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ATP-binding cassette transporter enhances tolerance to DDT in *Tetrahymena*

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The reuse of dichlorodiphenyltrichloroethane (DDT) as an indoor residual spray was permitted by the World Health Organization in 2007, and approximately 14 countries still use DDT to control disease vectors. The extensive exposure of insects to DDT has resulted in the emergence of DDT resistance, especially in mosquitoes, and the mechanism for this resistance in mosquitoes has been widely reported. Spraying can also introduce DDT directly into surface water, and DDT can subsequently accumulate in microorganisms, but the mechanism for the resistance to DDT degradation in microorganisms is unclear. Using whole-genome microarray analysis, we detected an *abcb15* gene that was up-regulated in a specific manner by DDT treatment in *T. thermophile*. The deduced ABCB15 peptide sequence had two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) to form the structure TMD-NBD-TMD-NBD, and each NBD contained three conserved motifs: Walker-A, C-loop, and Walker-B, which indicated the *T. thermophila* abcb15 was a typical ABC transporter gene. The expression of ABCB15 fused with a C-terminal green fluorescent protein was found to be on the periphery of the cell, suggesting that ABCB15 was a membrane pump protein. In addition, cells with abcb15 partially knocked down (abcb15-KD) grew slower than wild-type cells in the presence of 256 mg L⁻¹ DDT, indicating the tolerance of abcb15-KD strain to DDT exposure was decreased. Thus, we suggest that in *Tetrahymena*, the membrane pump protein encoded by ABCT gene abcb15 can enhance the tolerance to DDT and protect cells from this exogenous toxin by efficiently pumping it to the extracellular space.

Tetrahymena, DDT, ATP-binding cassette transporter, tolerance

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Dichlorodiphenyltrichloroethane (DDT), an organochlorine pesticide, was used to control insects that carried diseases (e.g., malaria and typhus) and destroyed agricultural crops worldwide from the 1940s to the 1960s. With high lipophilicity and a long half-life, DDT sprayed into the environment and accumulated in organisms, creates significant threats to wildlife and human health. For example, DDT has been implicated in a wide range of adverse effects on humans, such as liver cancer, pancreatic cancer, breast cancer, diabetes, pregnancy loss, poor sperm quality, and neurodevelopmental toxicity in children [1]. Thus, DDT was banned in many countries in the 1970s and was one of the 12 recognized persistent organic pollutants at the Stockholm Convention in 2001. However, currently DDT remains to be a significant threat to the health of wildlife and humans for two reasons: first, the prevalence of malaria markedly in-

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creased in some African countries after the banning of DDT. Therefore, the World Health Organization suggested the reuse of this pesticide in some areas in September 2006 [2]. Second, DDT concentrations are still growing in the northern hemisphere through long-range oceanic and atmospheric transport [3].

The extensive exposure of insects to DDT has resulted in the emergence of DDT resistance, especially in the mosquito. Currently, it is thought that the DDT resistance in the mosquito is mediated through two main mechanisms: first, the DDT resistance could be caused by the mutations in DDT target sites, such as the voltage-gated sodium channel, insensitive acetylcholinesterases, and the neurotransmitter γ -aminobutyric acid, which lead to low DDT binding capacity [4]. Second, the DDT resistance in mosquito could be due to alterations in the activities or levels of detoxification proteins, such as carboxylesterases, cytochrome P450 monooxygenases, and glutathione S-transferases. Since sprayed DDT can be released onto surface water directly or indirectly through dry and wet deposition from the atmosphere [5], microorganisms can also adapt to the presence of DDT under its long-term exposure, thus allowing its accumulation and serial transfer to higher levels in the food chain [6]. Therefore, analysis of the metabolism of DDT in aquatic microorganism (e.g., protozoa and bacteria) is very important.

Tetrahymena is a free-living ciliated protozoan distributed in freshwater environments around the world, and it has the conserved genes and basic metabolic pathways of a metazoan [7]. Studies in *Tetrahymena* have led to numerous scientific breakthroughs, and a number of molecular genetic technologies and genomic resources have been developed [8–10]. Using a microarray technique, we recently identified genes that were differentially expressed in *Tetrahymena thermophila* after exposure to DDT, and we found that an ATP-binding cassette transporter (ABCT) gene (*abcb15*) belonging to the B family may be associated with the robust DDT resistance of *T. thermophile* [11]. In the present study, the function of *abcb15* was analyzed to elucidate the mechanism of tolerance to DDT in *T. thermophile*.

