Cloning, Bioinformatics and Activity Analysis of the MnhB Gene from the Moderately Halophilic Bacterium Halobacillus Y5

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Abstract

Objective: The mnhb gene of Halobacillus Y5 was cloned to clarify its basic biological information. Methods: The genome of Halobacillus Y5 was extracted and the gene fragments were obtained by PCR, then 400–900 bp fragments were recovered and linked to vector pUC18. The constructed recombinant plasmids were transformed to competent E. coli KNabc by functional complementation, and the strain containing the gene of Na+/H+ antiporters was screened for bioinformatics analysis. The positive monoclonal strains were screened by specific medium. Results: mnhB gene has two CDS regions, respectively 305 bp and 128 bp, encoding 96 amino acids. The predicted relative molecular weight is 11048.16 Dt, the isoelectric point is 4.66, the fat coefficient is 130.40, and the instability coefficient is 29.15. It is a stable protein with a half-life of 30 h. Comparison of similarity between mnhb from Halobacillus Y5 and 5 sequences, the homology of wp_101843976.1, wp_082234107.1, wp_079529991.1, wp_128522668.1, wp_08502981.1 were 93%, 69%, 78%, 69%, 67%, respectively. Through phylogenetic tree analysis, the Na+/H+ antiporters from Halobacillus Y5 is in a separate branch, which may be a new member of Na+/H+ antiporter. Na+/Li+, K/H+ antiport activity was detected from everted membrane vesicles prepared from E. coli /pUC- mnhb but not those of /pUC18. Conclusion: A new mnhB gene was successfully cloned, which laid a theoretical foundation for the development and utilization of Halobacillus Y5.

Keywords: Moderately halophilic bacterium; mnhB gene; Na+/H+ antiporter; Membrane proteins; Cloning; Bioinformatics analysis.

INTRODUCTION

High concentration of Na+ will have a huge toxic effect on the growth, reproduction and development of microorganisms and other life activities. In order to adapt to this poisoning, microorganisms have formed two salt-appropriate mechanisms: [1] Internal salt strategy: The microorganisms accumulate high concentrations of NaCl as an osmotic agent to maintain the osmotic balance of the microorganisms. At present, this mechanism is only found in some aerobic archaea and a few extremely halophilic aerobic bacteria [2]. Salt rejection strategy. Most halophilic and salt-tolerant microorganisms use this strategy to resist the high-salt environment to maintain their normal growth. Under the hypertonic conditions of this kind of microorganisms that use salt rejection strategies to resist high salt stress, on the one hand, organic compatible solutes are accumulated in the body to adjust the osmotic balance inside and outside the cell; on the other hand, excess Na+ in the cell are expelled from the cell outside [1]. In this study, we first cloned the mnhB gene, and then used the functional complementation method to transform the ligase product into the defective E. coli KNabc competent cells, inoculated in a high-saline medium, and speculated that the Na+/H+ reverse of the mnhB gene transport function, this study laid a theoretical foundation for the development and utilization of the moderately halophilic bacterium Halobacillus Y5.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli KNabc (Δnha A, Δnha B, Δcha A) and plasmids pET19 (b), pUC18 were provided by the Microbiology Laboratory of Northeast Agricultural University, moderately halophilic bacteria Halobacillus Y5, negative control bacteria BL21/pET19 (plasmid pET19 is chemically transformed into the large intestine Escherichia BL21) and Escherichia coli...
BL21 were provided by the Bioengineering Laboratory of Heilongjiang Bayi Agricultural University.

**Main reagents**

Hind III and EcoR I, 1Kb DNA Ladder Marker, and T4 DNA ligase were purchased from Bao Bioengineering (Dalian, China) Co., Ltd.; Omega gel recovery kit was purchased from Harbin Shiguo Biotechnology Co., Ltd., and other reagents were of analytical grade.

