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Cloning and Knockout of YbhS belong to ABC Superfamily of *Escherichia coli*

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Abstract **Original Research Article**

ABC superfamily of bacteria is involved in the uptake of nutrients, excretion of metabolites, formation of bacterial biofilm, and is related to drug resistance. E. coli contains about 70 ABC transporters, equivalent to 5% of the genome. E. coli is predicted to have 11 export pumps, some of which are related to drug resistance. YbhFSR is one of them. In this paper, the permeable enzyme YbhS of the YbhFSR pump was systematically bioinformatics analyzed and gene knockout was carried out by Red recombination method, laying a foundation for subsequent studies on the function of YbhFSR transporter.

Keywords: ABC Superfamily, YbhFSR, YbhS, knockout.

INTRODUCTION

The ABC superfamily transporters are ubiquitous in almost all organisms from bacteria to yeast, nematodes, fruit flies, plants, mammals and so on. They are the protein family with the largest number and the most extensive functions known. Because the ABC transporter can pump foreign substances that have entered the cell out of the cell, it is the main cause of bacterial drug resistance and tumor cell multidrug resistance. Its gene expression level is related to the intracellular drug concentration and resistance. The degree of medicine is closely related. Both the uptake and export ABC transporters have similar structures, that is, two TMD (transmembrane domains) domains and two NBD (nucleotide-binding domains) domains. The TMD domain is a channel for material transport across the membrane. Among different ABC transporters, the amino acid sequence and structure of NBD are relatively conserved, while the TMD domain is less conserved, which is related to the function of the TMD domain involved in the transfer of different substrates [1]. A single TMD of most ABC family proteins is composed of 6 α-helices, and these 6 α-helices are parallel to each other. Of course, not all TMD domains of the ABC family are composed of 6 α-helices, such as E .coli hypothetical ABC family drug efflux pump YddA is predicted to be composed of 5 α-helices.

Compared with NBDs, TMDs have relatively few structural analyses. The only structural data available are from the complete E. coli lipid transporter [2] and vitamin B12 [3]. Although the TMD domain is less conserved, the TMDs of different ABC transporters can have different numbers of α-helices and some small sequence homology. The two available TMD structures (BtuD and MsbA) reported so far cannot model each other.

ABC transporters of Bacterial include single-drug resistance (SDR) transporter and multi-drug resistance (MDR) pumps. SDR transporters are used to transport a single or a group of closely related drugs. Almost all SDR transporters have $\{TMD\}_2$-$\{NBD\}_2$ arrangement, while MDR has $\{TMD\text{-}NBD\}_2$ arrangement. Several bacterial ABC transporters with multi-drug resistance have been identified through experiments, such as LmrA of L. lactis [4]. The four domains of LmrA have $\{TMD\text{-}NBD\}_2$ arrangements. Only a few ABC single-drug transporters have been reported in bacteria [5]. MacB is the first ABC family single-drug transporter experimentally confirmed in Gram-negative bacteria [6]. The YbhFSR transporter was preliminarily determined as a single-drug efflux pump by our early research, and there is no relevant report about permease YbhS of the YbhFSR pump.

E. coli is the simplest model organism, and the study of its ABC transporters can not only understand the transport mechanism of the ABC systems, but also provide theoretical reference for the related studies of other species. A part of the ABC family efflux pump of E. coli is related to drug resistance. Further research into these exports could help us in understanding bacterial resistance and even human anti-tumor drug resistance. The research on the structure and action

mechanism of microbial ABC proteins is of inestimable value for better understanding the function and structure of the whole superfamily.

MATERIALS AND METHODS

Bioinformatics Analysis of YbhS

FASTA format sequences were obtained from the National Center for Biotechnology Information (NCBI) for YbhS. Basic physicochemical properties of the protein were predicted using Protparam software (https://web.expasy.org/protparam/). The transmembrane domain was predicted using TMHMM2.0 software (http://www.cbs.dtu.dk/services/TMHMM/). ProtScale software (https://web.expasy.org/protscale/pscale/Bulkiness.htm) was used to analyze hydrophobicity.

