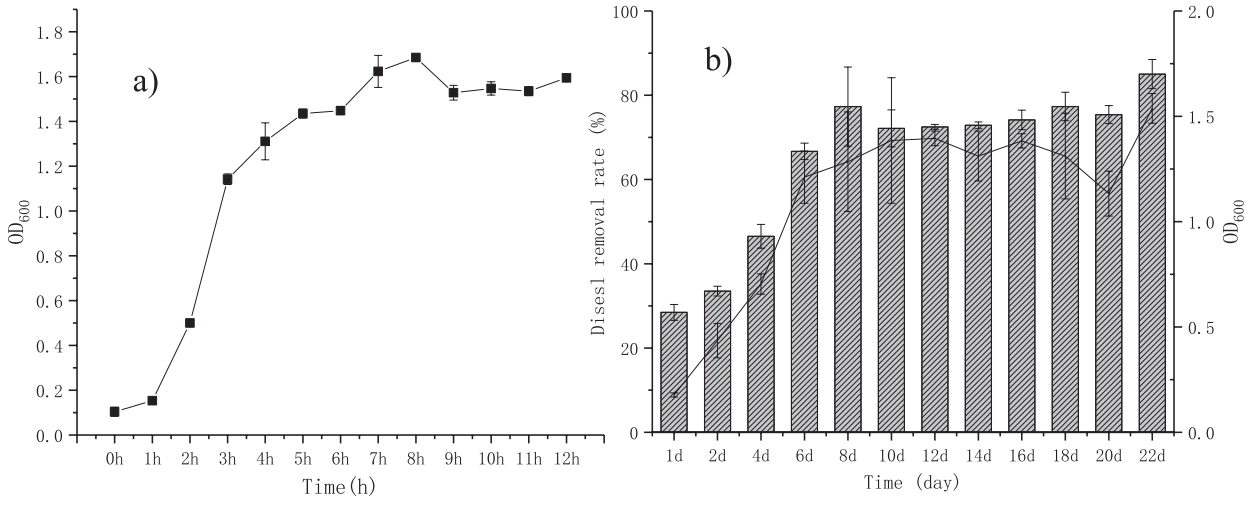

Fig. 1. Growth curve of strain RHZ01 and its removal effect on diesel a) Growth curve in NB media; b) Growth curve in MSM media and diesel removal rate.

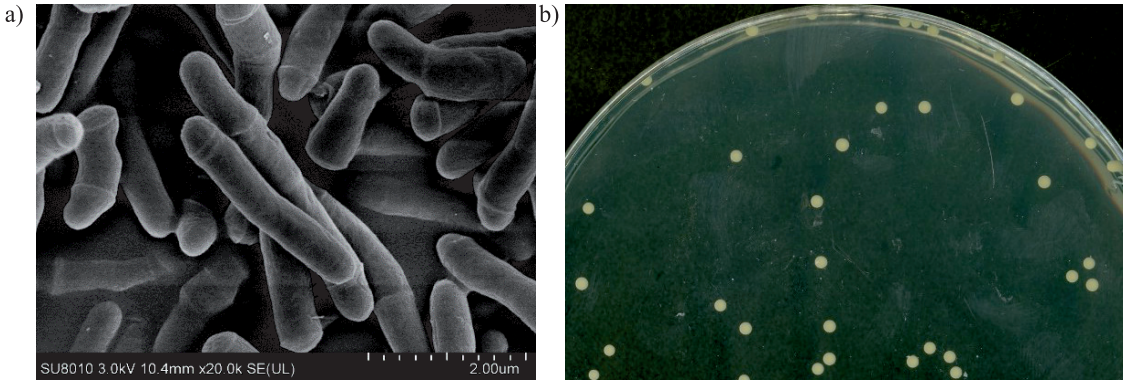

Fig. 2. Cell morphology of strain RHZ01under electron microscope and colony morphology on NB plate.

Rhodococcus spp. are widely distributed in various environments including contaminated soil, seawater and marine sediments, boreholes, groundwater, extreme conditions [38, 28]. Moreover, this genus was reported to contain many members that can degrade different kinds of organic and xenobiotic compounds, such as alkane, polycyclic compounds, heterocyclic [39, 40]. In this study, we isolated a strain, RHZ01, from the Shengli Oilfield in China. This strain was able to grow using diesel as the sole carbon source and degrade diesel very well. This strain is closely homologous to *Rhodococcus qingshengii* JCM 15477^T with 100% of 16S rRNA sequence similarity.

Effects of Nitrogen Source, Metal Ions, Ph and Temperature on Diesel Degradation

Strain RHZ01 was able to grow well in MSM with different nutrients as nitrogen source. It degraded more than 60% of diesel from MSM with NH_4NO_3 as the source of nitrogen The degradation rate of diesel was not significantly different among nitrogen sources in MSM ($P>0.05$) (Fig. 4a). Therefore, substitution of NH_4NO_3 in MSM with equivalent amounts of alternative nitrogen had no significant effect of diesel-degradation by RHZ01.

