A Bifunctional Cytochrome P450 Enzyme Involved in the *O*-Dealkylation or *N*-Dealkoxymethylation of Chloroacetanilide Herbicides in *Rhodococcus* sp. B2

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Abstract

Background: The chloroacetamide herbicides pretilachlor is an emerging pollutant, Due to the large amount of use, its presence in the environment threatens to human health. However, the molecular mechanism of pretilachlor degradation is unknown.

Results: Now, *Rhodococcus* sp. B2 was isolated from rice field and found to degrade pretilachlor. The maximum pretilachlor degradation efficiency (86.1%) was observed at a culture time of 5 d, 50 mg/L initial substrate, pH 6.98, and 30.1°C. One novel metabolite was identified by gas chromatography-mass spectrometry (GC-MS). Draft genome comparison demonstrated that a 32,147-bp DNA fragment, comprising a gene cluster (*EthRABCD*$_{B2}$), was absent in the mutant strain TB2 which could not degrade pretilachlor. The *Eth* gene system, encodes an AraC/XylS family transcriptional regulator, a ferredoxin reductase, a cytochrome P-450 monooxygenase, a ferredoxin and a 10-kDa unknown protein. Complementation of *EthABCD*$_{B2}$ and *EthABD*$_{B2}$, but not *EthABC*$_{B2}$ in strain TB2 restored its activity against chloroacetamide herbicides. The codon of *EthABCD*$_{B2}$ was optimized, expressed in *Escherichia coli*, and purified utilizing Ni-affinity chromatography. A mixture of EthABCD$_{B2}$ or EthABD$_{B2}$ but not EthABC$_{B2}$ catalyzed the *N*-dealkoxymethylation activity toward alachlor, acetochlor, butachlor, and propisochlor and *O*-dealkylation activity toward pretilachlor, revealing that EthD acted as a ferredoxin in strain B2. EthABD$_{B2}$ displayed maximal activity at 30 °C and pH 7.5.

Conclusions: This is the first report of a P450 family oxygenase catalyzing the *O*-dealkylation or *N*-dealkoxymethylation of pretilachlor and propisochlor. And the results provide microbial resource for the remediation of chloroacetamide herbicides-contaminated sites,

Introduction

Chloroacetamide herbicides are preemergence herbicides utilized to control broadleaf weeds and annual grasses in the cultivation of soybeans, corn, rice and many other crops [1, 2]. The main representative chloroacetamide herbicides, including acetochlor, alachlor, propisochlor, metolachlor, butachlor and pretilachlor are *N*-alkoxyalkyl-*N*-chloroacetyl-substituted aniline derivatives based on their structures. Due to their excessive application and chemical stability, chloroacetamide herbicides have been detected in the surface water, groundwater and drinking water in many countries [3–6]. Chloroacetamide herbicides have been reported to be highly deleterious to many aquatic organisms and are known to be carcinogenic to humans [7]$^{-}$[8].

Pretilachlor is a pre-planting or post-emergence herbicide used to eliminate broad-leaved weeds, grasses and sedges in the rice fields [9]. Pretilachlor residue in soil could damage rice leaves and exert toxicological effects on cyanobacteria [10, 11]. Pretilachlor exposure led to apoptosis, immunotoxicity, endocrine disruption and oxidative stress in gestating zebrafish [12] and hepatic P4502B subfamily-dependent enzyme activity in rat liver [13]. Therefore, great concerns have been raised about the degradation mechanism of chloroacetamide herbicides.

Microbial metabolism is considered as an important method for the removing chloroacetanilide herbicides in ecosystems [14, 15]. Now, many chloroacetamide herbicides-degrading microorganisms have been reported and the initial metabolism primarily involves two pathways: glutathione mediation [16, 17] and *N*-dealkylation [18, 19]. *N*-Dealkylation is primarily carried out by enzymes in the Rieske non-heme iron oxygenase (RHO) and cytochrome P450 families in living organisms [20, 21]. Chen et al. reported an RHO system, CndABC, that can execute the *N*-dealkylation toward acetochlor, alachlor and butachlor, but not toward propisochlor, pretilachlor or metolachlor, in Sphingomonad strains DC-2 and DC-6 [18]. Wang et al. showed that $EthBAD_{T3-1}$ (from *Rhodococcus* sp. T3-1) expressed in *Escherichia coli* acquired *N*-deethoxymethylase activity toward acetochlor resulting in the conversion of acetochlor to CMEPA, but not toward metolachlor or pretilachlor [19, 22]. Currently, the molecular mechanism for the dealkylation of propisochlor, metolachlor and pretilachlor by microorganisms remains unknown.

The strain *Rhodococcus* sp. B2 was isolated from a rice field in which pretilachlor had been applied for many years. Strain B2 could degrade pretilachlor with the initial reaction being *O*-dealkylation. In this study, the components of $EthRABCD_{B2}$ were cloned and verified to function in *Rhodococcus* sp. TB2, The codon of $EthABCD_{B2}$ was optimized and expressed in *Escherichia coli*, and purified utilizing Ni-affinity chromatography. A mixture of $EthABCD_{B2}$ or $EthABD_{B2}$ but not $EthABC_{B2}$ displayed *N*-dealkoxymethylation activity toward alachlor, acetochlor, butachlor and propisochlor and *O*-dealkylation activity to pretilachlor which indicated a new mechanism for initial degradation of chloroacetamide herbicide.

Materials And Methods

Chemical reagents, medium, isolation and characterization of pretilachlor degradation bacteria

Butachlor, alachlor, acetochlor, metolachlor, propisochlor, pretilachlor, CDEPA (2-chloro-*N*-(2,6-diethyl phenyl) acetamide) and CMEPA (2-Chloro-*N*-(2-methyl-6-ethylphenyl) acetamide) was from Alfa-Aesar (Shanghai, China). Acetonitrile (HPLC grade) was from Sigma-Aldrich (Shanghai, China). Other reagents using in this study were the AR grade. The composition of mineral salts medium (MSM), Luria-Bertani medium (LB), isolation, characterization and identificaton of pretilachlor degradation strain was in line with the description of liu et al. [23, 24].