1 Materials and methods

1.1 Cell culture and toxicity experiments

Two inbred strains of *T. thermophila* (SB210 and CU428) were kindly provided by Dr. Eduardo Orias (University of California, USA). Cells were grown as axenic cultures in super proteose peptone medium. The stock solutions of DDT and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) were described in our previous studies [11,12]. For toxicity analyses of growing cells, cells in the mid-logarithmic growth phase ($\sim 3 \times 10^5$ cells mL⁻¹) were inoculated into the

treatment medium of 4 mg L^{-1} DDT and 1 μ g L^{-1} TCDD for 24 h.

1.2 RNA extraction, cDNA synthesis, mRNA purification, SMART cDNA library construction, and 5' and 3' RACE cloning

Cells were collected after exposure to the medium for toxicity studies, and their total RNA was extracted using an RNeasy Protect Cell Mini Kit (Qiagen, Germany). RNA quality was monitored using spectrophotometry (Malcom, Japan) and electrophoresis. Total RNA was digested with DNase (Promega, USA) and reverse transcribed into double-stranded cDNA using M-MLV reverse transcriptase RNase H⁺ (TOYOBO, Japan) [13].

The cultures (5 mL) were incubated in the presence of 4 mg L^{-1} DDT for 24 h. Total RNA was then extracted and purified using PolyAtract mRNA isolation systems (Promega), and the purified mRNA was used to construct a SMART cDNA library (Clontech, USA) [14].

Using the SMART cDNA library as a template, 5'- and 3'-untranslated regions (UTRs) (5' and 3' rapid amplification of cDNA ends, RACE) of the abcb15 gene were PCR amplified using the primers 5' UT f (GCAGTGGTATCA-ACGCAGAGTG) and abcb15_r (GTACCCACTGAAAG-GATAACAAACTC), and 3' UT_r (GGCGGCCGACAT-GTTTTTTT) and abcb15_f (AGAATACAAATGTGAAG-TAAGCCTAAG), respectively [15]. The reactions were performed in a 25-µL mixture, containing 2.5 µL of 10× buffer, 0.5 μ L of 10 mmol L⁻¹ dNTP (Fermentas, Lithuania), 0.4 µmol L⁻¹ primers, 1 unit Taq Polymerase (BD advantage, USA), and 0.5 µL of template. The PCR cycling conditions were as follows: denaturation at 94°C for 5 min; amplification for 35 cycles of 30 s at 94°C, 30 s at 62°C, and 60 s at 72°C; extension at 72°C for 10 min. The PCR products were analyzed using electrophoresis with agarose gels and purified with a GlassMilk kit (Biostar, China). The purified products were ligated into pGEMT-Vectors (Promega) and transformed into Escherichia coli DH-5a cells. Positively screened clones were sequenced with an ABI model 377 Stretch automated DNA sequencer (PE Applied Biosystems, USA).

1.3 Subcellular localization of ABCB15

1.3.1 Predictions using biosoftware

The subcellular localization of ABCB15 was predicted from its derived protein using WoLF PSORT (http://wolfpsort. org/) [16] and TargetP 1.1 (http://www.cbs.dtu.dk/services /TargetP/) [17] with default parameters.

1.3.2 In situ localization

For in situ localization, ABCB15 was tagged with green

fluorescent protein (GFP) *in vivo*. Using cDNA (prepared in 1.2) as a template, the *abcb15* gene was amplified with PCR using primers TOPO_ABCB15_f (CACCTAAAAAATG-GAAGAAAAGTCATTATA) and TOPO_ABCB15_r (GC-AATTCTAATTTTAACTTTCCTGC), and the product was cloned into pENTRTM/D-TOPO (Invitrogen, USA) to form the plasmid pENTR_ABCB15. After the LR recombination reaction, pENTR_CYP5013C2 was introduced into pICC_gtw vectors (kindly provided by Professor Douglas L. Chalker, University of Washington, USA) to form the recombinant plasmid PICC_ABCB15 by replacing the *ccdB* coding region [18] (Figure 1). The reaction was performed in a 5-µL mix, containing 3 µL of pENTR_ ABCB15 plasmid, 1 µL of PICC_gtw, and 1 µL of Gateway LR Clonase II Enzyme Mix (Invitrogen).

The recombinant plasmid was introduced into Tetrahymena by conjugative electrotransformation [19]. Transformants were selected using serial transfers in 1× super proteose peptone medium supplemented with increasing amounts (from 100 to 1000 mg L⁻¹) of paromomycin (Sigma, USA) and then verified with whole cell PCR using the primers TOPO_ABCB15_f (CACCTAAAAAATGGAAG-AAAAGTCATTATA) and GFP_r (ATGTGGCTCCTT-ATCTTACCCTA) that flanked the *abcb15* and *gfp* genes. After adding 5 mg L^{-1} CdCl₂ to 2 mL of transformed cells $(3 \times 10^5 \text{ cells mL}^{-1})$, the *mtt1* promoter was induced. Three hours later, the cells were concentrated to a final concentration of 1 mgL^{-1} , fixed in 2% paraformaldehyde, and stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). An Axioplan 2 Imaging (Zeiss, Germany) microscope was used to detect the localization of the GFP fusion protein.