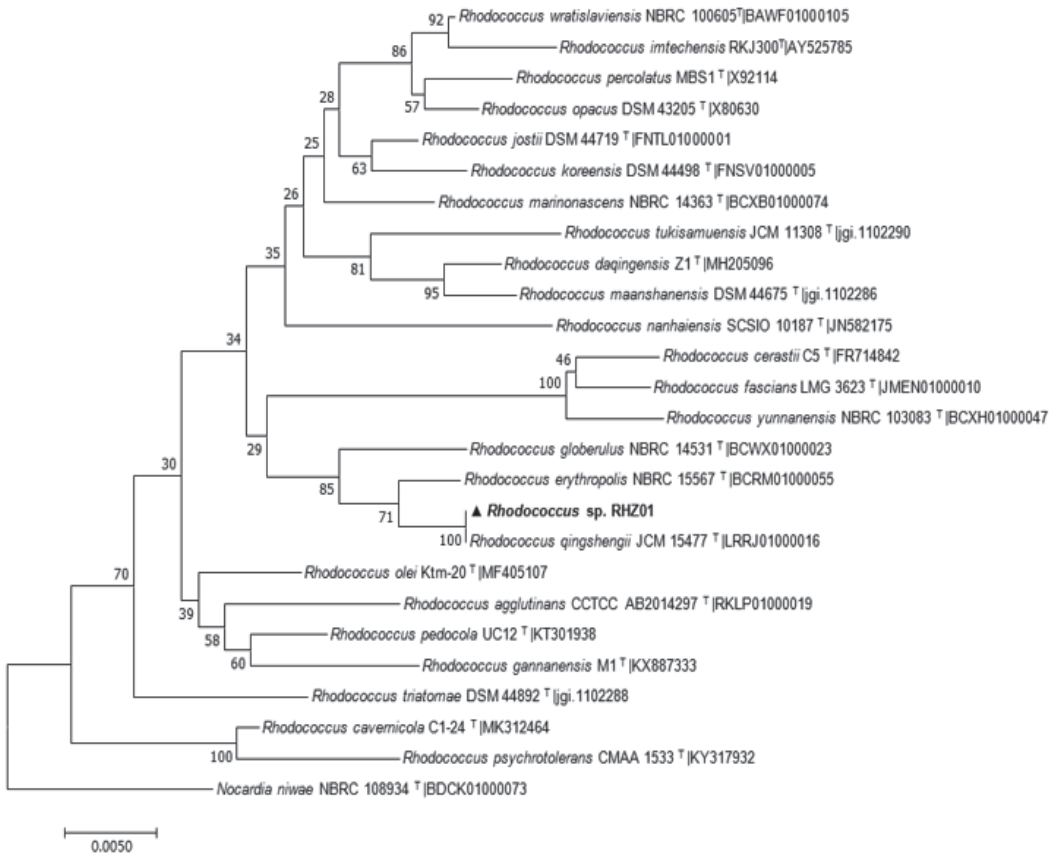

Fig. 3. Phylogenetic tree showing the relationship between strain RHZ01 and other related strains based on 16S rRNA gene sequences.

Substitution of Mg^{2+} in MSM with equimolar amount of Mn^{2+}, Zn^{2+}, Fe^{3+}, Ca^{2+} and Cu^{2+} had significant effect on growth curve and diesel degradation by RHZ01 (Fig. 4b). The growth was best in MSM with Mg^{2+} comparing with other metal ions and the degradation rate on diesel was 67.78%. In contrast, diesel degradation was significantly decreased when Mg^{2+} in MSM was substituted with equimolar other ions (one-way ANOVA, $F_{5,12}$ = 38.655, P<0.0001, Fig. 4b). In addition, substitution with Cu^{2+} resulted in slow growth of RHZ01 and no degradation with diesel.

Initial pH could affect the enzymatic activity within the microbial cells and biochemical process of the strain. It is unfavorable to microbial growth and metabolism in too acidic and alkaline condition. From (Fig. 4c), the initial pH value of 6-8.5, the diesel degradation rate was above 40%, and there was no significant difference among various treatments (P>0.05), indicating that the strain could grow and degrade diesel among different pH ranges, and could adapt to a wide range of environmental conditions in practical application. However, at pH> 9, the growth and degradation ability were decreased, indicating that the alkalinity has an inhibitory effect on the growth and diesel degradation ability of the strain (one-way ANOVA, F_6, 14 = 15.632, P<0.0001, Fig. 4c).

The culture temperature is closely related with the growth, reproduction and metabolic activity. Here, temperature had a significant effect on growth and diesel degradation by RHZ01, with optimal growth and degradation detected in the 20ºC -30ºC range. Diesel degradation was significantly inhibited further at 35ºC to 40ºC (one-way ANOVA, F4, 10 = 37.859, P<0.0001, Fig. 4d).