Optimization Of Pretilachlor-degrading Conditions

The effects of the cultivation conditions on pretilachlor-degrading were confirmed by the response surface methodology with a central composite design (CCD) procedure. The Design Expert software (version 12.0.3; StatEase, Inc. Minneapolis, USA) was used to design and carry out this experiment. Three factors, pH, temperature and inoculum size, were considered independent variables (Table 1), the degradation rate of 50 mg/L of pretilachlor by B2 in 5 days served as the response variable. A total of 20 runs with three replicates were performed. The uninoculated culture served as a control. Then the experimental data were used in an empirical model analysis (quadratic polynomial equation):

$Y = B + \sum B_i X_i + \sum B_{ij} X_i X_j + \sum B_{ii} X_i^2$ (1)

Y: predicted response; Xi and Xj: variables; B: constant; bi: linear coefficient; bij:

interaction coefficient; bii: quadratic coefficient.

Table 1
The design table of central composite design (CCD)

Symbols	factors	levels of variables				
		-alpha	Low(-1)	Medium(0)	High(1)	+alpha
A	pH	5.3	6	7	8	8.7
B	temperature(°C)	21.6	25	30	35	38.4
C	Inoculation (g/L)	0.03	0.1	0.2	0.3	0.37

Kinetics Of Pretilachlor-degradation By Strain B2

Batch experiments were performed to analyze the effects of the initial concentration of pretilachlor on the degradation efficiency, Degradation ability was detected at different pretilachlor concentrations (10, 20, 30, 50, 75, 100, 125, 150, 200, 250, 300 mg/L) under the optimum conditions. The pretilachlor degradation efficiency was detected by HPLC as following description. The data were fitted to Andrews model of substrate inhibition kinetics [25]:

$$q = \frac{q_{max} S}{S + K_S + (S^2/K_i)} \quad (2)$$

$$S_m = \sqrt{K_S \times K_i} \quad (3)$$

S: concentration of pretilachlor; *Ki*: inhibition constant; *Ks*: half- saturation constant;

and *qmax*: maximum degradation efficiency of pretilachlor; *Sm*: the critical inhibitor concentration of pretilachlor

Plasmids, strains and culture conditions

The plasmids and strains are shown in Table S1. The primers are described in Table S2. *E. coli* was cultivated at 37 °C with antibiotics added if necessary. Strain B2 was grown in LB medium supplemented with 100 mg/L pretilachlor at 30 °C. The derivatives and other strains were all cultivated at 30 °C in LB medium.

Genome Sequencing, Annotation, And Genome Analysis

The DNA extraction was in line with the method of Sambrook et al [26]. Genome sequencing of strains B2 and TB2 was carried out using the Illumina MiSeq system by GENEWIZ Co., Ltd. (Suzhou, China). The BLAST program, combining the databases of Nr protein, KEGG, COG and Swiss-Prot, was utilized for annotation. To determine the absent DNA fragment in strain TB2, genome comparison of strain B2 and TB2 was carried out using the software Mauve1.2.3 [27]. Chromosome walking was performed by SEFA-PCR [28]. Protein sequences were aligned with the ClustalX program [29], then MEGA 7.0 [30] was used to build phylogenetic tree.

Functional complement in strains TB2 and R-XP

A 4,964-bp fragment *EthRABCD*$_{B2}$ (containing the native promoter), a 4,597-bp fragment *EthRABC*$_{B2}$ (containing the native promoter) and a 3,785-bp fragment *EthABCD*$_{B2}$ (containing the native promoter), were cloned from the genome of strain B2, was constructed. A 3,388-bp fragment *EthABD*$_{B2}$ (containing the native promoter) was amplified from the plasmid pQEth3. Fragments, *EthRABCD*$_{B2}$ and *EthRABC*$_{B2}$, were ligated into the HindIII-SpeI sites of the *Rhodococcus-Escherichia coli* shuttle vector pRESQ [31], yielding pQEth1 and pQEth2, respectively. Fragments, *EthABCD*$_{B2}$ and *EthABD*$_{B2}$, were cloned into the shuttle vector pRESQ by a Gibson Seamless Assembly Kit (HaiGene Co., Ltd). The constructed vectors were first transformed to *E. coli* DH5α and then introduced into strains TB2 or R-XP by electrotransformation [32]. All the recombinant plasmids were confirmed by sequencing. The abilities of *E. coli* DH5α and strain TB2 containing the recombinant plasmids to degrade pretilachlor were detected by whole-cell biotransformation experiments according to the method of Liu et al. with some modifications [33]. Briefly, centrifugal collection the post-log phase transformants, then washed, resuspended the transformants in 20 mL MSM to a final OD_{600nm} of 1.0. Each substrate was injected to the cell suspensions at a final concentration of 100 mg/L and cultivated at 160 rpm and 30 °C. Samples were harvested at appropriate intervals, the degradation metabolites were monitored by HPLC as described below.

Expression of EthABCD and purification of the recombinant proteins

To express *EthABCD* and *EthABC* under T7 promoter, a 3,234-bp fragment and a 2,867-bp fragment without native promoter were cloned from strain B2 with the primers pET-EthF1/pET-EthR, pET-EthF2/pET-EthR. The PCR product was cloned into plasmid pET-29a(+) to construct recombinant plasmids pET-EthABCD and pET-EthABC. *E. coli* BL21(DE3) harboring pET-EthABCD and pET-EthABC was grown at 37 °C until OD_{600} = 0.6, and then incubated at 16 °C for 12 h after adding 0.5 mM IPTG. Then, undergone whole-cell transformation with centrifugal collection cells according to above description.

The gene cluster *EthABCD*, synthesized by GenScript company depending on *E. coli* codon usage form, were amplified with the primers of Table 2. The products were ligated into the corresponding site of expression plasmid pET29a(+) and transformed as recombiant plasmids into *E. coli* BL21(DE3)pLysS. Each recombinant plasmid was verified by sequencing. The gene expression and recombinant proteins purification were performed according to the description of Hussain et al [34]. SDS-PAGE was used to

determine the protein molecular weight, and the protein concentrations were calculated by the Bradford method [35].