1.4 Construction of the ABCB15 knockdown strain

Using total genomic DNA as template, the 5' and 3' flanking regions were amplified with PCR using primers abcb15_5f (TTATTAAAGTTATGATTGTCCTGCTA) and abcb15neo 5r (GTGTATTTAAATTAAAGGAGTTATT-CTCTGAAACCCATTAGTCAAAGTAG, overlapping sequences are underlined), and abcb15neo_3f (CAAATT-TTTACTGGAAAAATGCATGGAAAATGGCAACATAG TCG, overlapping sequences are underlined) and abcb15 3r (ACTTAAAACCAAGCTCAAAAGACAG), respectively. The neo4 cassette DNA was obtained with PCR amplification using the plasmid pNeo4 (kindly provided by Professor Mochizuki, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Austria) as a template and primers neo4_f (GAATAACTCCTTTAATTTAAA-TACAC) and neo4_r (GCATTTTTCCAGTAAAAAT-TTG) [20]. The 5' flanking region, neo4 cassette, and 3' flanking region were linked through overlapping PCR using the primers abcb15nest_f (AAAGTTATGATTGTCC-TGCTATTA) and abcb15nest_r (TAAAACCAAGCTC-AAAAGACAG) to form the knockdown target construct. The PCR cycling conditions were as follows: denaturation for 10 min at 94°C; amplification for 35 cycles of 30 s at 94°C, 1 min at 50°C, and 5 min at 72°C; extension for 10 min at 72°C.

The PCR product was analyzed using electrophoresis with agarose gels and purified with a GlassMilk kit (Biostar). The purified product was transformed into starved B2086 cells using a Bio-Rad biolistic gun [21]. Transformants were selected with electrotransformation. During the selection process, endogenous macronuclear *abcb15*



Figure 1 The target gene *abcb15* is placed into the PICC_gtw vector using the Gateway homologous recombination system.

alleles were partially replaced with the *abcb15* knockdown alleles using phenotypic assortment (Figure 2). The knockdown strain of *abcb15* was named *abcb15*-KD strain.

Cells with *abcb15*-KD and wild-type strains in mid-logarithmic growth phase ($\sim 3 \times 10^5$ cells mL⁻¹) were inoculated into the treatment medium of 4 mg L⁻¹ DDT for 24 h, and total RNA was then extracted and subsequently reverse-transcribed into cDNA. The presence of the *abcb15* knockdown construct was detected with real-time PCR using the template of the above cDNA and the primers abcb15_f and abcb1_r; the 18s rRNA gene, as an endogenous control, was amplified with primers of 18s_f (CCTGGGAAGGTACGGGTAAT) and 18s_r (AAGGT-TCACCAGACCATTCG). Real-time PCR reactions and the calculations of the relative expression levels of *abcb15* were performed as described in our previous study [13].

1.5 Growth models in T. thermophila

Cells in the mid-logarithmic growth phase ($\sim 3 \times 10^5$ cells mL⁻¹) of the B2086 or *abcb15*-KD strains were inoculated into 20 mL of fresh super proteose peptone medium, and the final density was $\sim 1 \times 10^4$ cells mL⁻¹, with 256 mg L⁻¹ DDT and 0.05 µg mL⁻¹ CdCl₂. The samples were collected at 3, 4.5, 8, 10.5, and 14.5 h, and the cells were counted (Beckman Coulter, USA). To adjust to the new environment after inoculation, cells underwent a lag phase of 0–4.5 h. During this transition, the first-order growth model was postulated as $N_B=N_A \times \exp(k \times t)$, where N_B is cell density in stage B, N_A is cell density in stage A, k is the growth rate parameter (h⁻¹), and t is a specific time. Once activated, cells increased by binary division, with the corresponding growth model as $N_C=N_B \times 2^{t/G}$, where G is generation time (h) and N_C is cell density in stage C [22].

2 Results

2.1 The characteristics of T. thermophila abcb15 gene

Our sequencing results showed that the full-length of

abcb15 gene in *T. thermophila* was 4937 bp, containing 9 exons and 8 introns (Figure 3). The mRNA length was 4157 bp, with an open reading frame (ORF) of 3957 nucleotides for 1318 aa. There were 49 nucleotides in the 5'-UTR and 151 nucleotides in the 3'-UTR, including a conserved consensus polyadenylation signal element AATAAA [23]. The deduced ABCB15 peptide sequence had two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) to form the structure TMD-NBD-TMD-NBD [24]. In addition, each NBD contained three conserved motifs: Walker-A, C-loop, and Walker-B (Figure 3). All these results indicate that *abcb15* in *T. thermophila* is a typical ABC transporter gene.

