Characterization and Expression Analysis of Extradiol and Intradiol Dioxygenase of Phenol Degradation Haloalkaliphilic Bacterial Isolates

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Research Article

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Abstract

Haloaklophilic bacteria have a potential advantage as a bioremediation organism for high pH oil polluted and industrial wastewater. In the current study, Haloalkaliphilic isolates were obtained from Hamralake, Wadi EL-Natrun, Egypt. The phenotypic features, biochemical characters, and 16S rRNA sequence comparison were used to identify the bacterial isolates including *Halomonas HA1* and *Marinobacter HA2*. These strains showed high requirement of NaCl for growth specially HA1 strain that essentially required NaCl for its growth. The isolates are capable of degrading phenol at optimal pH values between 8 and 9 with the ability to grow in levels of pH up to 11, like what was seen in *Halomonas HA1* strain. Both isolates represent two different mechanistic pathways for phenol degradation. *Halomonas HA1* exploits the 1,2 phenol meta cleavage pathway while *Marinobacter HA2* using the 2,3 ortho cleavage pathway indicated by universal primer sets for 1,2 and 2,3 CTD genes. Phenol degradation showed a comparable pattern between both isolates, while *Marinobacter HA2* isolate can eliminate the added phenol within an incubation period of 72 h, The *Halomonas HA1*isolate required 96 h to degrade 84% of the same amount of phenol. The phylogenetic analysis of the amino acid sequence of 1,2 CTD (catechol dioxygenase) of *Halomonas HA1*showed an evolutionary relationship between 1,2 dioxygenases of both *Halomonadaceae* and *Pseudomonadaceae* while 2,3 CTD of *Marinobacter HA2* shared the main domains of the closely related species. Semi-quantitative RT PCR analysis proved the constitutive expression pattern of both dioxygenase genes.

Introduction

Phenol is an important industrial chemical that is utilized as an intermediate substance for chemicals production such as xylenols and oil refining (Chandrasekaran et al. 2018). Phenolic compounds constitute one major source of industrial pollutant because of their toxicity (Wasi et al. 2013). Due to their biological inhibition effect, phenol-contaminated hyper saline effluent are treated through different chemical protocols (Chinalia et al. 2008). The high expense of such labor and complicated techniques elucidate the need for biological treatments with more economical cost (Lefebvre and Moletta 2006). Several reports demonstrated the efficiency of aerobic microbial phenol degradation under hypersaline conditions (Bonfá et al. 2011; Lu et al. 2015).

Aerobic phenol degradation involves two highly conserved enzyme systems, both of them use NADH as an electron donor and molecular oxygen to cleave aromatic ring. These cleavage pathways are known as ortho pathway or (intradiol enzymes) that cleave the ring between two hydroxyl groups and meta pathway or (extradiol enzymes) that cleave the ring between one hydroxyl group and its adjacent non-hydroxylated carbon (Harayama and Rekik 1989). Both enzyme systems of dioxygenase have to use nonheme iron to establish the functional enzymatic structure that required for substrate binding (Ballou and Broderick 1999). Formation of catechol ring is the initial product of monooxygenases oxidation of phenol ring which is followed by oxidative cleavage either through meta cleavage (generates 2 hydroxymuconicsemialdehyde) or ortho cleavage (generates muconic acid). The ultimate product of further oxidation is beta-ketoadipate, which enters the tricarboxylic acid cycle (Xu et al. 2019).

In this study, we aimed to isolate and identify phenol-degradation haloalkaliphilic bacteria from Hamra-lake depression in Wadi El-Natrun which located in the Sahara desert, 90 km north-west of Cairo, Egypt. This alkaline and hypersaline lake aggregate has pH value between 8.5 and 11 and considered as a hypersaline and alkaline aquatic ecosystem which are rich with sulfate, chloride, carbonates, and sodium (Taher 1999). In general, Haloalkaliphilic bacteria possess special adaptation mechanisms to survive and grow under salinity and alkaline pH. These properties of dual extremity make them interesting from both, fundamental research and biotechnological points of view (Feng et al. 2005; Joshi et al. 2008).

Material And Methods

Samples collection

Isolation of haloalkaliphilicphenol degrading bacteria and culture conditions

Water samples were collected from Hamralake in Wadi El Natrun, Egypt where the pH was 10.0 and water salinity was 300 g/l. Halophile growth medium (HGM) was prepared according to (Bonfá et al. 2013), the pH 9 was adjusted using $NaHCO_3$. The media was supplemented with 3M NaCl and 2.5 mM phenol as a sole carbon and energy source. The water sample was added to HGM media with 1:10 volume ratio and incubated at 30 C∘ for 2 weeks. Two more successive culturing were carried out using 50 µl of diluted culture spread on an agar plate of the same media. Single colonies were plated on new agar plates with the same media for biochemical characterization.