Cloning of ybhS

The ybhS sequence was obtained from NCBI. We designed specific primers (ybhS -F and ybhS -R, Table 1) for PCR amplification. Genomic DNA of WT E. coli was used as a template for PCR amplification of the ybhS gene. The amplified fragment was cloned into the pMD-18T carrier to transform into E. coli DH5α competent cells. Positive clones were screened on ampicillin plates. Positive clones were verified by PCR amplification and sequencing.

Table-1: Primers used in this experiment

primer	Sequence (5'to3')	Size(bp)
ybhS-F	CGCGGATCCATGAGTAACCCGATCCTGTCCT	31
ybhS-R	CCCAAGCTTCTAATCCAGCCGACGTTTGG	29
K- ybhS F	AAGCCTTTATTCAGTTGATCCACGACTGGGATAAG GAGCAT AGCATTGAGCGATTGTGTAGGCTGGAG	68
K- ybhS R	TCTTTGCGGATTAACGTCCATAAGCGATGAAACAT GCTCTTCTCCTAACGGCTGACATGGGAATTAGC	68
T- ybhS F	ATGGAGCAAGCCTTTATTCA	20
T- ybhS R	CGCCGTTATCTTCATCGTAG	20
Cm-F	TGAAACTCACCCAGGGATTG	22
Cm-R	ATAAATCCTGGTGTCCCTGT	22

Knockout of gene and verification of gene disruption

A long fragment primer was designed to extend the target fragment (Table 1). The primer was designed according to Datsenko. PCR amplification used pKD3 plasmid as templates. The PCR product was purified by gel recovery, digested with DpnI, and then re-purified again. Finally, the recovered products were dissolved in water. The concentrations of the PCR products after purification were required to exceed 100 ng/μL. The pKD46 plasmid was cloned into the E. coli K-12 strain and detected with primers (Table 2). Then 5 μL of target fragment and 50 μL of electroconversion-sensing cells were added to a 0.2 cm chamber and subjected to electric shock with a Bio-Rad electroporation system. After the electric transfer, the product was coated on the corresponding resistance plate for screening after incubation at room temperature overnight. Positive clones on the plate were selected for PCR verification. Two pairs of primers were designed, and four pairs of primers were cross-checked for PCR verification (Table 1). The lengths of the four pairs of primer amplification fragments from the positive strain differed from those of the strain without knockout. PCR positive strains were sent to Shanghai Biological Engineering Co., Ltd for sequencing verification.

RESULTS

Bioinformatics Analysis of YbhS

TMHMM 2.0 software was used to analyze the transmembrane domain of YbhS. The transmembrane sites of YbhS proteins are shown in Table 2 and Figure 1. The results showed that YbhS had six transmembrane alpha-helical structures, and both NH2-terminal and COOH- terminal located in the cytoplasmic matrix.

Table-2: Helix positions of YbhS

TMSs	Total	6
TMHMM Server	TMS1	27-49
	TMS2	182-204
	TMS3	231-253
	TMS4	263-285
	TMS5	292-314
	TMS6	348-370

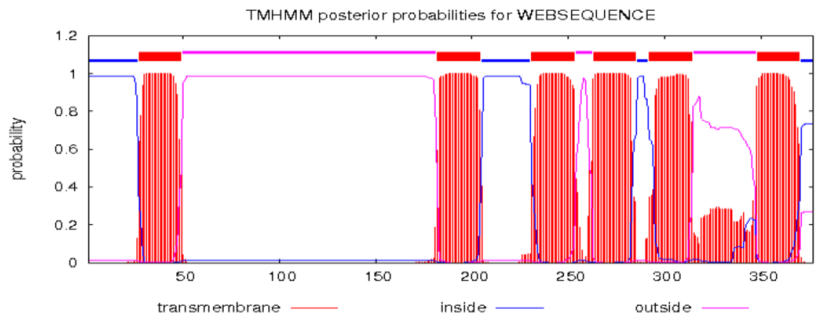

Fig-1: The TMHMM 2.0 software was used to predict the transmembrane domain of YbhS protein