Table 2
Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of Squares	degrees of freedom	Mean Square	F-value	p-value	
Model	1489.53	9	165.5	21.17	< 0.0001	significant
A-pH	54.42	1	54.42	6.96	0.0248	
B-T	5.29	1	5.29	0.6762	0.4301	
C-I	89.97	1	89.97	11.51	0.0069	
AB	87.12	1	87.12	11.14	0.0075	
AC	45.51	1	45.51	5.82	0.0365	
BC	4.62	1	4.62	0.591	0.4598	
A^2	1118.5	1	1118.5	143.06	< 0.0001	
B^2	152.73	1	152.73	19.53	0.0013	
C^2	8.27	1	8.27	1.06	0.3279	
Residual	78.19	10	7.82			
Lack of Fit	60.88	5	12.18	3.52	0.0969	not significant
Pure Error	17.31	5	3.46			
Cor Total	1567.72	19				

Enzyme activity assays

The enzyme activity toward the degradation of several chloroacetanilide herbicides was assessed at 30°C for 1 h in a 1 mL mixture (20 mM Tris-HCl buffer pH 7.5, 0.15 μg $EthB_{B2}$, 0.58 μg $EthA_{B2}$, 0.17 μg $EthC_{B2}$, 0.12 μg $EthD_{B2}$, 1 mM NADH, 0.1 mM NaCl, 0.5 mM Fe^{2+}, and 1 mM $MgCl_2$, 1 mM 2-Mercaptoethanol). The reaction was started after adding the substrates at a final concentration of 0.5 mM, The assays were stopped by adding 2 mL of dichloromethane, and the disappearance of the substrates was monitored by HPLC. The metabolites were determined by GC-MS analysis as described below. One unit of enzymatic activity was defined as the enzyme required to generate of 1 nmol product per minute.

The optimal pH for the reaction mixtures at 30 °C was determined in four different bufers: 20 mM citrate buffer (pH 3.8–5.8); 20 mM Na_2HPO_4-citric acid (pH 6.0–8.0); 20 mM Tris-HCl buffer(pH 7.5-9); and 20 mM glycine-NaOH buffer (pH 8.5–10.0). The optimal reaction temperature was evaluted at the optimal conditions and diferent temperatures (10–70 °C). The effects of potential inhibitors or activators on the enzymatic activity were determined by adding various metal ions and chemical agents to the reaction systems and inculation at 30 °C for 60 min. Enzyme activity without any additive compounds was defined as 100%.

Chemical analysis

The sample was extracted according to a previously description [23]. HPLC analysis with the Kromasil 100-5C18 column (250 mm × 4.6 mm × 5 μm) was utilized. The injection volume was 20 μL. Acetonitrile/water (80:20, v/v, 0.8 mL/min) was used as the mobile phase, and the measuring wavelength was 215 nm. The identification of metabolites were accomplished by GC-MS (Shimadzu QP2010 Plus) with a RTX-5MS column (15 m length × 0.25 mm id × 0.25 μm internal diameter) in split mode (1:20). The temperature program was as follows: 100 °C for 1.5 min, ramp at 50 °C/min to 260 °C (7 min hold), ramp at 50 °C/min to 300 °C (1 min hold). The detectation mass range was from 70 m/z to 350 m/z. Helium (1.0 ml/min) was the carrier gas.

Nucleotide Sequence Accession Number

The GenBank accession numbers of the *Rhodococcus* sp. B2 16S rRNA gene sequence and the gene cluster $EthRABCD_{B2}$ are KM875453 and KJ946935.

Results

Isolation, and identification of the pretilachlor-degrading strain B2

A pure culture named B2 was isolated. The bacterial cells were gram-positive, and nonmotile. Colonies were convex, opaque and red on LB agar after two days. Urease and catalase were positive in strain B2, but nitrate reduction, oxidase and starch hydrolysis were negative. The 16S rRNA gene sequence showed that strain B2 had a 100% similarity with strain *R. erythropolis* DSM43066^T and 99.93% similarity with *R. erythropolis* NBRC100887^T, and formed a subclade with these two strains (Fig. S1). Strain B2 was preliminarily identified as *Rhodococcus* sp. basing on its characteristics.

Optimization Of The Cultivation Conditions For Pretilachlor-degradation

Three factors pH, temperature, and inoculum size which have significant effects on the microbial degradation were chosed as the cultivation conditions to optimize using the CCD model. The data in table S3 was used in multiple regression analysis, and response variable Y can be obtained from the quadratic polynomial model equation:

$$Y = 84.31 + 2.00A - 0.622B + 2.57C + 3.3AB - 2.39AC + 0.76BC - 8.81A^2 - 3.26B^2 - 0.76C^2 \quad (4)$$

The ANOVA analysis results for the quadratic response surface model are shown in Table 2. The regression model for pretilachlor degradation was statistically significant ($P < 0.05$) with $R^2 = 0.9501$, and the results showed A, C, AB, AC, A^2, B^2 significantly affected the degradation of pretilachlor by strain B2. Thus, pH, inoculum size had significant effects on the degradation rate. Based on the p values of AB and AC (0.0075 and 0.037), the pH-temperature and inoculum size-pH interaction effects on the degradation of pretilachlor were highly significant. Therefore, the response surface analysis was conducted to determine the impacts of the interaction between pH and temperature on pretilachlor-degradation by strain B2 (Fig. 1). These results revealed the maximum rate of pretilachlor degradation by strain B2 was 86.1% under the optimal conditions of pH 6.98, 30.1 °C, and inoculum size 0.3 g/L.

Kinetics of pretilachlor degradation by strain B2

The effect of the initial pretilachlor concentration on the degradation of pretilachlor was calculated via nonlinear least squares regression analysis using Origin 9.0 pro software and the results are shown in Fig. 2. The kinetic parameters were as follows: $q_{max} = 3.28\ d^{-1}$, $K_S = 53.51$ mg/L, and $K_i = 25.38$ mg/L. the Sm was 36.84 mg/L, which indicates that the theoretical degradation efficiency of pretilachlor was highest at this concentration, when the concentration of pretilachlor was more than 36.84 mg/L, the inhibition of strain B2 by the pretilachlor became obvious. This might be attributed to the toxicity of pretilachlor to the strain. The Andrews model was as follows:

$$q = \frac{3.28S}{S + 53.51 + S^2/25.38}$$

Statistical regression results revealed the parameters of the pretilachlor degradation kinetics (Table. S4). The correlation coefficient $R^2 = 0.8285$, indicates that the model was an excellent fit to the experimental data. the pretilachlor degradation activity of strain B2 increased at low pretilachlor concentrations, but decreases at higher pretilachlor concentrations. This kinetic model is helpful for predicting the microbial bioremediation of pretilachlor by strain B2.