Genomic Characteristics of the Isolate RHZ01

Table 1 and Fig. 5 showed the genome properties of the strain RHZ01. The genome of *R. qingshengii* RHZ01 was 6,506,318 bp in total length, with only comprised one circular chromosome. The genome has 62.48% G+ C content which is near the recognized range for *Rhodococcus* species (62-69%) [28, 41]. The genome was annotated with six databases (NR, Swiss-Prot, Pfam, EggNOG, Go and KEGG), which predicted a total of 6384 protein-coding sequences, 54 tRNAs, and 15 rRNAs. And according to the clusters of orthologous genes (COG) designation [42], 603 genes are responsible for cellular processes and signaling, 2,008 genes for metabolism, and 1,812 genes are function unknown (Fig. 6). The complete genome sequence of *R. qingshengii* RHZ01 was deposited in GenBank under the accession number CP085084.1. The BioProject and BioSample numbers are PRJNA768639 and SAMN22059269, respectively.

The genomic data of six *Rhodococcus* spp. from the NCBI database were downloaded and comparatively analyzed with RHZ01 in order to further understand the characteristic of the genome (Table 2). Among those strains, *R. qingshengii* F2-2 was isolated from oil-spilled soils from “Festivalnoe” oil field in Western

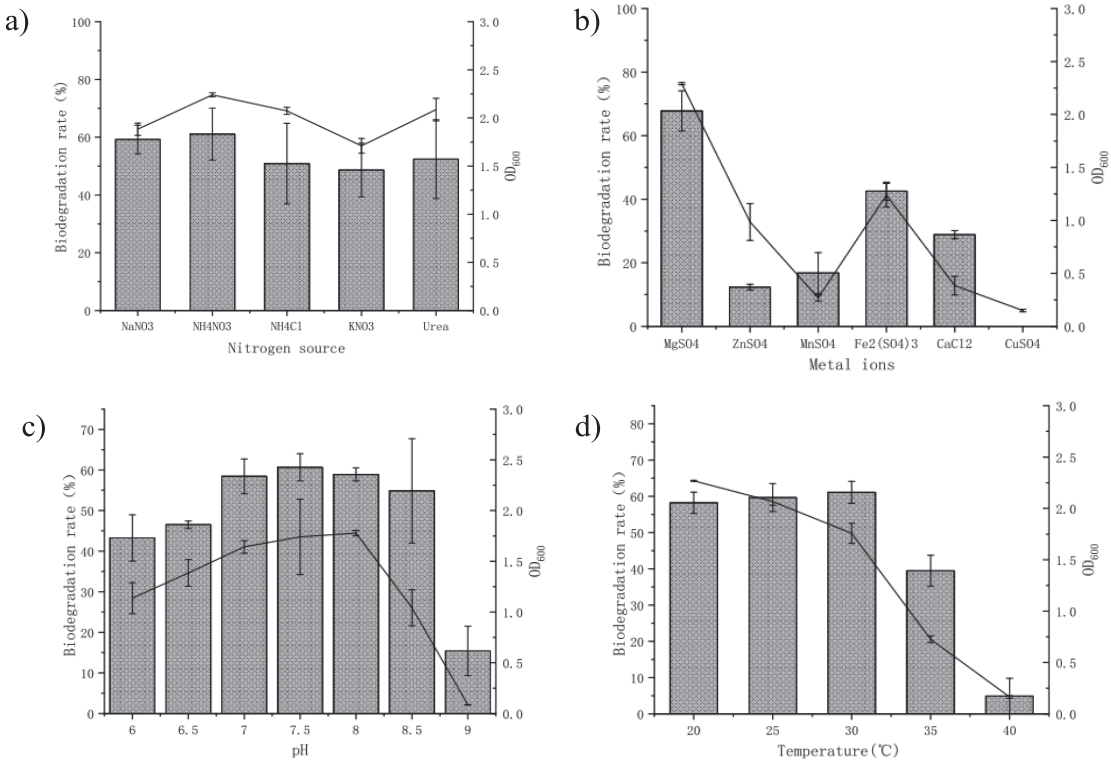

Fig. 4. Effects of nitrogen source a), metal ions b), pH c) and temperature d) on strain RHZ01 growth and diesel degradation.

Table 1. Genome properties of the isolate RHZ01.