2.2 The ABC transporter gene *abcb15* is up-regulated by DDT in *T. thermophila*

It has been shown by a microarray study that 75 genes are up-regulated more than two fold by the DDT treatment, including ABC transporter genes related to DDT detoxification [11]. When we carefully examined three physiological/developmental stages (growth, starvation, and conjugation) and different (DDT or TCDD) treatments, we found that only DDT treatment resulted in a remarkably increase in the expression of *abcb15* (Figure 4). Combined with the microarray data at different life cycle stages [8], we conclude that *abcb15* (TTHERM_00240450) was up-regulated in a specific manner by DDT treatment in *T. thermophile*.

2.3 ABCB15 protein is localized on the cell membrane in *T. thermophila*

Based on the WoLF PSORT and TargetP 1.1 software computational analyses, ABCB15 was localized on the cell membrane. This localization is further confirmed by fluorescent studies. When *abcb15* was fused with C-terminal GFP, the expression of this ABC transporter chimeric protein under CdCl₂-inducing conditions was found to be on the periphery of the cell (Figure 5).



Figure 2 Schematic representation of the targeted *abcb15* knockdown construct and the strategy for *in vivo* replacement.

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$^{-1}$	T Q S R L A S Q S S Q T L N T E Q E F L	
60	AATGAAGCAGGAAAGTTAAAAATTGAATTGCTAAAAATTGATTTAGCAATTTTAAAACTT M K Q E S Q N Q N C Q K L I Q Q F Q N L	2520
120	GAATAATATTAAAGAAGAAGATGATGATGAATGAAGAAGCCTAAAATAAAGCAAT	2580
180	AAGAAGTTCTTTTAGCAGCTAAACTTAAAAACACCCCCAAAACAAATAGAGATAGTAGAATA	2640
240	AAATATTATTGATTTATAGAATATTTAGAATACAAATGTGAAGTAAGCCTAAGAAAAGAC	2700
300	AAACGATTAAGGATTATAGAAAAGACTTTTTTTTTTTTATAATGAGAATAAACCATCAATTA	2760
360	TGTGCTTGGTTTTCTTTTTGCTATTGGCAATGGAGTTTGTTATCCTTTCAGTGGGTACGT	2820
420	V L G F L F A I G N G V C Y P F S G Y V	
120	GCTTGGTAAAATCAGTGATGTGTTGCTTGATAGAACAAGAAGTGATTTTAGAGAACAAAG	2880
480	LGKISDVLLDRTRSDFREQS	
	CAATTTGTAAAGTCTTTATTTTTGATTTTGGGTTTAGCATAACTCTTGACATGTACTTT	2940
540	N L Q S L Y F L I L G L A Q L L T C T F	
	CTAATTTTACTTCTTCGAGAGTAGCTGAATAACTTACATTCAAGTTACGTAAAGATCT	3000
600	Q F Y F F S R V A E Q L T F K L R K D L	
	CTTTTAAAAATATTTTGAAAATGCCTATTTCTTGGTTTGATCACCCTCATAATACTCCTGG	3060
660	F Q K Y L K M P I S W F D H P H N I P G	2100
-		3120
720	<u>S L I Q R L S I D C Q A V N N M I S S I</u>	2190
790		5160
180	aattttattcaataaagCTTTCTAATGTAAGCTCTTTGGTCAGTGCTTTAGCTTTGGCTT	3240
840	LSNVSSLVSALALAF	
010	TTTCAGCAGATTGGAGAACAACCCTAGTTGGTTTAAGTCTTATGCCTTTGATGGTTTTGA	3300
900	<u>S A D W R T T L V G L S L M P L M V L S</u>	
	GTTAGGCTTGGTATATGAGCAGAATGGAAGGCTTTGGAGAAAAAACTGATGCCGCATTTA	3360
960	Q A W Y M S R M E G F G E K T D A A F R	
	GGGATTCCACCAATATGATAAATGAAGCTGCTTGTAATATAAGAACCGTTACTAGTTTTG	3420
1020	D S T N M I N E A A C N I R T V T S F G	
1080	GAAACAATTAGCAATTAGTCCAAAATTTTACATAAATTTTAGATAAAAATATCAAAGAGA	3480
1140	N N Q Q L Y Q N F T Q I L D K N I K E I TAAAAAAATCTGCTCTGGAAGCAGGTCTTGCTATAGCATGCACAAATTTCATTTTGTTTG	3540
1200	K K S A L E A G L A I A C T N F I L F A	
	${\tt g} {\tt t} {\tt a} {\tt t} {\tt t} {\tt t} {\tt t} {\tt t} {\tt a} {\tt t} {\tt a} {\tt a} {\tt a} {\tt t} {\tt a} {\tt a} {\tt t} {\tt t$	3600
1260	<u> </u>	
	TCTATGGTACAATCTTTTATGCAGGATCTACTTTTCATAGGGATTATGATCTTTCGATTG	3660
1320	YGTIFYAGSTFHRDYDLSIV	
	${\tt TTGATATGTTTATTTCTATTTAATGCCTAATGTTTGCTGCTATTGGTATAGgtaatatat$	3720
1380	<u>DMFISIQCLMFAAIG</u> IG	
	aagat catttttagag tat ctattaagaattttagat acattttaatttttatgat catttaattttagagat at taga at ta	3780
1440	gaaattatettitacaaatattaetaattateaaaagGCTCAAACAGTCATTATTTGGGA	3840
1500	5 N 5 H Y L G	3900
1560		3300
1000		3960
1620	ENQLNILNFDNQDIHGEIQF	0000
1020	AAGAACGTTACTTTTAAATACCCACAAAGAGATTAGATT	4020
1680	K N V T F K Y P Q R D Q I I L K D V S F	
	ACCATTOCTOCACCATACAAACTTCCTTTCCTTCCACCACCACCAAATCTTCT	4090