Phylogenetic analysis of the 16S ribosomal RNA gene

Genomic DNA was extracted from the pure culture using GeneJET Genomic DNA Purification Kit (Thermo Scientific). PCR amplification of the 16S rRNA gene carried out using Bact 27f (5'-AGAGTTTGATC(A/C)-TGGCTCAG-3') and Bact 1492r (5'-TACGG(C/T)-ACCTTGTTACGACTT-3'), (Chang et al. 2000; Khalil H et al. 2017). The amplified products were sequenced using a 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols (Abd El Maksoud et al. 2020). The obtained sequence of 16S rRNA gene was compared using the BLASTN program against the nucleotide sequences collection (nr/nt) database, available through the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree relative to high scoring BLAST hits was performed using the (MEGA 7.0.26) software.

Bacterial growth and phenol degradation

HGM medium amended with 2.5mM phenol as a sole carbon source was used to follow phenol degradation by bacterial isolates. Different temperature, NaCl concentration, and pH (using $NaHCO_3$ or $Na2CO_3$) were applied separately to determine best conditions for bacterial growth on phenol. The primary culture was prepared by growing bacteria on mineral salt media described above that supplemented with 0.3% yeast extract, then cells were harvested by spin down at 3000 rpm and 5 °C for 15 min. Cells were washed twice with 50 mM phosphate buffer (pH 7) and resuspended in liquid HGM

media supplemented with 2.5 mM phenol as a sole carbon source with an initial optical density (OD_{600}) of 0.05. Triplicate Samples were separately centrifuged at regular intervals over an incubation period with optimal environmental conditions and the OD_{600} were measured in parallel. Phenol concentrations in the samples were measured using a modified aminoantipyrine method (Lu et al. 2015). One mL of each sample was centrifuged at 13,000g for 10 min. A volume of 300 ul of the supernatant was added to 6 µL 4-aminoantipyrine (2%, w/v) and 6 µL potassium ferricyanide (8%, w/v). After incubation period of 10 min, the solution was mixed with 2 mL of chloroform. The organic phase phenol was estimated by the absorbance level at 505 nm. Phenol concentration was calculated according the standard curve with different concentrations of phenol.

Amplification of intradiol 1,2 and extradiol 2,3 dioxygenase genes

The presence of intradiol 1,2 CTD was detected in isolated strains using degenerative primer for highly conserved region (~ 400bp), cat1 (5'-ACCATCGARGGYCCSCTSTAY-3') and cat3 (5'-GTTRATCTGGGTGGTSAG-3') (R=A or G; S= C or G and Y= C or T), previously described by (de Lourdes Moreno et al. 2011). The detection of extradiol 2,3 CTD was carried out using degenerated primers C23O-F (5'AGG TGW CGTSAT GAA MAA AGG 3') and C23O- R (5'TYAGGT SAK MAC GGT CAK GAA 3') (K= G or T; M= A or C and W= A or T), to amplify (~ 934bp) of 2,3 CTD gene, previously described by (Junca and Pieper 2003). PCR mixture contained 10 µl of PCR master mix (Biovision), 50 pmol of each primer and 100ng of genomic DNA. The total volume was completed to 20 µl using sterile distilled water. The PCR conditions consisted of an initial cycle of 5 min at 95ºC, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 30 sec, and extension at 72ºC for 1 min. The complete gene sequence of 1,2 CTD of *Halomonas HA1* isolate was obtained using universal primers DOG F (5'-TGACTGTTAAAATTTATGACACCCCTGAAG-3') and DOG R (5'-TTATGGACGCGCTTGCAGCTC-3'). Those primers were deduced depending alignment of high similarities 1,2 CTD genes. The amplified program was 94ºC for 30 sec, annealing at 60ºC for1 min, and extension at 72ºC for 1 min. Complete 2,3- cat gene sequence of *Marinobacter HA2* isolate was isolated using C5 and C31 primers which designed according conserved sequence alignment of high similarity 2,3 cat genes. The primers sequence were C5 ('5-ATGAAAAAAGGTGTAATGCGTCC-3') and C31 ('5-GTTCAGYRYVCGRTCGTGG TAG-3') (V= A, C or G). The amplified program was 94ºC for 30 sec, annealing at 58ºC for 30 sec, and extension at 72ºC for 1 min. The PCR product was electrophoresed using 1% ethidium bromide agarose gel and visualized with UV illumination. The DNA sequence was carried out using the amplified products were sequenced using a 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols. Conserved domain analysis performed using program of the National Center for Biotechnology Information (NCBI), (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Expression analysis of 1,2 CTD and 2,3 CTD genes of *Halomonas* and *Marinobacter* isolates