Characteristics	Value	Characteristics	Value
Genome size (bp)	6,506,318	GI	14
CDS number	6,384	Prophge	6
Longest CDS length (chr) (bp)	16,470	CRISPR-Cas	4
Average CDS length (chr) (bp)	934.89	VFDB numbers	519
GC content (%)	62.48	Antibiotic resistance genes	298
Coding Region (chr) (bp)	5,968,320	NR annotation	6,260
5s rRNA	5	Swiss-Prot	4,265
16s rRNA	5	Pfam	4,965
23s rRNA	5	COG	5,212
tRNA	54	GO	4,196
sRNA	35	KEGG	2,440

Siberia that showed strong degradation ability on crude oil [43]. *R. erythropolis* PR4 obtained from the Pacific Ocean, which was capable of utilizing diesel oil, normao-, iso- and cycloparaffins and aromatic compounds [44, 45]. The strain R7, capable of utilizing variable-chain-length n-alkanes, were obtained from a polycyclic aromatic hydrocarbon contaminated soil [41]. This bacterium has the largest genome with five plasmids compared with other strains. *R. jostii* RHA1 characterized as a biphenyl degrader was able to grow on C10-C19 n-alkanes [46, 47]. *R. erythropolis* KB1 was a petroleum-degrading bacterium isolated from the desert soil [48]. *R. erythropolis* X5 showed effective n-alkane destruction at low positive temperature [29]. All the strains in Table 2 are 6.47-10.12 Mb and harbor more than one plasmid except for RHZ01.It is speculated the strain RHZ01 maybe have no plasmid of its own or has lost during isolation process. The highest dDDH values were obtained in the comparison of RHZ01 with *R. qingshengii* F2-2 and *R. erythropolis* X5 (85.4% and 85.1% dDDH, respectively).

Table 2. Genomic characteristics of several *Rhodococcus* spp.

Characteristics	RHZ01	F2-2	PR4	R7	RHA1	KB1	X5
Genera	*R. qingshengii*	*R. qingshengii*	*R. erythropolis*	*R. opacus*	*R. jostii*	*R. erythropolis*	*R. erythropolis*
Genome size (Mb)	6.47	7.02	6.90	10.12	9.70	6.86	7.00
Chromosome number	1	1	1	1	1	1	1
Plasmid number	0	3	3	5	3	3	1
GC content (%)	62.48	62.39	62.29	66.88	66.97	62.47	62.31
Protein-coding genes	6,384	6,371	6,327	8,882	8,556	6,066	6,326
Total RNA genes	104	71	72	62	67	73	71
rRNA number	15	15	15	9	12	15	15
tRNA number	54	53	54	53	50	54	53
Alkane monooxygenase number	5	5	4	3	1	5	5
dDDH estimate (%,GLM-based, Formula I)	—	85.4	81	16.1	16.4	83	85.1
GenBank No.	CP085084.1	CP092101.1	AP008957.1	CP008947.1	CP000431.1	CP050124.1	CP044284.1