T I P A G Q K V A F V <mark>G P S G A G K S</mark> S

ATTATCCAATTGATATAAAGATTTTATGATAACTACTAGGGAGAAATTTTATTAGATGGT

IQLIQRFYDNYQGEILLDG GTTGATATAAAGAACTATGATTTGTTGAAATACCGTTCTAAATTTGGAGTTGTATCGTAA

V D I K N Y D L L K Y R S K F G V V S Q GAGCCAACACTTTTTACGGGAACTATCAAAGAAAATATAATTTACAATACTGAAAATGTA

<u>E P T L F T G T I K E N I I Y N T E N V</u> AATGAATAATAAATAGAATCCATAACTAAATAAGTAAATGCCTATGATTTCATTACTAAT

<u>NEQQIESITKQVNAYDFITN</u>

aaagATGGCGTTAATGGATTTGATAGATAAGTGGGTTTGAAAGGAAATTAATTAAGTGGT

G Q K Q R I A I C R A M I K Q P K I M L CTAGATGAAGCAACAAGTGCTTTAGATTCACAAAATGAAAAAATAGTCTAAGAATCCCTA

L D E A T S A L D S Q N E K I V Q E S I AACGAAGCAATGAAACAAAAAACTAGTATTTGTGTGGCCCACAGAATATCTACTATTAAA

N E A M K Q K T S I C V A H R I S T I K

GATTCAGATATGATCTATGTGATGGAAAATGGCAACATAGTCGAGCAAGGAAAATATGAT

D S D M I Y V M E N G N I V E Q G K Y D

 ${\tt AATGAAAGAATGCTATAAAAAAATTATTGACATAACAA\underline{{\tt AATTAA}}{\tt TATTTTAAATTTCTTG}$