Identification of the metabolites resulting from pretilachlor degradation by strain B2

The products of pretilachlor degradation by strain B2 were detected by GC-MS. And a new metabolite appeared at a retention time of 11.1 min. In GC-MS, this product produced ions at m/z 120.0, 162.1, 176.0, 220.0, 237.9 and 269.0 (Fig. 3), which correspond to *N*-hydroxyethyl-2-chloro-*N*-(2,6-diethyl-phenyl)-acetamide. The metabolite during pretilachlor degradation was never reported, which indicted a novel product. However, the metabolite could not be further metabolized by strain B2. Therefore, pretilachlor degradation process by strain B2 was *O*-dealkylation, representing a new mechanism of initial chloroacetamide herbicide degradation.

Screening Of A Mutant, TB2, Defective Degradation In Pretilachlor

When grown on LB agar adding 100 mg/L pretilachlor, the colonies of strain B2 could produce a visible transparent halo, and N-hydroxyethyl-2-chloro-N-(2,6-diethyl-phenyl)-acetamide, which is more water-soluble than pretilachlor, was formed. In our study, we found that a few cells of strain B2 did not generate a transparent halo after continuous streaking on fresh LB agar plates, one such isolate was named TB2. Resting cell transformation experiments illustrated that TB2 could not metabolize pretilachlor (Fig. S2), suggesting that the related gene responsible for O-dealkylation in pretilachlor degradation was deleted in the mutant TB2.

Genome Comparison Of Strains B2 And TB2

The draft genomes of strains B2 and TB2 were sequenced with the Illumina MiSeq system. The draft genomes of strains B2 and TB2 were 6,873,325 bp and 6,728,834 bp in length. Genomes comparison of B2 and TB2, a fragment from scaffold 51 of strain B2 was absent in the genome of mutant TB2. This absence fragment was verified using PCR. Subsequently, the flanking regions of scaffold 51 were confirmed by SEFA-PCR. Finally, a 115,851-bp fragment was acquired. Sequence comparison combined with PCR demonstrated that a 32,146-bp part of this fragment was absent in mutant TB2 (Fig. 4A).

ORF analysis of the absent fragment in strain TB2

A gene cluster consisting of five genes, *EthR, EthA, EthB, EthC and EthD*, was found by the analysis of all ORFs, and the encoded amino acid sequence of the missing fragment was identified in NCBI (Table S5). *EthR*, encoding the AraC/XylS family regulator (37 kDa), shares the highest similarity with the EthR from *Rhodococcus sp.* T3-1 (100%). *EthA*, encoding a ferredoxin reductase (43 kDa), shares the highest similarity with EthA from *Rhodococcus sp.* T3-1 (100%). *EthB*, encoding a cytochrome P450 oxidase (44 kDa), shares the highest similarity with EthB from *Rhodococcus sp.* T3-1 (97.5%). *EthC*, encoding a 2Fe-2S ferredoxin (11 kDa), shares the highest similarity with EthC from *Mycobacterium* sp. CH28 (99.06%). EthD, encoding a protein of unknown function (10 kDa), shares the highest similarity with EthD from *Mycobacterium* sp. CH28 (90.29%) (Fig. 5). The gene *EthB* was termed $EthB_{B2}$ (EthB from strain B2), and its inferred amino acid sequence was aligned that of $EthB_{T3-1}$ from *Rhodococcus* sp. strain T3-1, as shown in Fig. 4B. Ten amino acid differences were observed between the two proteins, which may confer different physical properties to $EthB_{B2}$. The high similarity of the two proteins indicated a horizontal gene transfer event (Fig. 4A). The upstream *eth* gene cluster contains two gene fragments (*tnpA1*, and *tnpA2*) encoding the proteins displaying > 99% sequence identity with the Tn3 family transposase (TnpA) and one fragment (*tnp1*) belongs to the IS30 family transposase (Table S5). However, a transposase was not found downstream of the *eth* gene cluster. In contrast, two transposons, IS3-type class II, flanked the *EthRABCD* gene cluster of *R. ruber* IFP 2001 [36].

Functionally complementing Eth gene cluster in strain TB2

To determine the function of the gene cluster $EthRABCD_{B2}$, the shuttle plasmid pQeth1 containing $EthRABCD_{B2}$ was introduced into strain *E. coli* DH5α and strain TB2. TB2 (pQeth1) acquired the capability

to generate a visible transparent halo in LB agar supplemented with 100 mg/L pretilachlor, which was similar to strain B2. HPLC showed that TB2 (pQeth1) could degrade pretilachlor, demonstrating the *O*-dealkylation activity of EthRABCD$_{B2}$ (Fig. S2). A similar phenomenon was found in strain *Rhodococcus* sp. R-XP(pQeth1). However, *E. coli* DH5α(pQeth1 or pQeth2) and strain TB2(pQeth2) failed to degrade pretilachlor, revealing that *EthRABCD*$_{B2}$ was expressed at a low level or that the native promoter could not be distinguished in *E. coli*, and EthD$_{B2}$ was essential for degradation. The *EthABCD*$_{B2}$ and *EthABC*$_{B2}$ under the control of T7 promoter in vector pET-29a(+) were introduced into *E. coli* BL21(DE3). Whole-cell transformation assay results showed that the IPTG-induced suspension of *E. coli* BL21(DE3) harboring *EthABCD* (not EthABC) was able to degrade alachlor and acetochlor but with low activity (data not shown). Considering that *EthR*$_{B2}$ and the *EthC*$_{B2}$ gene were not essential, *EthABCD*$_{B2}$ and *EthABD*$_{B2}$ with native promoters were reconstituted to analyze the degradation of chloroacetanilide herbicides. The HPLC results showed that strain TB2 (pQeth3, and pQeth4) could convert pretilachlor, butachlor, alachlor, acetochlor and propisochlor to the corresponding metabolites, indicating *EthD* was probably a ferredoxin gene.