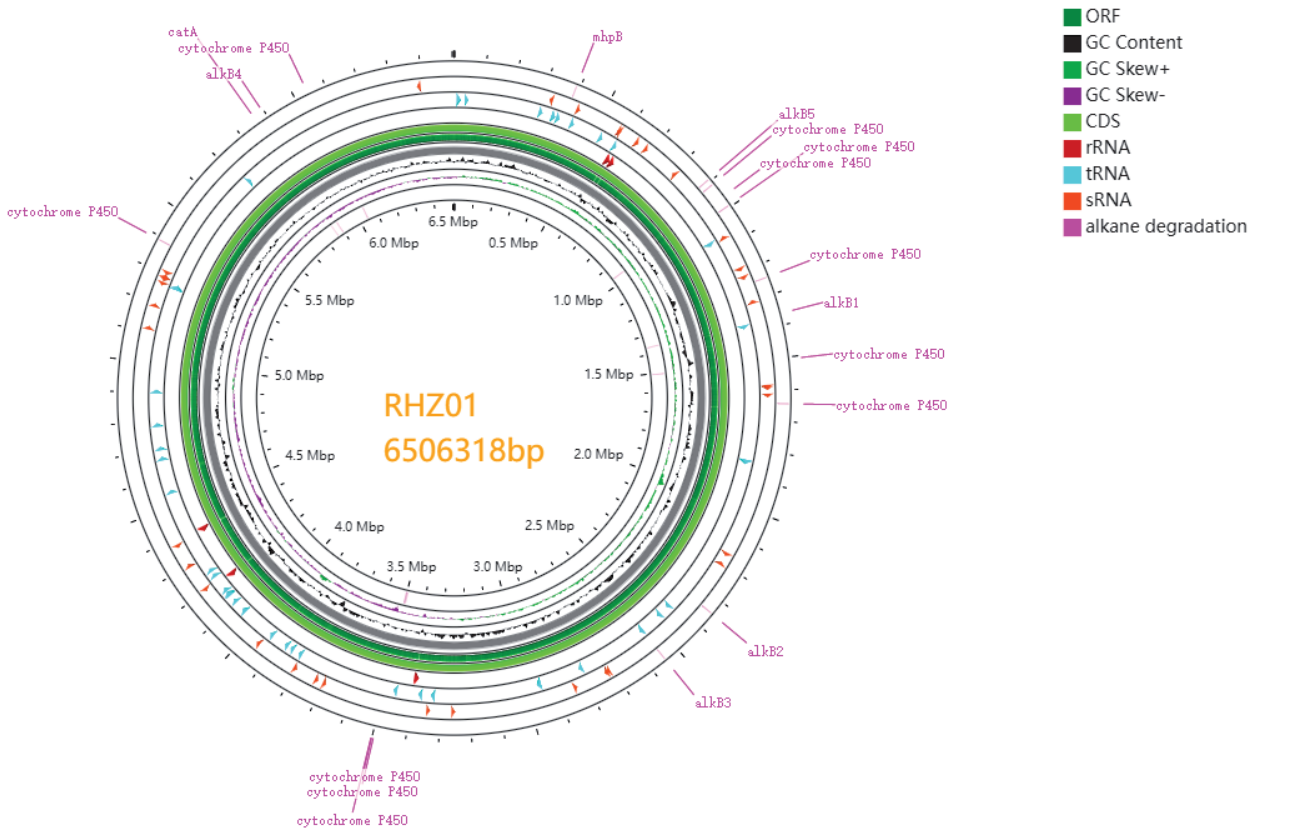

Fig. 5. Circular representation of genome and features of strain RHZ01. The contents of the featured rings (starting with the outermost ring to the centre) are as follows, Ring 1: genes involved in hydrocarbon degradation, combined forward and reverse strand: Ring 2, 3 and 4: RNA(including sRNA, tRNA and rRNA) combined forward and reverse strands; Ring 5: CDS in forward and reverse strands; Ring 6: Combined ORFs in forward and reverse strands; Ring 7: plot of GC content; Ring 8: GC skew plot, values above average is depicted in green and below average in purple; Ring 9: sequence ruler. The figure was produced using Proksee (https://proksee.ca/).

Alkane Monooxygenases and the Encoding Gene

One of the major fraction in diesel oil are alkanes. Many *Rhodococcus* spp. were known for their ability to degrade short- and long-chain alkanes, and other organic pollutants [49, 50]. Here, the genome of *R. qingshengii* RHZ01 encodes 72 putative oxygenases, including 34 monooxygenases and 25 dioxygenases. Among those oxygenases, five putative genes encoding alkane monooxygenase were found to be involved in alkane degradation process compared to other alkane

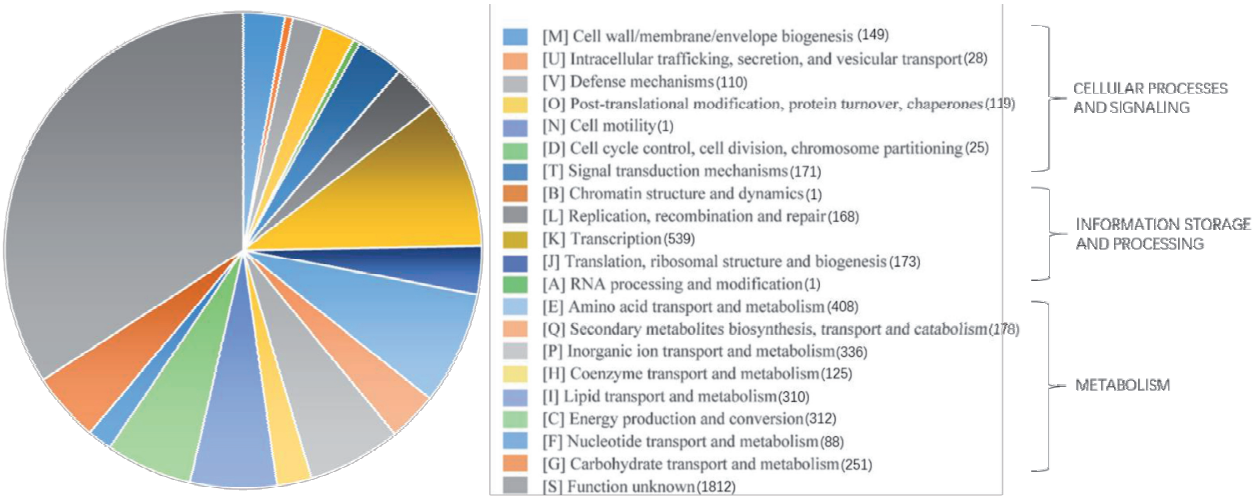

Fig. 6. Distribution of genes in annotated genome of strain RHZ01 based on COG classifications.