TAAAATATATTTTATACATATCAATCTATTATGATTTTAATTTCAAGTTATCTTTTAATA

<u>Q L M N L K N</u> N F Y K L Q L G S S Q *

ΤΑΑΑΤΤΤΑΑΤΤΑΑΤΑΤΑΤΑΤΤΤΑΑΑΑΑΤΤΟ

G V N G F D R Q V G L K G N Q L S G GGATAGAAATAAAGAATTGCTATTTGTAGAGCTATGATTAAATAACCAAAAATTATGCTA 4140

4200

4260

4320

4380

4440

4500

4560

4620

4680

4740

4800

4860

4888

TAATCATAATTAAGGAAGAAGCTATAATTTTTCTGATGTATTAACTGCATTTTTAGCAAT	1260
N H N Q G R S Y N F S D V L T A F L A I	
TATGATGAGCAGCTTCGAATTAGGCTAAGCTATGAACTCTATTAAGGCTTTCACTCAGGC	1320
M <u>MSSFEL</u> GQAMNSIKAFTQA	
${\tt TCGTCAAGCTGGATTTAACATGTTTTAAgtaaataaattagccagttaattgattttta$	1380
R Q A G F N M F Q	
a attagatgttta a attattgctta ca atta att	1440
ccattcaatatttaaaattttttcaataattaaataaaaaa	1500
I L N R K S K	
AAGTAGATTTAAATGAGAACGGTATTGACTTAACTAAAAAATAAAT	1560
V D L N E N G I D L T K K Q I N G E I K	
AATTTGAAAATGTAGATTTCTCTTACCCTACTCATCTTGATACAAAGATTCTGAAAAAATT	1620
FENVDFSYPTHLDTK <u>ILKNL</u>	
TAAATATCTCAATATAACCTCACAAAAAAAAAAACAGCTTTTGTAGGTGAATCAGGAAGgtatt	1680
N I S I Q P H K K T A F V G E S G S	
ttacttaatcctcttctaaaataggatgtatttaaattttatttttttcaaaattagTGG	1740
G	
AAAATCAACTATAGTTTAGCTCATAGAGAGATTTTATGACCCATAATTTGGCAATATTTA	1800
K S T I V Q L I E R F Y D P Q F G N I Y	
TTTGGATGGTGTCAATCTAAAGGACTTTAAATTAACTTCTCTTCGTTAATCTATCGGATA	1860
L D G V N L K D F K L T S L R Q S I G Y	
TGTAGGATAAGAACCCGTTCTTTTGCTACTACTATTAGAGAAAAATCTATTATATGGTAA	1920
V G Q E P V L F A T T I R E N L L Y G K	
AAGAGATGCAACTGAAGAACAAATGATTGAAGCTCTCAAATAGGCAAACGCTTGGTAATT	1980
R D A T E E Q M I E A L K Q A N A W Q E	1000
TATTGAAAAATTAGAGAAAAGGTTTAGATACTTATGTTGGTACATCTGGTGCTTAATTCTC	2040
I F K I F K G I D T Y V G T S G A O F S	2010
AGGAGGCTAAAAGTAGCGTATTAGCATGCAAGAGCTATTTTAAAAAATCCAAAAAATCC	2100
G G O K O R I S I A R A I I K N P K I I	2100
TTTCCTTGATGAAGCTACTACTACTACTAGACCCTAAAAATGAAGCTCAAAATTTAAAGCAC	2160
	2100
	2220
	2220
	2220
	2200
TCATCATTATTAAAAAAACAATCCTCCTTATTTTAAAATTCTACAAAAAA	2240
	2340
	9400
	∠400
E K E Q E K Q A A H N N K T L C L L G E	0.100
AACATAATCAAGATTAGCATCTTAGTCTTCTTAAACTCTAAATACAGAACAAGAATTTTT	2460

GAATAGCTTAAACATATATAAACGATTAAACTGAAAGCATATATAAAAA ATGGAAGAAAAGTCATTATAAAACTTAGAAAAACCCACTTAAAAATTATGAAGATTAAGAA M E E K S L Q N L E K P T Q N Y E D Q E GTGGCAGATGGTAAAATACCATTTTGGCATCTATTTAAACTAGCATCGAAAAACTGATATC VADGKIPFWHLFKLASKTDI ATTTTAGTTGTTTTAGGAAGCTTAGCTTCTGTTATAAATGGTTGTCTGTAGCCACTTTTT I L V V L G S L A S V I N G C L Q P L F GGTCTACTATTTGGTGAAATGGCATAAAAATTTAGCCCTGGATATAGCGCCGATGCAGTT GLLFGEMAQKFSPGYSADAV GTGGATAACTGCAGGACTATTGCATTATGGTTTGTTGGAATTGGAGCAAgtaatttttt V D N C R T I A L W F V G I G A I ttagttgataattttgatctcatcataatggtagagataagaggataaaatatgcctacatcttttaataattttgttaagtatatatcaaactttactatttttatattaaaagTATCATTTATACTTTCAATCTTTATGATGTACTTTTGGATCAGCGTGGGTTAGAGATAGGCAATA I L S I F M M Y F W I S V G Q R Q A I AAATTTAGATTAGAGTACTTTAAGTCTTTGCTCAAATAAGAAGTAGGTTATTTTGATTAA K F R L E Y F K S L L K Q E V G Y F D Q ATTTAGGCAAATGAGCTTTCATCTAAAGTATCTACTGAATGCTTCAAAATATAGTCAGCA

<u>SSKVSTECFKIQS</u>

LQNMMF

TTGGGAGAAAAAACATGCATCTTTATTTATTCATTGAGTATGTTTATTGGTAGTCTTATA LGEKTCIFIYSLSMFIGSLI ATAGCCTTTATTAGGGGATGGCAAATTGCTCTTGTGGCAATTGCTGTAACACCACTTATA I A F I R G W Q I A L V A I A V T P L I GCTTCAGCAGGCTACTTTGACTAATGGGTTTCAGAAGGAATTGTAAAAAAAGACAAGCTCA S A G Y F D Q W V S E G I V K K T S S GCCTATTCATCTGCAGGAGGTATATCTGAGTAGGCCATAAGCGCTATTAGAACAGTAAAA Y S S A G G I S E Q A I S A I R T V K GGTTTAAATGGTTAAGATTTTGAATAAAATAAATATAAAGCATGATCAAGAAAGCATTC G Q D F E Q N K Y Q S M I K K A F TAAGTCTCTTTAAAGTTTTCTATCTATCAAGGTGTTGGATTAGgtaaatcattaatttat

ttatcttactcaataaataataaaaaatatcacacagaaaaattgtatcaaataggttattagtatttatttgatgtaatagtcaataattgaactatttttatatttaccaaaagattt ${\tt ttaaactaattaattttgaataattctctttacttaaaaag{\tt GACTTTAAAATATGATGTT}$