About 5 ml of exponential phase (OD_{600} of 0.2) cultures of *Halomonas HA*1 and *Marinobacter HA2* isolates were collected from a culture grown on minimal medium containing phenol (2.5 mM), glucose (5 mM) or phenol and glucose (2.5 mM and 5 mM respectively) for RNA isolation. RNA was extracted using

the GeneJET RNA Purification Kit (Thermo Scientific) and eluted in 50 µl of RNase-free water (Khalil et al. 2017, 2019). The extracted RNA was treated with DNase I (Thermo Scientific), according to the manufacturer instructions. First strand cDNA was carried out using 1µg Total RNA, 2 µl of Maxima Enzyme Mix Reverse Transcriptase from (Thermo Scientific), 200 pmol of cat3 gene primer and 4 µl of the supplied buffer in 20 µl total volume. Serial dilutions of 10^{-3}, 10^{-4} and 10^{-5} of cDNA were prepared in sterilized distilled water. For Semi-quantitative RT-PCR 5 µl of each dilution was used in PCR reaction under the conditions that were previously described for cat1 and cat3 primer (1,2 CTD conserved region of *Halomonas HA1*) and primers C5 and C31 (2,3 CTD gene of *Marinobacter HA2*).

Results

Identification and growth characters of phenol degrading isolates

The morphological investigation showed that both isolates, HA1 and HA2, were Gram negative rods. The biochemical characters demonstrated a distinguished pattern of urease production which was negative for HA1. The full-length 16S rRNA (1500 bp) of both strains were sequenced and deposited under GenBank accession number KT223026 (HA1) and KU323642 (HA2). The phylogenetic analysis of HA1 demonstrates similarity of 98% with the 16S rRNA gene sequence of *H. salifodinae* BC7 while, HA2 rRNA sequence analysis revealed 98.2% similarity with *M. alkaliphilus*. Growth parameters showed HA1 isolate showed obligatory salt requirement of 1% for HA1 and optimal growth concentration of 8% NaCl however, the strain can grow up to 20% NaCl. In addition, the pH tolerance of the strain extended to pH 11 with optimal growth at pH 9. The optimal growth temperature is 35 ˚C with ability to grow up to 50 ˚C. In contrast, *Marinobacter HA2* isolate showed a lower tolerance for adverse growth conditions. The best growth layout was obtained at 30 ˚C, pH 7 and 4% NaCl (Figure 1).

Genomic screening for phenol degrading genes

Only phenol meta-cleavage pathway with 2,3dioxygenase can accumulate 2-hydroxymuconic semialdehyde from catechol reagent which gives a yellow color (Nogales et al. 2017). The absence of 2-hydroxymuconic semialdehyde in the *Halomonas HA1* isolated culture indicated the ortho-cleavage pathway of phenol degradation. In contrast, the obtained yellow color by applying catechol regent on cells of phenol growing *Marinobacter HA2* indicated the presence of meta-cleavage pathway of phenol. Degenerative primer sets for conserved region of 1,2 CTD genes (cat1 and cat 31) and 2,3 CTD genes (C23O- F and C23O- R) were used to detect the expression levels of corresponding genes in both isolates. Interestingly, the 2,3 CTD gene segment was disappeared in *Halomonas HA1* while, 1,2 CTD gene segment was not detectable in *Marinobacter HA2*. This makes both strains as a good system to compare the two different mechanisms of phenol degradation. Under optimal growth conditions of pH, salinity and temperature both isolates were grown on minimal salt media; the isolates can eliminate phenol at different time intercourse. *Marinobacter HA2* isolate can eliminate added phenol within incubation period of 72 h, whereas *Halomonas HA1* isolate required 96 h to degrade 84% of the same amount of phenol .

Analysis of the catabolic genes

Universal primers for the 1,2 CTD gene were used to isolate a gene sequence encoded for 303 amino acid residues. The Amino acid sequence of isolated 1,2 CTD demonstrate the main characters of the of the conserved 1,2 CTD domains. The conserved domain of substrate binding sites of Leu 73, Ile 105 and Gly 107 showed high corresponding to all intradiol dioxygenases. Other similarities to halophilic 1,2 CTD include Val 200 replacement of the Tyr 200 residue and the presence of Gly 77 instead of Ala as previously reported by (de Lourdes Moreno et al. 2011). The presence of non-heme Fe binding residues of Tyr 164, Tyr 198, His 222 and His 224 suggested the trigonal bipyramidal geometry which is indistinguishable to other intradio ldioxygenases structures which have been considered by crystallographic examines (Earhart et al. 2005).The sequence alignment analysis with other database 1,2-CTD showed the highest similarity (91%) of protein identity with *P. putida* (Family *Pseudomonadaceae*) and (73.7%) with *H. daqingensis* (Family *Halomonadaceae*). The 1,2-CTD of *H. organivorans* showed the lowest similarity with *Halomonadaceae* 1,2 CTD genes (Figure3).The Sequence with 299 amino acid of *Marinobacter* 2,3 CTD was obtained using degenerative primer set. The identified protein shares the main conservative domains with other extradiol dioxygenases. As shown in figure 4 the Fe II binding sites were recognized in residues of His 153, His 214 and Glu 265. Substrate binding residues were identified as His 246 and Tyr 255 (45). The sequence showed protein identity of 99% with 2,3 CTD protein of *M. shengliensis* and 93% with *M. hydrocarbonoclasticus* 2,3 CTD protein (Figure 5).