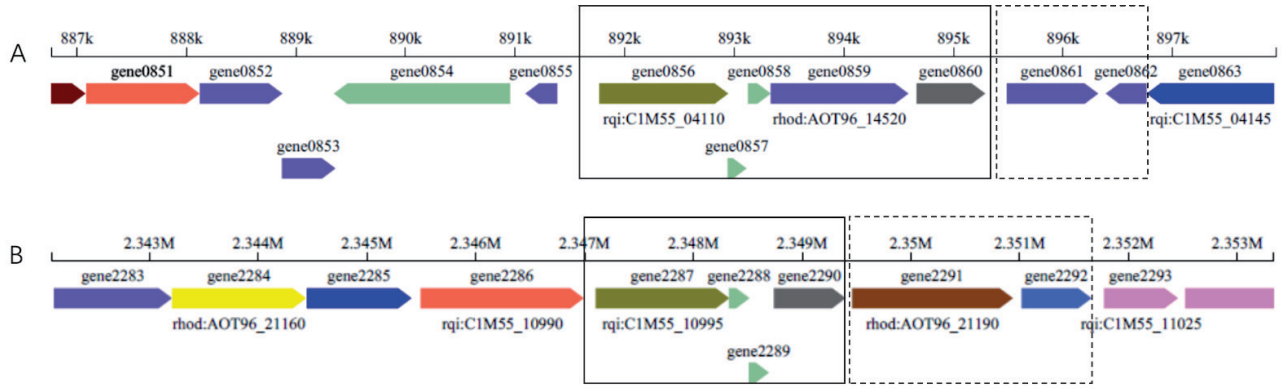

Fig. 7. Genomic map of *alk*B1 and *alk*B3 in *R. qingshengii* RHZ01. A, *alk*B1 gene cluster in black box. And gene0856, gene0857, gene0858, gene0859 and gene0860 were *alk*B1, two rubredoxin genes, FAD-dependent oxidoreductase and TetR/AcrR family transcriptional regulator, separately. Gene0861 and gene0862 in dotted line box encode two proteins whose functions are unknown. B, *alk*B3 gene cluster in black box. And gene2287, gene2288, gene2289 and gene2290 were *alk*B3, two rebredoxin genes and TetR family transcriptional regulator. Gene 2291 and gene2290 in dotted line box encode adenosylhomocysteinase and dTMP kinase.

Table 3. Amino acid sequences-based analysis of physiochemical features of alkane monooxygenase of strain RHZ01.

Gene ID	Code	AA	MW(kDa)	PI	II	AI	EC	GRAVY	TPR	TNR	Half-life (h)
Gene0856	AlkB1	391	44.37	9.01	42.02	95.83	111060	-0.028	39	33	10
Gene1310	AlkB2	383	43.12	9.44	52.21	100.44	110475	0.058	36	28	10
Gene2287	AlkB3	408	46.49	8.94	53.04	99.85	116560	-0.019	41	35	10
Gene2480	AlkB4	365	41.71	9.16	54.41	94.38	123090	0.002	38	31	10
Gene5849	AlkB5	388	44.16	8.80	56.84	91.31	121600	-0.090	39	35	10

AA, number of amino acids; MW, Molecular weight; PI, Isoelectric point; II, The instability index; AI, Aliphatic index; EC, Extinction coefficient; GRAVY, Grand average of hydropathicity; TPR, Total positively charged residue; TNR, Total negatively charged residue

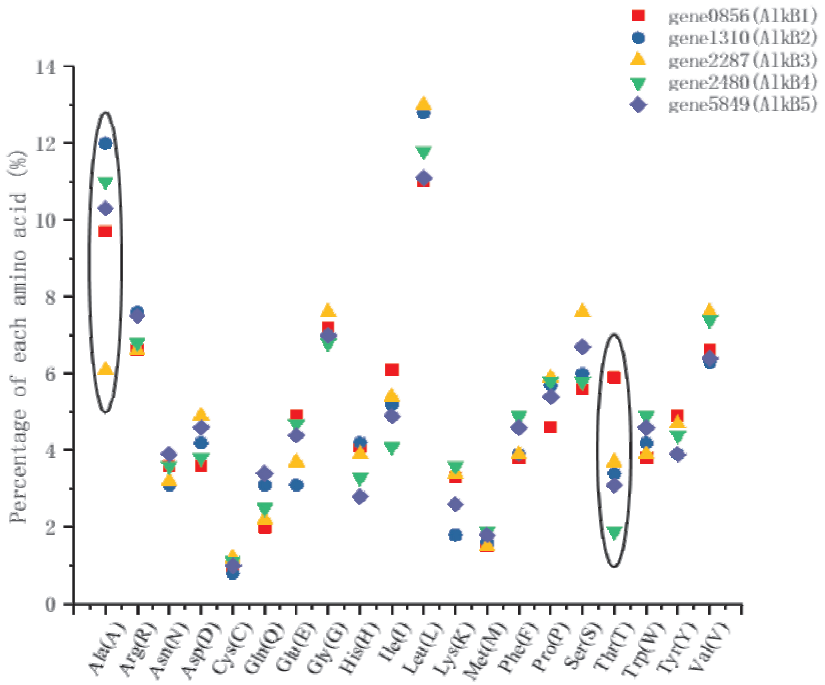

Fig. 8. The amino acid composition of alkane monooxygenases in strain RHZ01.