TTTCTTTGATTTTGCTTTGACTTTTTGGGTAGGATCTAAATTTATTGAAGACGAAGTTTA FFDFALTFWVGSKFIEDEVY

Q V S L K F S I Y E G V G L G

QANEL

Figure 3 Full-length and deduced amino acid sequences of *Tetrahymena* gene abcb15. The transmembrane and nucleotide-binding domains are marked with straight and wavy lines, respectively. Lowercase letters represent intron regions. Walker-A, C-loop, and Walker-B motifs are labeled with boxes. The possible transcription termination signal in the 3' untranslated region is also labeled with a straight line.

YSKN



Figure 4 Comparison of the expression levels of *abcb15* during three physiological/developmental stages (growth, starvation, and conjugation) and during treatment with DDT and TCDD. L indicates growing cells with $\sim 1 \times 10^5$ cells mL⁻¹. For starvation, $\sim 2 \times 10^5$ cells mL⁻¹ cells were collected at 0, 9, and 24 h (referred to as S0, S9, and S24, respectively). For conjugation, equal volumes of B2086 and CU428 cells were mixed, and samples were collected at 0, 10, and 18 h after mixing (referred to as C0, C10, and C18, respectively) (data from TGED http://tged.ihb.ac.cn/).



Figure 5 The subcellular localization of ABCB15. A, Fluorescence micrograph of the subcellular localization of ABCB15-GFP expressed from the construct depicted above the micrograph. B, Phase-contrast micrograph of the same cell. C, The macro- and micronucleus of the cell stained with DAPI. Scale bar, 20 μm.

2.4 Knock down of *abcb15* results in a lower tolerance to DDT in *T. thermophila*

To analyze the function of *abcb15*, we generated the *abcb15* knockdown (*abcb15*-KD) strain by partially replacing the endogenous *abcb15* gene in the macronucleus of *T. thermophila* with 45 copies of the neo4 cassette. After treatment with DDT (4 mg L⁻¹) for 4 h and 24 h, the expression level of *abcb15* in the *abcb15*-KD strain was reduced 5–11-fold compared with that in the wild-type (WT) strain using real-time PCR (Figure 6), suggesting that *abcb15*-KD strain.

Under conditions of DDT treatment (256 mg L⁻¹), there was no significant difference ($P \ge 0.05$) in growth rate (k) values between the *abcb15*-KD strain (k=0.3511) and the WT strain (k=0.3267) within 4.5 h after inoculation, when cells were adjusted to the new environment (Table 1). Since then, cells entered the logarithmic growth period, and the mean generation time (GT) for the *abcb15*-KD strain (GT=3.4951 h) was longer than that for the WT strain (GT=2.7232 h) (P<0.05) and reached 14.5 h. This result



Figure 6 Reduced expression level of *abcb15* analyzed with real-time PCR. The wild-type (WT) and *abcb15* knockdown (KD) strains were treated with 4 mg L⁻¹ for 4 and 24 h, respectively (n=3). *, P < 0.05.

Table 1 Growth models developed for the lag phase of different strainsof *T. thremophila* under 256 mg mL⁻¹ DDT treatment

Staging	Growth models		
Suallis	0 – 4.5 h	4.5 – 14.5 h	
B2086	$N_B = N_A \times \exp(0.3267 \times t)$	$N_{C}=N_{B}\times 2^{t/2.7232}$	
abcb15-KD	$N_B = N_A \times \exp((0.3511 \times t))$	$N_C = N_B \times 2^{t/3.4951}$	

indicated that the growth rate of the *abcb15*-KD strain was inhibited by DDT treatment.