The physicochemical properties and components of the amino acid sequence encoded by YbhS protein in E. coli K-12 MG1655 were analyzed by ProtParam program. The results showed that YbhS protein was encoded by 377 amino acids, with a molecular weight of 42.06 kDa. The theoretical isoelectric point (PI) of YbhS protein was 8.42, which was alkaline. The protein encoded by ybhS is a water-transporting protein. Among the 20 amino acids encoded by YbhS gene in E. coli K-12 MG1655 strain, aromatic amino acids (phenylalanine, tyrosine, tryptophan) accounted for 9.9% of the total amino acids.

Cloning of ybhS

The ybhS gene was amplified using genomic DNA of E. coli K-12 as template, and a 1134 bp fragment was found by 1% agarose gel electrophoresis, consistent with the expected fragment size. The fragment was recovered and purified and ligated to the pMD18T vector. Single colonies picked from plates were verified by PCR (Figure 2A) and sequencing verification. Results were consistent with NCBI sequences.

Gene disruption of ybhS

This protocol is illustrated as Kirill A. Primers in the PCR amplification used for targeting fragment preparation were 68 nt long fragments including a 45-nt target gene flanking sequence and a 23-nt resistance gene sequence, using pKD3 as a template. The target fragments of 1.1-bp were prepared separately (Figure 2B). The pKD46 plasmid was transformed into E. coli K-12 competent cells, and plasmid positive cells were screened according to ampicillin resistance and confirmed by PCR amplification. After arabinose induction, E. coli K-12/pKD46 cells expressed Gam, Bet and Exo- three λ-phage recombinant enzymes.

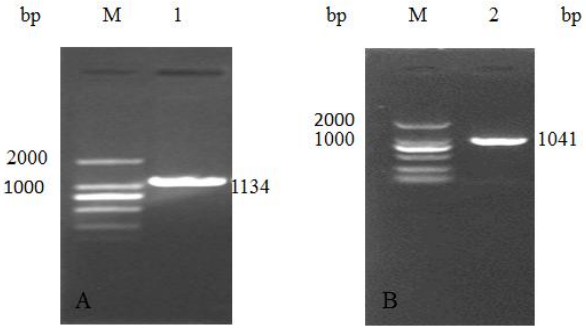

Fig-2: Identification of recombinant pMD-18T (+)-ybhS and target fragment of ybhS(1041bp)

We designed two pairs of identification primers, which were combined into four primer pairs to verify the positive clones by PCR. For positive clones, we used four pairs of primers to demonstrate that all mutants had the correct amplification size. The wild-type fragment size of ybhS was 1,371 bp. After electroconversion, ybhS was replaced with chloromycetin 1,341 bp. In addition, three other primers were used to identify the recombinant strains. The sizes of the amplified fusion recombinant fragments were as expected. The results showed that ybhS had been replaced by the chloromycetin resistance gene (Figure 3). After further sequencing and identification, we determined that the linear targeting sequence was fully consistent with the theoretical sequence.