Transcription analysis

RNA was isolated from bacterial culture grown on saline minimal media supplemented with phenol as a sole carbon source, glucose as a sole carbon source and phenol with glucose. Primers for the conservative 1,2 CTD domain (*Halomonas*) and 2,3 CTD (*Marinobacter*) were used for RT-PCR. As shown in figure 6 A and B, amplified fragment of about 400 bp of the 1,2 CTD gene and 934 bp of 2,3 CTD were obtained with the all used carbon sources. This result showed a constitutive gene expression pattern.

Discussion

Both genera of *Halomonas* and *Marinobacter* contain several members of haloalkaliphilic species and many of them are known with their ability to degrade aromatic compounds at high pH (Nojiri et al. 2004; Ben Ali Gam et al. 2007). The optimized growth parameters for strains isolation demonstrate the high tolerance properties for extreme conditions of *Halomonas HA1* isolate. The high tolerance for salt concentration is a common attribute in this family which may refer to a particular proton translocation system in which NaCl is used for glucose adsorption (Zhao et al. 2019). Special ectoine expression system is another mechanism by which some *Halomonase* species can be adapted in a high salt concentration (Kindzierski et al. 2017). Under aerobic conditions, phenol degradation pathway is introduced by the action of monooxygenase enzymes and leads to catechol formation. The action of dioxygenases for latter pathway of dearomatization of catechol is included undergo ortho-, meta- or para-cleavage. Ortho-cleavage, catalyzed by catechol 1,2-dioxygenase (intradiol-type dioxygenases) using

Fe(II) as a cofactor, which known as the β-ketoadipate pathway (Harwood and Parales 1996).The meta-cleavage is catalyzed 2,3-dioxygenase (extradiol-type dioxygenase), using Fe(III) as a cofactor (Suenaga et al. 2014). The metagenomic analysis of the phenolic sediment isolates showed that the isolates contain either 1,2 CTD or 2,3 CTD gene and the absence of 1,2/ 2,3 CTD in positive strains (Silva et al. 2013; Tian et al. 2017). However, other studies claim that *P. putida* has both cleavage pathways as reported by Basak et al., (Basak et al. 2014). In the same ecosystem of Hamra Lake (Wadi EL-Natrun), the two isolates represent two different model pathways for phenol degradation, *Halomonas HA1* exploits the 1,2 phenol meta cleavage pathway while *Marinobacter HA2* follows the 2,3 ortho cleavage pathway as demonstrated using universal primer sets for 1,2 and 2,3 CTD genes. *Marinobacter HA2* isolate can eliminate the added phenol within incubation period of 72 h. In comparison, *Halomonas HA1* isolate required 96 h to degrade 84% of the same amount of phenol. The presence of phenol meta cleavage pathway of *Marinbacter HA2* could interprets the variation of phenol degradation rate of both strains. Functionality of 2,3 catechol dioxygenase pathway in phenol degradation may clarify the gene outspread among microbial isolates in environments of phenol contaminated sediments (Heinaru et al. 2000). According to the ecological advantage the *Halomonas HA1* isolate showed more growth potential under the same stress conditions. *Halomonas* genus was represented in the isolated phenol decomposer consortium (Naghoni et al. 2017). The degradation potential of a halophilic bacteria *H. organivorans* was proved to catabolize different concentrations of low molecular weight aromatic compounds at 10% NaCl concentration (García et al. 2005). The phylogenetic-related strain *H. salina* showed phenol degradation efficiency of 66% at 10% NaCl. The catechol 1,2 CTD gene sequence of isolated *Halomonas HA1* provides evidence as evolutionary gene divergence between *P. putida* (Family *Pseudomonadaceae*) and *H. daqingensis* (Family *Halomonadaceae*), class *Gammaproteobacteria*. The bacterial phylogenetic estimation based on 1,2 CTD sequence was previously reported (Táncsics et al. 2008; Shen et al. 2009). In this context, our isolate has two different phylogenetic status based on its 16S rRNA or 1,2 CTD gene sequence. This evidence could indicate the presence of separate evolutionary origin ofthe isolated 1,2 CTD genes. Many reports demonstrated the role of transposons in the evolution of bacterial dioxygenases (Nojiri et al. 2004; Liang et al. 2012). In comparison with 16S rRNA, the dioxygenase gene sequence provides a sufficient method to interspecies differentiation among the bacterial genera (Táncsics et al. 2008; Shen et al. 2009). The sequence analysis of 299 amino acid of *Marinobacter* 2,3 CTD was obtained using degenerative primer set. The identified protein shares the main conservative domains with other extradiol dioxygenases. The isolated sequence shows high similarity with taxonomically related *M. excellens* and other members of *Microbacteriaceae* as shown in figure 3. In comparison to intradiol 1,2 CTD enzymes, the extradiol 2,3 CTD enzymes show longer stretch of conserved domains. The expression analysis of *Halomonas HA1* 1,2 CTD and *Marinobacter HA2* 2,3 CTD showed a constitutive gene expression pattern. Many phenol degrading operons in *proteobacteria* expression are activated by inducers substrate the targeted pathway (Shingler et al. 1993; Pérez-Pantoja et al. 2008). Phenol and benzoate are the common inducer of 1,2 catechol dioxygenases (Caposio et al. 2002; Suzuki et al. 2002). The 2,3 meta cleavage dioxygenase mechanism of *P. pseudoalcaligenes* is mainly utilized with phenol and salicylate (Jõesaar et al. 2017). In some cases both constitutive and induced expression pattern are exhibited by different dioxygenase genes in the same strain (Comte et al.