Expression of the gene cluster EthABCD $_{B2}$ and reconstitution of the chloroacetanilide herbicide degradation enzyme in vitro

The components of the gene cluster *EthABCD*$_{B2}$ were expressed in *E. coli* BL21(DE3)pLysS and the recombinant proteins were purified using Ni-affinity chromatography. The *Mw* value of the four proteins were consistent with the theoretically calculated values (Fig. S3). Purified EthC$_{B2}$-His6 and EthD$_{B2}$-His6, were mixed with EthAB$_{B2}$-His6 in vitro. The results showed that the EthABD$_{B2}$-His6 and EthABCD$_{B2}$-His6 (not EthABC$_{B2}$-His6) mixture showed activity toward degrading pretilachlor indicating that EthD was a ferredoxin. The catalytic activities of EthABD$_{B2}$ for pretilachlor was 3.41 ± 0.4 μmol/min/mg, EthABD$_{B2}$ carried out *N*-dealkoxymethylation to acetochlor, alachlor, propisochlor butachlor, *O*-dealkylation to pretilachlor, but it was unable to degrade metolachlor. The GC-MS results showed that strain EthABD$_{B2}$ could convert butachlor, alachlor, acetochlor and propisochlor to the corresponding metabolites CDEPA (for butachlor and alachlor) or CMEPA (for propisochlor, acetochlor) (Fig.S4-S7). These metabolites result from C-N bond cleavage by *N*-dealkoxymethylation, and based on the comparison of the chemical structures of chloroacetanilide herbicides, the number of C-atoms between N and O in the side chain affected the degradation. According to these results, the metabolic mechanism of pretilachlor (*O*-dealkylation) was different from that of the other four chloroacetanilide herbicides(*N*-dealkylation), and the degradation pathway of chloroacetanilide herbicides by EthABD$_{B2}$ was proposed (Fig. 4C). The recombinant strain TB2 (pQeth4) could not degrade metolachlor, which has a chiral carbon, suggesting that steric hindrance blocks enzyme-substrate interactions. EthB$_{B2}$, a cytochrome P450 monooxygenase of the multicomponent system, plays a key role in degradation, and EthABD$_{B2}$ from strain B2 has a broader substrate spectrum than that from strain T3-1. EthABD$_{B2}$ is a better enzyme for practical bioremediation of chloroacetanilide herbicides.

Characteristics of EthABD$_{B2}$

The effects of different environmental factors on enzyme activity have been determined (Fig. S8). The enzyme activity was detected at 10–65 °C, with an optimum temperature of 30 °C (Fig. S8B). And the enzyme showed high activity at pH 7.0-8.5, with an optimum pH of 7.5 in Tris-HCl buffer (Fig. S8A). Greater activity was lost at pH values below 4.0 or above 10.0. Metal ions play an important part in the enzyme activity. As shown in Table S5, Fe^{2+} and Mg^{2+} could strongly enhance enzyme activity, while Ca^{2+} could also slightly rise enzyme activity. However, divalent cations Ba^{2+}, Co^{2+} and Zn^{2+} slightly decresed enzyme activity, while Ag^{+}, Cu^{2+}, Hg^{2+}, Ni^{2+} Cr^{2+} and Mn^{2+} showed significantly inhibition on enzyme activity. Chemical agents EDTA severely inhibited the enzyme activity, indicating that metal ions were required for its enzymatic reaction.

Discussion

Glutathione S-transferase and cytochrome P450 play an important function in the detoxification of chloroacetanilide herbicides in plants and mammals [37, 38]. In microorganisms, a monooxygenase system belonging to RHO family *N*-dealkylase and catalyzing the *N*-dealkylation of butachlor, acetochlor and alachlor, CndABC, was cloned from strains DC-2 and DC-6 [18]. $EthBAD_{T3-1}$ from *Rhodococcus* sp. T3-1, expressed in *E. coli*, showed *N*-deethoxymethylase activity against acetochlor, but not against pretilachlor or metolachlor [19, 22]. At present, we identified and characterized the function of the cytochrome P450 monooxygenase system $EthABD_{B2}$ cloned from strain B2. $EthABD_{B2}$, ten amino acids difference with $EthBAD_{T3-1}$ system, acquired the ability to catalyze the *O*-dealkylation or *N*-dealkoxymethylation of the chloroacetanilide herbicides pretilachlor, propisochlor, alachlor, acetochlor and butachlor. The different functions of the two proteins indicte that the key amino acid mutant broadens the substrate spectrum, which indicting the amino acid mutants of this gene could be attempted to degrade metolachlor in the further research.

EthRABCD was first identified in the fuel oxygenate-degrading strain *R. ruber* IFP 2001 which has the *O*-dealkylation activity toward tert-amyl methyl ether(TAME), ethyl tert-butyl ether(ETBE) and methyl tert-butyl ether(MTBE) [36]. The Eth system, also found in gram-negative strain *Aquincola tertiaricarbonis* L108, which lacks *EthR*, enables the efficient metabolization of MTBE, diethyl ether, TAME, ETBE, diisopropyl ether and tert-amyl ethyl ether(TAEE). However, this Eth monooxygenase system can not catalytize the metabolization of the synthetic ethers, including phenetole, and isopropoxybenzene, inferring that the nonreacting side chain link with the ether molecules may not exceed the size of a the tert-amyl group [39]. Herein, we enriched the function of Eth system in dealkylating O atom or dealkoxymethylating of N atom in synthetic compounds with larger residues, including pretilachlor, acetochlor, propisochlor, and butachlor.