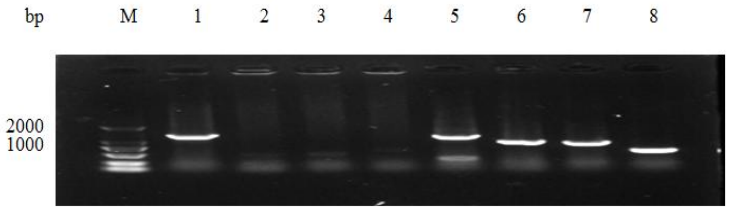

Fig-3: Results of positive clones by PCR of ybhS

M. DL2000 DNA marker； 1, 2, 3, 4 was E.coli K-12.5, 6, 7, 8 wasΔybhS

DISCUSSION

In recent years, the research on the drug resistance of bacteria is very hot. Scholars at home and abroad have conducted a lot of in-depth research on the role of transporter expression in different bacteria and different malignant tumors. Although these transporters were originally discovered to be proton-powered pumps, another type of multi-drug efflux transporters driven by ATP hydrolysis appeared in the mid-1990s, namely the ABC family transporters, which carry multiple substrates for ABC transport. Diverse, including peptides, sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic ions and glutathione conjugates, etc. [7]. ABC transporters differ greatly in substrate selection, some ABC Transporters are highly specific to substrates, while some ABC transporters can transport several structurally unrelated compounds. The difference in substrate selection of ABC transporter may be closely related to its special structure.

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The role of TMD is to form a channel that recognizes and mediates the passage of the substrate through the cell membrane. For ABC transporters that transport hydrophilic molecules, TMD separates the substrate from the phospholipid bilayer by providing a hydrophilic pathway across the cell membrane. In most cases, these permeases have specific substrate specificities and may require periplasmic binding proteins to facilitate transport. On the contrary, TMDs used for drug and lipid transfer can directly identify and remove a large number of chemically unrelated lipids and toxins from the cell membrane. Many of these transporters transport useful substrates, such as anticancer drugs [8]. The YbhFSR system of the ABC family of Escherichia coli is composed of three functional proteins. YbhF has two NBD domains, while YbhR and YbhS each contain a TMD domain. Therefore, the YbhFSR transporter forms a $(\{TMD\}_2\text{-}\{NBD\}_2)$ organization structure. Therefore, according to the domain arrangement classification of ABC drug efflux transporters in single-drug efflux and multi-drug efflux, YbhFSR is closer to single-drug efflux transporters. This is consistent with the previous research results of our laboratory. The transmembrane domain of the YbhFSR transporter is encoded by two proteins, YbhS is one of them. The functional analysis and gene knockout of the ABC family drug efflux pump permease are used to study the substrate binding of the ABC family drug efflux pump TMDs Lay the foundation for the functions in the transport.

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REFERENCES

1. Doerrler W T, Raetz C R H. (2002).ATPase Activity of the MsbA Lipid Flippase of Escherichia coli[J], Journal of Biological Chemistry, 277(39):36697-36705.
2. Locher K P. (2002).The E. coli BtuCD Structure: A Framework for ABC Transporter Architecture and Mechanism[J], Science, 296(5570):1091-1098.
3. Marchler-Bauer A, Bo Y, Han L, et al. (2017).CDD/SPARCLE: functional classification of proteins via subfamily domain architectures[J], Nucleic Acids Research, 45(D1):D200-D203.
4. Agboh K, Lau C, Khoo Y, et al. (2018).Powering the ABC multidrug exporter LmrA: How nucleotides embrace the ion-motive force[J], Sci Adv, 4(9):s9365.
5. Joanne Y, Barry H I. (1999).ABC transporters: bacterial exporters-revisited ¢ve years on[J]. Biochimica et Biophysica Acta, 1461:177-200.
6. Tikhonova E B, Devroy V K, Lau S Y, et al. (2007). Reconstitution of the Escherichia coli macrolide transporter: the periplasmic membrane fusion protein MacA stimulates the ATPase activity of MacB[J], Molecular Microbiology, 63(3):895-910.
7. Moussatova A, Kandt C, O'Mara M L, et al. (2008). ATP-binding cassette transporters in Escherichia coli[J], Biochimica et Biophysica Acta (BBA) - Biomembranes, 1778(9):1757-1771.
8. Chang G R. (2003). Multidrug resistance ABC transporters[J], FEBSLetters, 555:102-105.