3 Discussion

In the present study, we showed that in T. thermophile, abcb15 was up regulated by DDT treatment in a specific manner (Figure 4), which is consistent with our previous studies [8,11]. The deduced amino acid sequence from our sequencing data revealed that ABCB15 from T. thermophile possesses the typical characteristics of the functional ABC transporters, i.e., it is formed in a TMD-NBD-TMD-NBD structure. The two TMDs form the translocation pathway and two NBDs hydrolyze ATP. These domains contain a Walker-A motif that binds the nucleotide, a C-loop motif that contacts the nucleotide in the ATP-bound state, and a Walker-B motif that provides a conserved glutamate residue to orchestrate the nucleophilic attack on ATP via a water molecule [24]. Through hydrolyzing ATP to ADP, ABC transporters acquire energy and transport a wide variety of substrates across membranes, such as ions, sugars, amino acids, polypeptides, toxic metabolites and xenobiotics, and drugs. Therefore, these transporters not only provide nutrients for cells, but also protect cells from numerous toxic compounds [25]. Since the ABC transporters are one of the most highly conserved gene super families in prokaryotic and eukaryotic organisms, ranging from bacteria, fungi, and protists to plants and animals, it is very likely the ABCB15 protein from T. thermophile also functions in protecting cells from toxic DDT by transporting it out.

The deduced *T. thermophile* ABCB15 shares a 32.3% amino acid identity with a human p-glycoprotein (P-gp) encoded by *abcb1*, also known as a multidrug resistance *mdr* gene [26], which is higher than that shared by STE6 in yeast (26.2%) [27]. This information suggests that the *T. thermophile* ABCB15 protein may also function as a P-gp. The human *p-gp* gene is constitutively present in various normal tissues and expressed primarily in the liver, colon, kidney, adrenal gland, and pancreas. The localization of P-gp mainly on secreting and excretory cells corresponding to its function, i.e., transporting endobiotics and xenobiotics –such as anthracyclines, vinca alkaloids, taxanes, epipodo-phyllotoxins and topotecan, and protecting cells from toxic agents [28].

It has been shown that human P-gp recognizes DDT as a substrate and performs as an efflux pump to protect cells. For example, 10 μ mol L⁻¹ DDT treatment resulted in a 2–3-fold increase in the expression level of *mdr1* in HepG2 cells, and rhodamine staining indicated that DDT was a substrate for the MDR pump. In addition, DDT induced a two-fold increase in *mdr1* gene expression in HeLa cells, whereas changes in the expression levels of three additional

ABC transporters (*bcrp*, *mrp1*, and *mrp2*) were not detected [29]. In the present study, the expression level of *abcb15* was markedly increased by exposure to 4 mg L^{-1} DDT, without being increased by a variety of other physiological conditions or by TCDD treatment, indicating that ABCB15 also recognized DDT as a substrate.

As an important membrane pump, the ABC transporter protein distributes on the plasma membrane and membranes of organelles. Among 11 ABC transporter genes in yeast (Schizosaccharomyces pombe), two were localized on the plasma membrane, two on mitochondrial membrane, four on vacuolar membrane, one on endosomal and Golgi membranes, and two on endoplasmic reticular membrane [30]. Computational analyses indicated that T. thermophile ABCB15 protein was localized on the plasma membrane. In addition, the chimeric ABCB15-GFP protein was evenly distributed in the cell periphery (Figure 5), which is consistent with the results of a study examining ABC transporters in yeast [30]. These results indicate that ABCB15 protein may function as a membrane pump to transport DDT out of the cell and enhance the tolerance of T. thermophile to DDT. This hypothesis is supported by our data from abcb15-KD strain. When abcb15 was knocked down (Figure 6), the tolerance of *T. thermophile* to DDT exposure decreased, which is manifested by the decreased growth rate.

T. thermophila has two types of nuclei with distinct functions: a micronucleus that contains five pairs of centromere-bearing chromosomes in diploid germlines, and a macronucleus with ~225 acentromeric chromosomes in somatic nuclei that contain ~45 copies for most genes [10]. In this study, the *abcb15*-KD strain was constructed in somatic cells by replacing the neo4 cassette at the macronucleus *abcb15* coding sequence.

In the presence of 4 mg L^{-1} DDT, the *abcb15*-KD strain (GT=3.5 h) grew more slowly than WT cells (GT=2.7 h), demonstrating that *abcb15* plays an important role in the resistance of T. thermophila to DDT. In P-gp (abcb1) knockout mice, cerebral concentrations of amitriptyline and its metabolites were higher than those in controls [31]. Previous studies showed that the following two mechanisms are responsible for the multidrug resistance of human P-gp: (i) TMDs recognize and bind drugs that diffuse into the cell and then transport them out of the membrane, utilizing the energy from the ATP hydrolysis by NBDs, and reduce the concentration of drugs in the cell; (ii) some lipophilic drugs that diffuse very slowly across the lipidic cytoplasmatic membrane are recognized and bound by TMDs and are thus pumped out of the membrane [32,33]. The finding that the abcb15-KD strain grew slower than WT cells in the presence of DDT indicates that ABCB15 is a powerful pump that transports DDT out of the cell and protects the cells from the damage caused by DDT.

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