The cytochrome P450 monooxygenase system $EthRABCD_{B2}$ of *Rhodococcus* sp. B2 suffers from transposon-mediated recombination, causing *eth* loss mutants, e.g., *Rhodococcus* sp. TB2, which were unable to degrade pretilachlor. The deletion mechanism was similar to that from *R. ruber* IFP 2001 (IS3-type transposon element) but was different from that from *A. tertiaricarbonis* L108 (rolling-circle IS91

type). The sequence similarity of the gene cluster *EthRABCD*$_{B2}$ in the reported bacterial strains was > 90%, implying that the *Eth* gene cluster was horizontally transferred and that the transposons were likely the reason for high mobility. Transposons in strains are rapidly adapted toward environmental transitions, such as substrate changes. This is also true of enzymes for the degradation of man-made xenobiotics degradation, such as chloroacetanilide herbicides which have been applied globally for less than one hundred years [40]. The *Eth* gene cluster has been discovered in gram-positive strains, such as *R. ruber* IFP 2001, *Rhodococcus* sp. T3-1 and gram-negative strains, such as *A. tertiaricarbonis* L108, suggesting that this gene cluster was highly conserved and has a complex transfer history.

EthABCD and EthABD, but not EthABC, showed activity against chloroacetanilide herbicides. A similar phenomenon was found in *Rhodococcus* sp. T3-1, which deduced that EthD is a ferredoxin. EthC was predicted to be a ferredoxin, and we inferred that EthABC was a possible a system for the degradation of other compounds. Interestingly, neither chloroacetanilide herbicides nor fuel oxygenates could be recognized as the natural inducers of the Eth system. The natural substrates should be the chemicals with *O*-alkyl or *N*-alkyl and has been present in the nature all the time. The identification of this novel function of the *Eth* gene cluster can be exploited for the biodegradation of soil contaminated by gasoline ethers and chloroacetanilide herbicides.

Conclusions

One strain named *Rhodococcus* sp. B2 was isolated from herbicide contaminated field, the optimum conditions (culture time, 5 d; initial substrate concentration, 50 mg/L; pH, 6.98; temperature, 30.1°C) for degradation pretilachlor efficiency (86.1%) were acquired from a response surface methodology. One novel product were detected during the biodegradation and identified. An absent DNA fragment containing the functional gene cluster (called *EthRABCD*$_{B2}$, first reported to be responsible for the *O*-dealkylation of the fuel oxygenate chemicals) was identified by genome comparison between strain B2 and its mutant strain TB2. The recombinant enzyme EthABD$_{B2}$ could degrade chloroacetanilide herbicides and catalyze the *N*-dealkoxymethylation activity toward alachlor, acetochlor, butachlor and propisochlor and *O*-dealkylation activity toward pretilachlor. The broad substrate spectrum of EthABD$_{B2}$ indicate the potential for bioremediation of environments contaminated by gasoline ethers and chloroacetanilide herbicides.

Declarations

Authors' contributions

HL and LS conceived and designed the experiments. HL, ML, AL and LS performed the experiments and analyzed the data. HL and GZ wrote the paper. All authors read and approved the final manuscript

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Availability of data and materials

All materials described within this manuscript, and engineered strains are available on request.

Ethics approval and consent to participate

Our manuscript does not report data collected from humans or animals.

Consent for publication

Our manuscript does not contain any individual person's data in any form.

Competing interests

The authors declare that they have no competing interests

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approved for publication. Approval does not signify that the contents necessarily reflect the views or policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. 1. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 1999, **443:**183-221.

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Figures

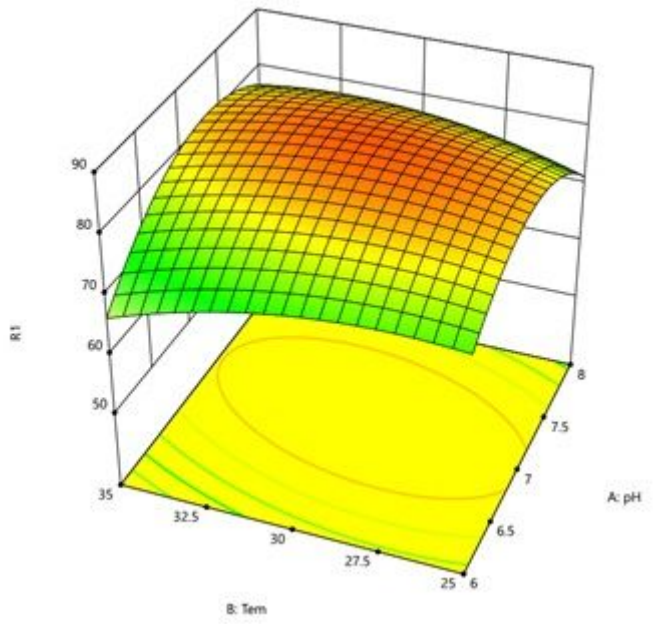


Figure 1

Response surface plot for the interaction effect of pH and temperature on the biodegradation activity of strain B2.

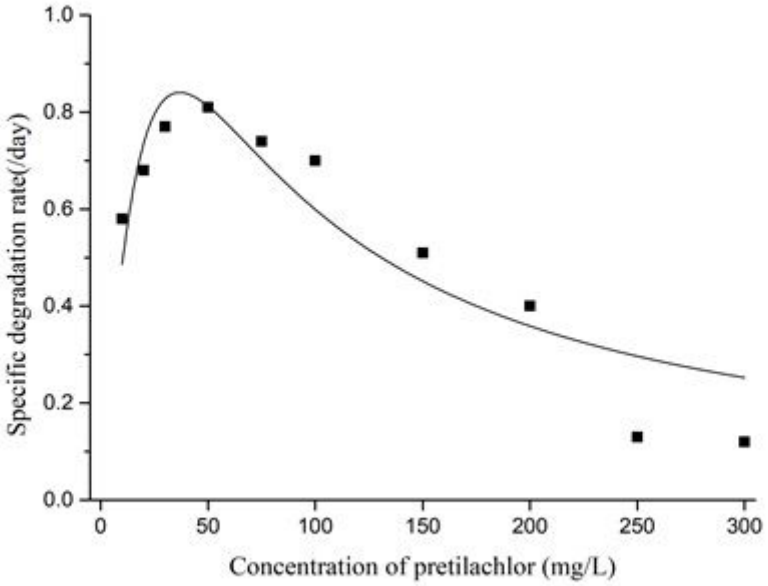


Figure 2

Relationship between initial pretilachlor concentration and specific degradation rate by strain B2