2013). The constituent activation of xenobiotic degrading genes could be a consequence of the presence of internal promoter that can alter the inducing nature of these clusters. The alternation of transcriptional aspect of phenol degrading clusters could be evolutionary advantage by which bacteria can adapt with xenobiotic polluted environment (Cui et al. 2016). An evidence indicated the role of class I transposons in Patchwork Assembly of 3-chloro-catechol degradation cluster in *P. stutzeri* (Liu et al. 2011).

Declarations

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Conflict of interest statement

The authors declare no conflicts of interest.

Availability of data and material

All data are included in the manuscript

Code availability

Not applicable

Author's contributions

NA, HH, and SS established the study design. NA and HK provided the research strategy and supervised overall the research plan. NA, HH and AE performed the experiments. NA, HH and HK interpreted the data and provided the scientific and statistical analysis. NA and HK prepared and wrote the manuscript. All authors read and approved the manuscript.

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Figures

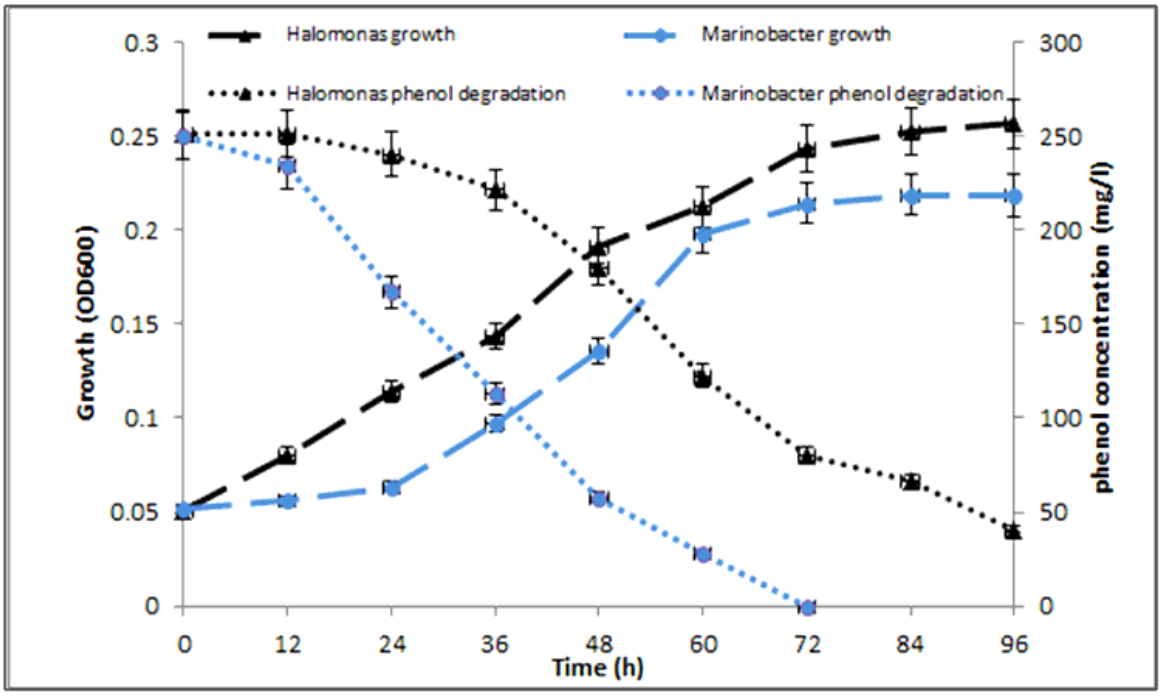

Figure 1

Biodegradation of phenol and growth curve of Halomonas HA1 and Marinobacter HA2 bacteria on phenol as the sole carbon source.