monooxygenases-encoding gene existed in other *Rhodococcus* strains (Fig. 5). Of the five genes encoding alkane monooxygenases, only *alk*B1 (gene0856) and *alk*B3 (gene2287) are followed by two rubredoxin genes. They are gene0857 and gene0858, as well as gene2288 and gene2289 (Fig. 7). The *alk*B monooxygenases genes are usually associated with rubredoxin genes, which play an important role in electron transport during alkane degradation process [45]. The *alk*B1 locus contains a FAD-dependent oxidoreductase (gene0859) and a TetR/AcrR family transcriptional regulator (gene0860), which is the regulator family known as transcriptional repressors, regulating themselves and the adjacent operon [45, 51]. And there are two genes next to this transcriptional regulator, gene0861 and gene0862, encoding 277- and 123- amino acid proteins, whose function are not well understood (Fig. 7a).

Another gene cluster, gene2287-gene2290, mainly consists of an alkane monooxygenase (gene2287), two rubredoxin gene (gene2288 and gene2289), one TetR family transcriptional regulator (gene2290). Compared to gene0856 operon, downstream to the alkB3 operon contains adenosylhomocysteinase and dTMP kinase (Fig. 7b). The relationship between these proteins and alkane degradation is unclear. About other three alkanes monooxygenase encoding gene (gene1310, gene2480 and gene5849), reductase was not found.

To better understand alkane monooxygenases in RHZ01, five alkane monooxygenase protein sequences were characterized by physicochemical features (Table 3). The analysis suggested that isoelectric point (pI) is 8.5-9.5 for enzyme which means the protein is more alkaline and is very similar to other pI value of alkane monooxygenase among *Rhodococcus*