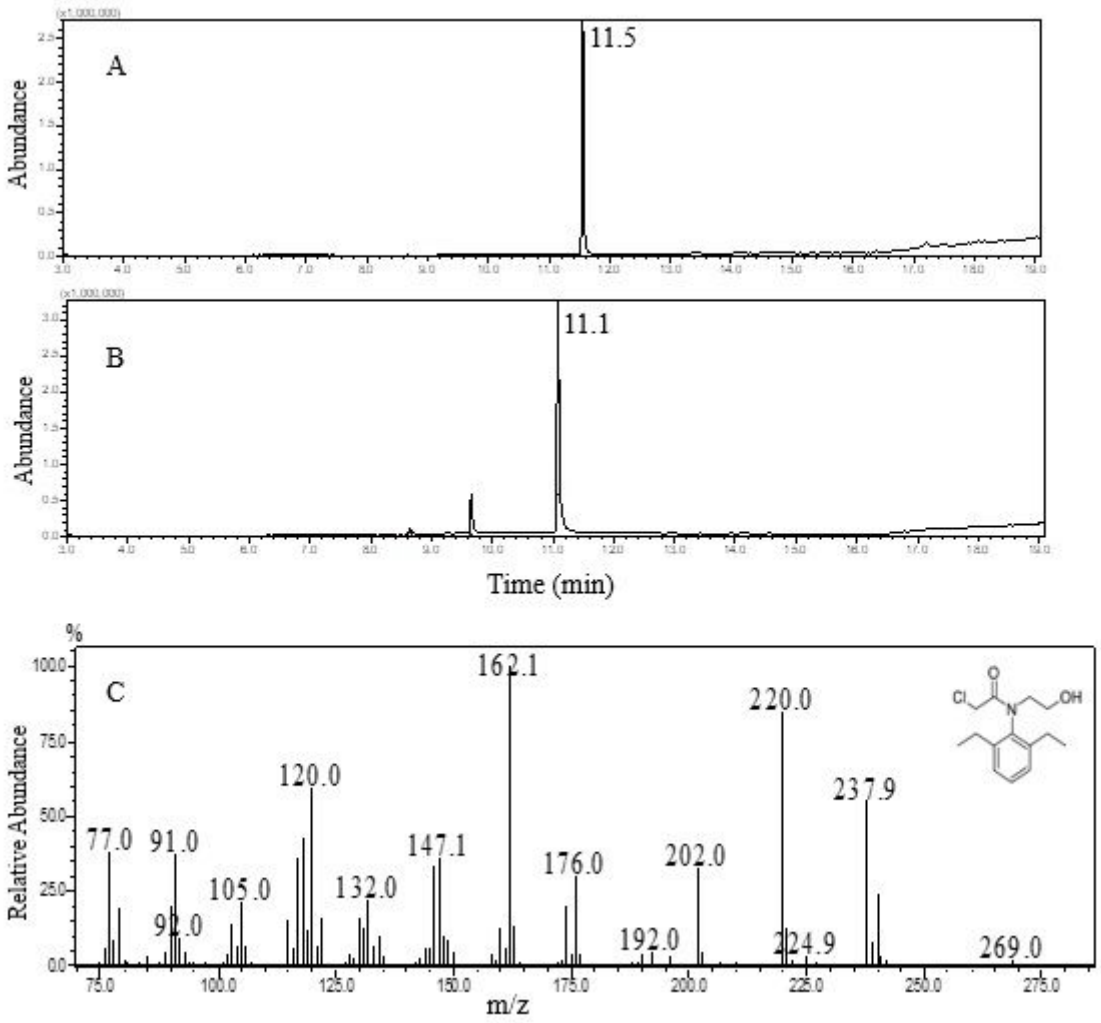
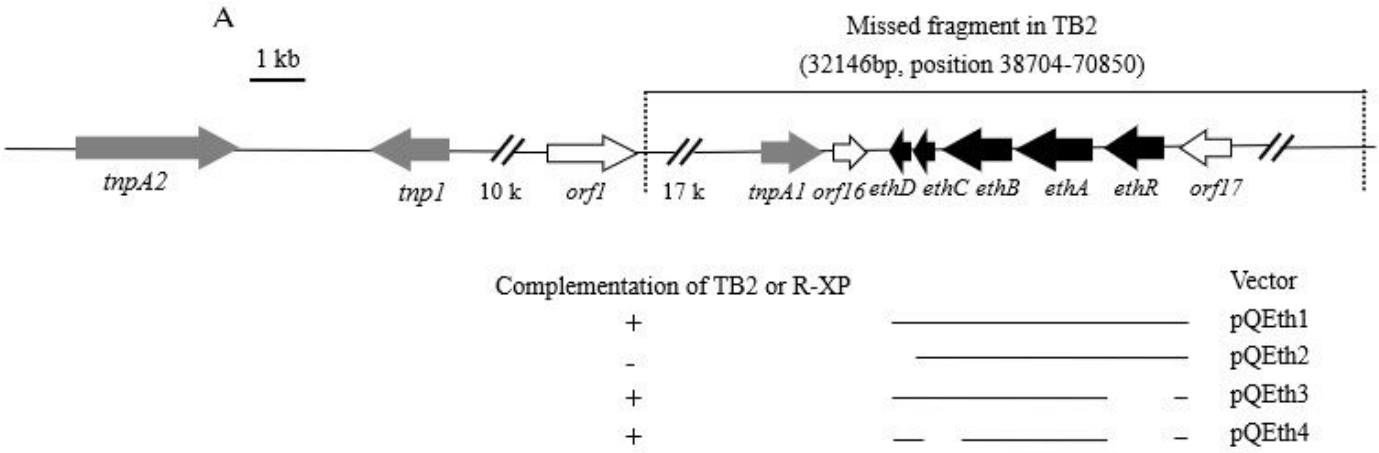


Figure 3

GC-MS profile of the metabolite produced during pretilachlor degradation by strain B2. A and B, the GC spectra of the pretilachlor extract obtained from control sample and whole cell transformation of strain B2, respectively; C, mass spectra for the peak at 11.1 min.