Figure 2

Multiple sequence alignment of conserved 1,2 CTD protein domain. Residues in boxes show the lipid-binding sites. Residues with arrows show the active sites. Residues in discontinuous boxes indicate the Fe-ligands. Bacterial names are indicated in letters as demonstrated: (A) A. lwoffii, (B) H. pacifica, (C) H. lutea, (D) C. salexigens, (E) Halomonas HA1, (F) P. putida, (G) P. fluorescens.

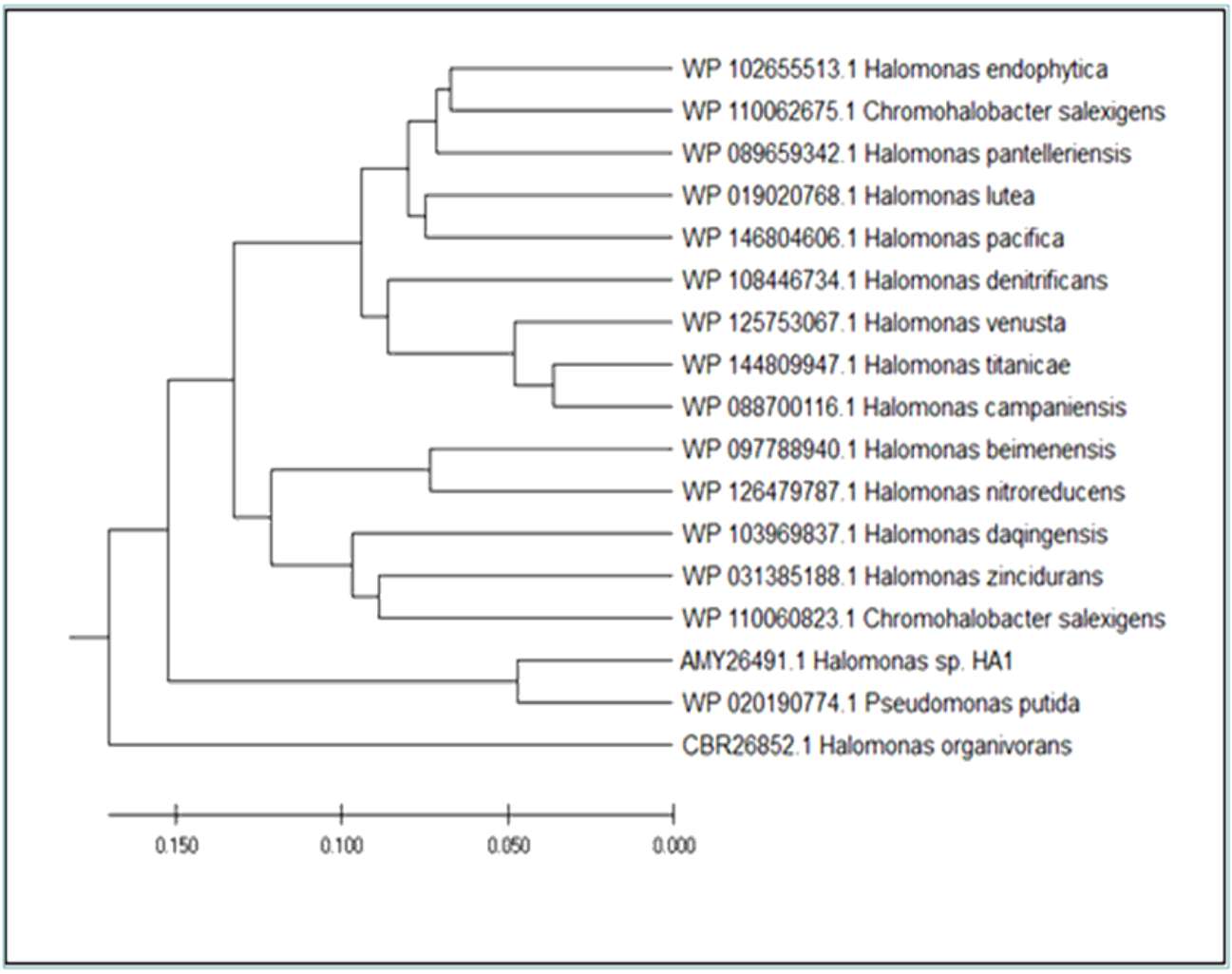

Figure 3

Phylogenetic tree of 303 aa residues of 1,2 CTD enzyme from Halomonas HA1 in comparison with other 1,2 CTD enzymes from related bacteria. The trees are constructed using MEGA software and the UPGMA method. Branch lengths are proportional to the interfered phylogenetic distances.