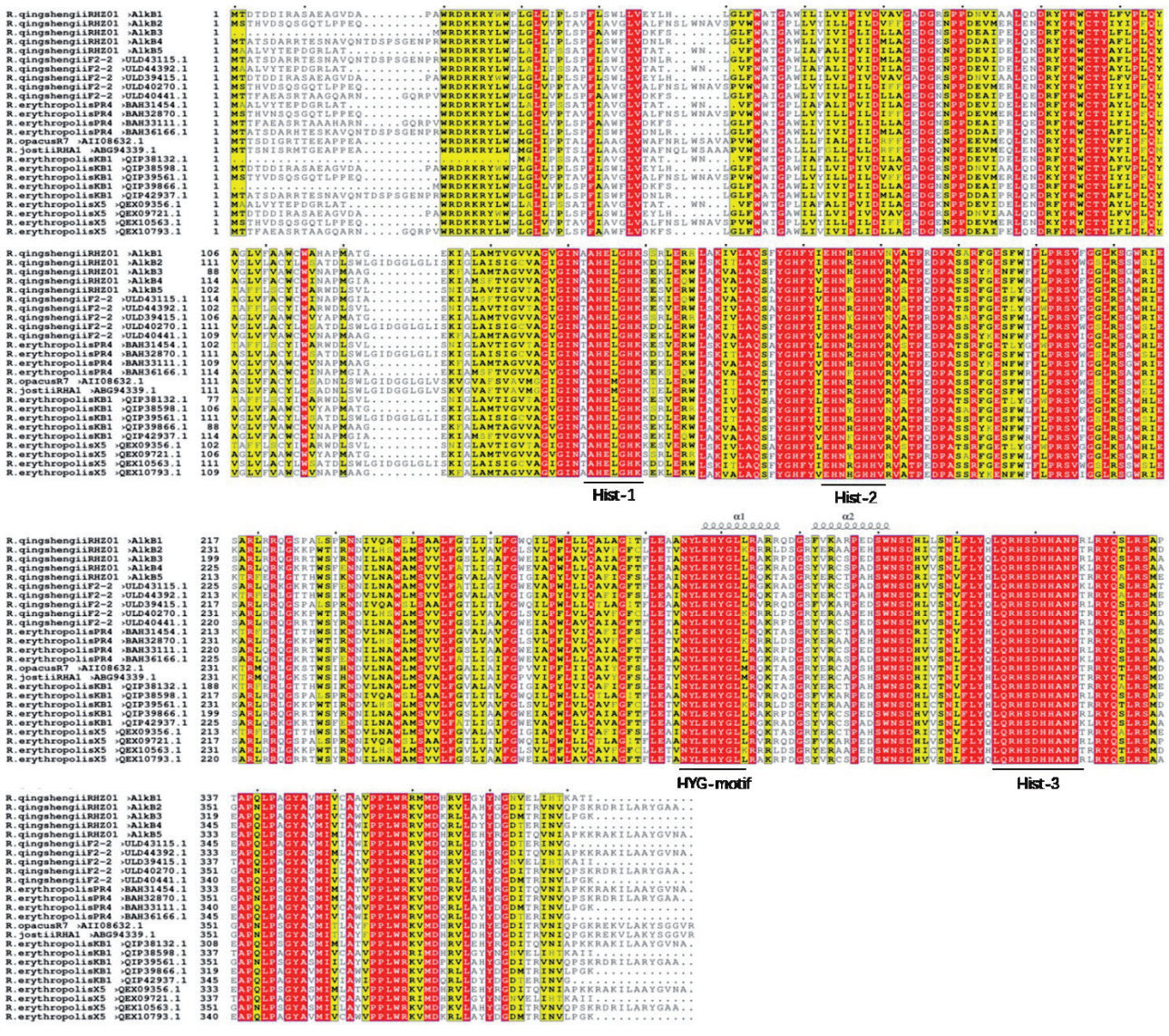

Fig. 9. Multiple alignments of full-length alkane monooxygenases from RHZ01 and other *Rhodococcus* strains. The three conserved histidine boxes (Hist-1, 2 and 3) and the additional HYG-motif are underlined. Two putative α-helix structures are labeled above the amino acids.

species [52]. The prediction results showed that all of alkane monooxygenases were unstable protein (the instability index>42). In addition, the aliphatic index of AlkB1 to AlkB5 were 95.83, 100.44, 99.85, 94.38, 91.31, separately, which indicated that these enzymes were thermostable. Thermal stability is thought to be related to application, such as biocatalysts in industrial process, and highly thermostable enzyme is the key research for application purpose [53]. Assuming all pairs of cysteine residues form disulfide bonds, the extinction coefficients (EC) were calculated at 280 nm in water. The EC range from 110,475 to 123,090 $M^{-1}cm^{-1}$ and the average molecular weight of the protein is 43.97 kDa. According to the Grand Average of Hydropathicity (GRAVY), AlkB1, AlkB2 and AlkB3 are hydrophilic protein while the other proteins were hydrophobic. However, it was reported that the alkane-1-monooxygenase of *Rhodococcus* had better interactions with water molecules [53]. The amino acid composition of alkane monooxygenases in strain RHZ01 was shown in Fig. 8. Here, alanine content in AlkB3 is only 6.1% which is less than that in other alkane monooxygenase, but the alanine content is relatively high in AlkB2 and AlkB4, which are two hydrophobic proteins. Another amino acid, threonine, its content was the highest in AlkB1 and the lowest in AlkB4.

Sequence alignments of alkane monooxygenases in RHZ01 with other published AlkB sequences downloaded from NCBI indicated that all of these alkane monooxygenases share several conserved sequences (Fig. 9). From the conserved sequences, one conserved HYG-motif (NYLEHYGL[L/K(M)]) and three highly conserved regions of the Hist boxes (Hist1, HE[L/M] GHK; Hist2, HN[H/R(F)]; Hsit3, LQRHSDHHA) were similar to alkane hydroxylase from other strains, such as *Pseudomonas aeruginosa* SJTD-1 [54], *Alcanivorax* sp. 2B5 [55], *Dietzia* sp. DQ12-45-1b [56], which means those sequences are highly conserved in most bacterial alkane monooxygenases. And the Hist3 box is the longest perfectly conserved fragment in *Rhodococcus* spp. [57]. About HYG-motif, it is also conserved in related hydrocarbon monooxygenase [57]. It is of great significance to study these conserved sequences because they can be used as probes to detect the activities of related degradation enzymes in the process of alkane degradation. And It can further reflect the level of microbial activity.

Conclusion

In this study, a diesel-degrading bacterium RHZ01 was isolated from oil-contaminated soil in Shengli Oilfield and identified as *Rhodococcus qingshengii* based on the morphology, physiology, biochemistry and molecular biology. *R. qingshengii* RHZ01 could grow well in MSM with diesel as the sole carbon source and the diesel removal was 85.02%. Diesel degradation by this isolate was not significantly affected by substituting NH_4NO_3 with other nitrogen source ($NaNO_3$, NH_4Cl, KNO_3 and Urea). Substitution of Mg^{2+} in MSM with other metal ion (Mn^{2+}, Zn^{2+}, Fe^{3+}, Ca^{2+} and Cu^{2+}) had significant effect on growth curve and diesel degradation by RHZ01. And the optical initial pH and culture temperature for growth and diesel degradation were 6-8.5, 20-30ºC, respectively. The genome annotation analysis of *R. qingshengii* RHZ01 revealed potential COG encoding alkane monooxygenases, catechol dioxygenase, cytochrome P450, and other genes related to hydrocarbon degradation. Five putative alkane monooxygenases encoding genes were analyzed, and protein sequences in *R. qingshengii* RHZ01 were characterized and alignment with other published AlkB sequences. These suggest that the strain RHZ01 will elucidate the genetic basis of bioremediation features and has good potential for future bioremediation for diesel polluted soil.

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Conflict of Interest

The authors declare no conflict of interest.

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