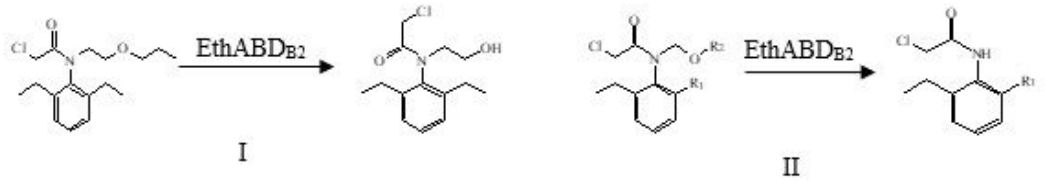


B

```
B2      70 LGDWQTFSSAQGNTMSTALNEATKESSGSKEGADHLRMRKLMLQDLGPKA 110
T3-1    70 LGDWQTFSSAQGNTMSTALNEATKDFSGSKEGADHLHMRKLMMQDLGPKA 110

B2     191 DKLQSWALENITPETAREGSVAASTWEAVERGDVTDVQAVAILAGYVTAG 240
T3-1   191 DKLYSWALENITPETAREGSVAASMWEAVERGDATDVEAVSILAGYITAG 240
```

C



I II

Note: I, pretilachlor

II, Alachlor, R1= CH_3CH_2-, R2= CH_3-; Acetochlor, R1= CH_3-, R2=CH_3CH_2-;

Butachlor, R1= CH_3CH_2-, R2=$CH_3CH_2CH_2CH_2$-: Propisochlor, R1= CH_3-,R2=

$(CH_3)_2CH$-.Strain B2 cannot further metabolize three metabolites.

Figure 4

Physical map of the putative transposable element containing EthRABCD gene cluster in Rhodococcus sp. B2 and degradation pathway of chloroacetanilide herbicides. (A) Arrows indicate the sizes, locations, and directions of transcription of the ORFs. Complementation of the EthRABCDB2-disrupted mutants with different regions is illustrated below the physical map. (B) The difference of the amino acid sequences of

EthB from strain B2 and T3-1. Highlighted characters represent the different residues. (C) Proposed degradation pathway of pretilachlor, alachlor, acetochlor, propisochlor and butachlor by EthABDB2.

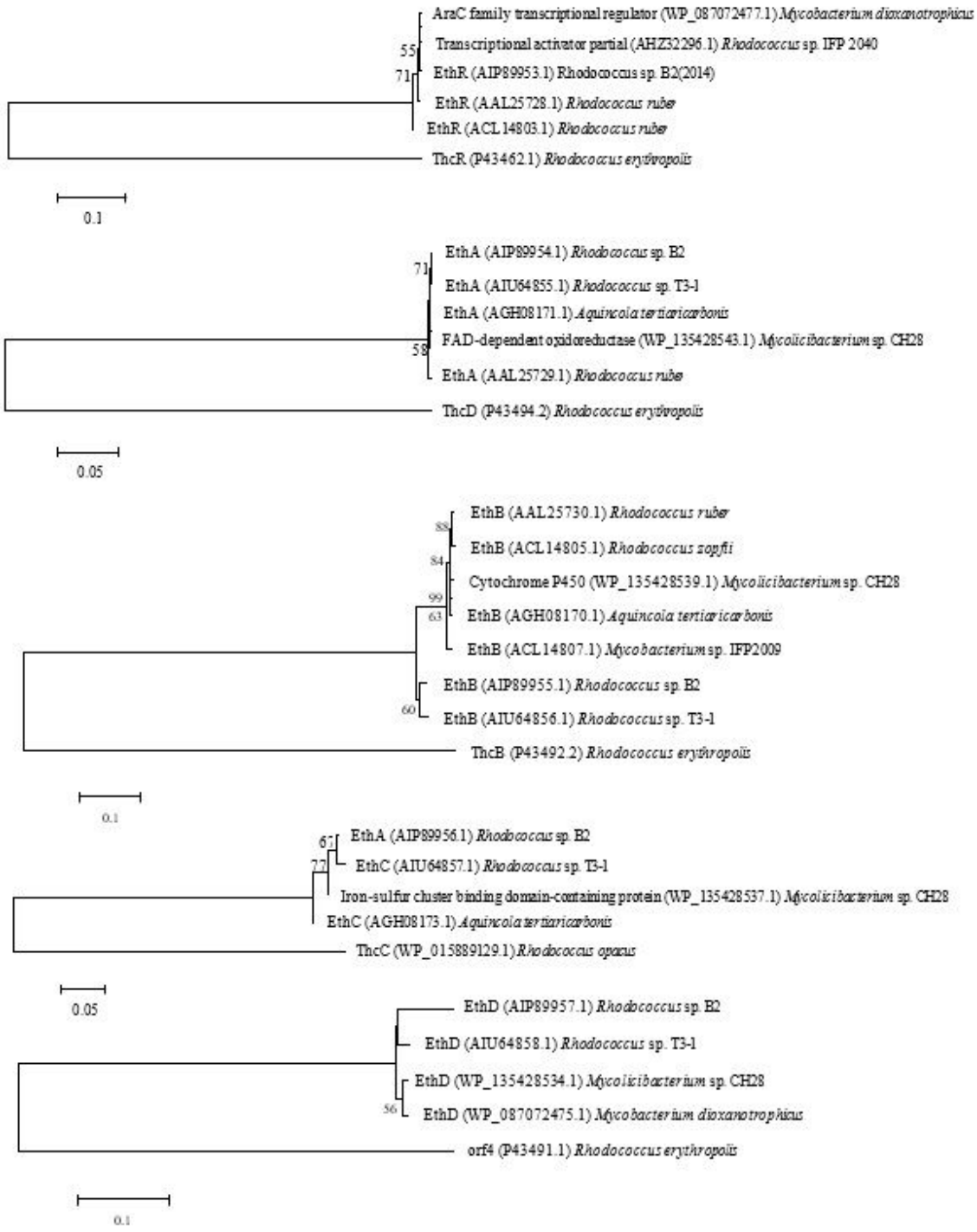


Figure 5

Phylogenetic tree of EthRABCDB2 and related proteins constructed by the neighbor-joining method. The branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. Name of the strains, proteins and their GenBank numbers are displayed in the phylogenetic tree. Thc gene

cluster was used as an out-group The scale bar indicates amino acid residue substitutions per amino acid

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