Figure 4

Multiple sequence alignment of conserved 2,3 CTD protein domain. Residues with arrows show the active sites. Residues in discontinuous boxes indicate the Fe-ligands. Bacterial names are indicated in letters as demonstrated: (A) P. putida, (B) P. stutzeri, (C) G. bacterium, (D) Marinobacter sp. HA02, (E) Marinobacter excellens.

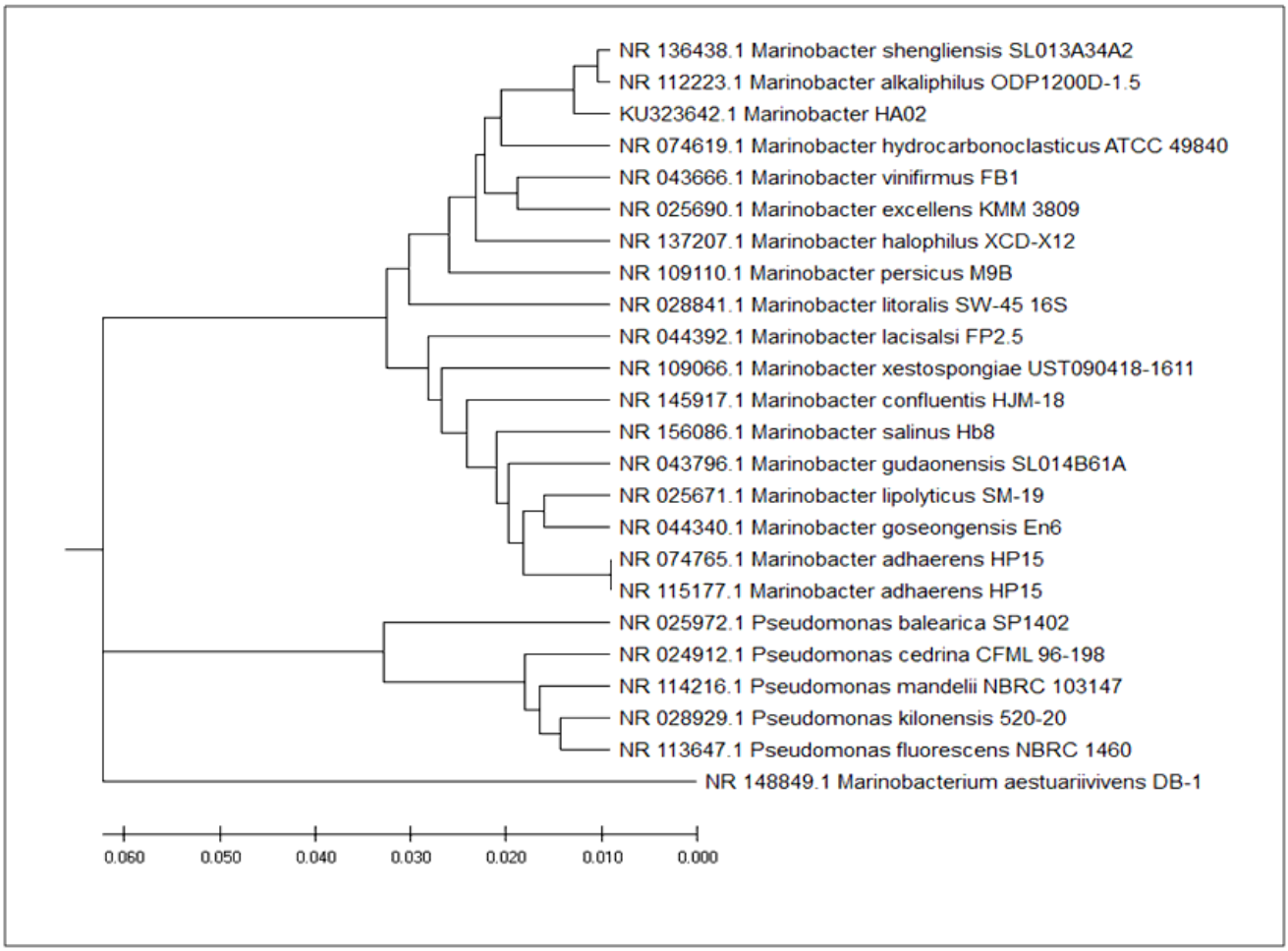

Figure 5

Phylogenetic tree of 299 aa residues of 2,3 CTD enzyme from Marinobacter HA2 in comparison with other 2,3 CTD enzymes from related bacteria. The trees are constructed using MEGA software and the UPGMA method. Branch lengths are proportional to the interfered phylogenetic distances.

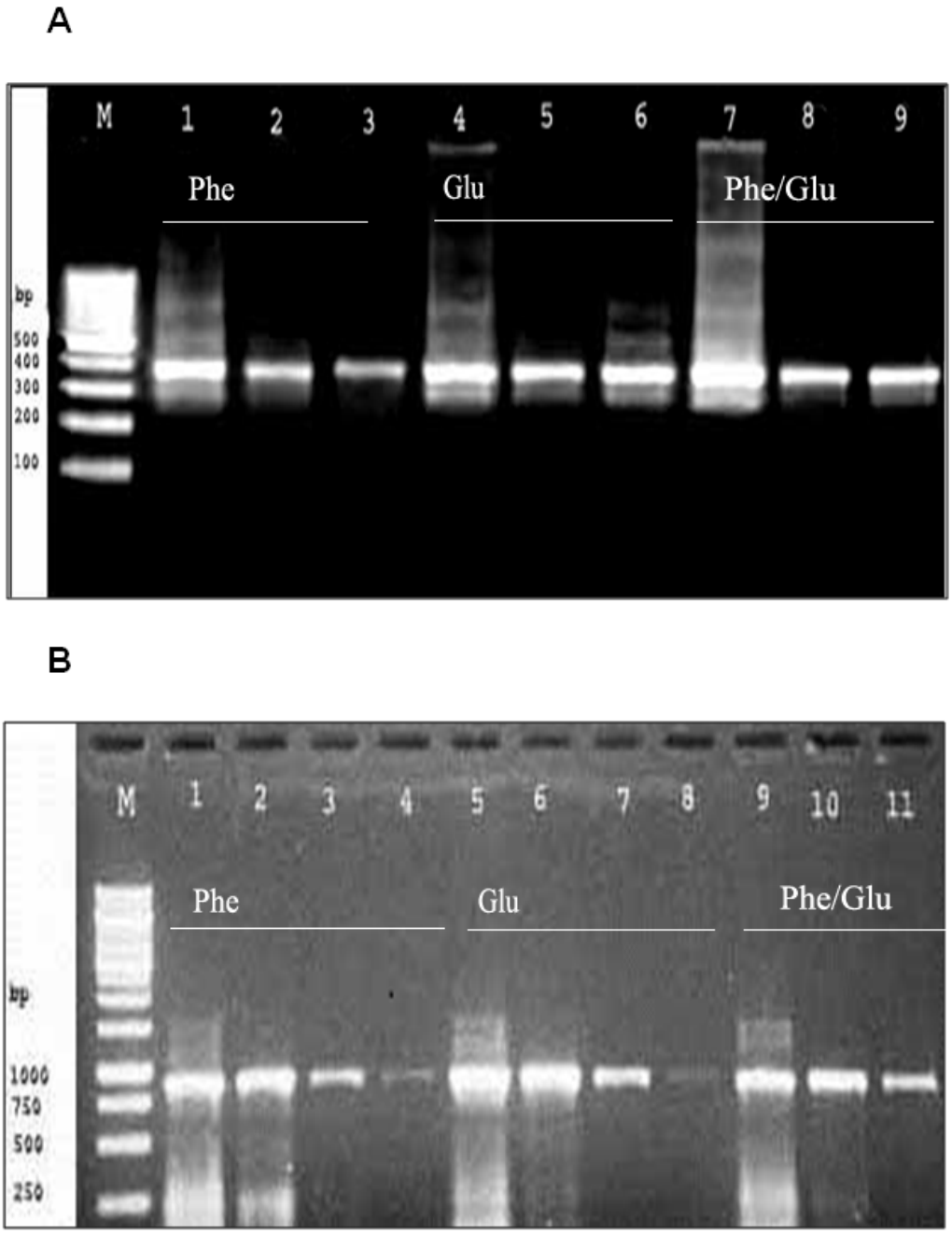

Figure 6

Semi-quantitative RT-PCR detecting the expression pattern. (A) (A) Halomonas HA1 1,2 CDG gene in presence of phenol (1-3) or glucose (4-6) as a sole carbon source and phenol with glucose (7-9). (M) is 100 bp DNA marker (Fermentas). (B) Marinobacter expression 2,3 CDG gene in presence of phenol (1-4) or glucose (5-8) as a sole carbon source and phenol with glucose (9-11). (M) is 1 Kb DNA marker (Fermentas)