**Medium**

LB medium and LBK medium[2]: The extraction of Halobacillus Y5 genomic DNA is activated with LB medium, the specific steps are: 1% inoculum is inoculated into 500 mL LB medium, 37°C, 16 800×g culture 16~18 h; centrifuge at 9 600×g for 5 min at 4°C, collect the bacteria, and wash the bacteria with sterile water. The total genome was extracted and stored at -20°C after electrophoresis [3].

**Amplification of mnhB transporter gene**

Premier 5.0 was used to design primers [4], mnhB transporter gene F primer sequence: 5′-CCGGAATTCTGGTCGTATCGTTACAATAG-3′ (the underlined part is EcoRI restriction site), R primer sequence: 5′-CCCAAGCTTTTAATGATGA TGATGATGATGATG-3′ (The underlined part is the Hind III restriction site). The primers were synthesized by BGI (Beijing) Biotechnology Co., Ltd. The fragments of 400~900 bp were recovered by gel and ligated with Hind III and EcoR I fully digested pUC18 vector to construct recombinants. The ligated vector and target fragments were electro-transformed into EP432 competent cells [5], and coated Inasolid LBK medium with a Na+ concentration of 0.5 mol/L and 50 μg/mL Amp, cultured at 37°C for 24 h to select positive clones.

**Bioinformatics Analysis**

DNAMAN biological software for open reading frame (ORF), amino acid sequence analysis and nucleic acid alignment; use BLAST in the NCBI website for nucleic acid and amino acid sequence homology analysis; use the website http://web.expasy.org/protparam/Perform protein relative molecular mass and theoretical isoelectric point analysis; use http://www.ncbi.nlm.nih.gov/services/TMHMM/ to predict the transmembrane structure of protein sequences; apply http://web.expasy.org/protscale/ for hydrophobicity analysis; MEGA is used 5.0 software, select the Neighbor-Joining method to construct a phylogenetic tree; use the website https://swissmodel.expasy.org/ to simulate the three-dimensional structure of mnhB and related sequences to analyze the structural similarity.

**Assay of Na+(Li+)/H+ antiport activity**

The Na+(Li+, K)/H+ antiport activity of everted membrane vesicles was determined according to the extent of the collapse of a transmembrane proton gradient, with acridine orange as the pH indicator, as described by Rosen [6]. Fluorescence was monitored with a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) at excitation and emission wavelengths of 495 and 530 nm, respectively.

**RESULTS AND ANALYSIS**

Sequence analysis of mnhB gene of Bacillus halophilus

PCR fragments by agarose gel electrophoresis to check the size of the amplified gene mnhB. The results showed that its length was between 400 and 900 bp, which were in line with the expected length of 433 bp, and the length of the fragment was recovered by the gel [6, 7]. Import the sequencing result to the vector sequence into DNAMAN software. After comparing them, it was found to have 97% homology with the MSMB of Halobacillus sp. Marseille-P3879. Then use NCBI's online ORF program to determine its CDS region, its ORF1 is 305 bp, ORF2 is 128 bp, a total of 96 amino acids are encoded in Table 1. Compare the similarity of mnhB from Halobacillus Y5 with 5 related sequences. The results of the comparison are shown in the figure below. It is with login WP_ 101843976.1, WP_082234107.1, WP_079529999.1, WP_128522668.1, WP. The homology of 085029880.1 protein was 93%, 69%, 78%, 69%, 67%, respectively (Fig. 1).

![Alignment of mnhB with associated Na+/H+ antiporters](image-url)
Analysis of physical and chemical properties of protein

*mnhB* protein was composed of 96 amino acids, of which Leu and ALA were 14.9% and 11.9% respectively (Table 1). The isoelectric point was 4.66, the negatively charged amino acid residue number (ASP + clu) was 5, the positively charged amino acid residue number (Arg + clu) was 7, and the whole protein was negatively charged. The protein molecular formula is c517h813o137n119s5, fat coefficient is 130.40, instability coefficient is 29.15, extinction coefficient is 0.809, half-life is 30 h. 994, which is a hydrophobic protein (Fig. 2).

| Amino acid | Amount | Percentage (%) | Amino acid | Amount | Percentage (%) |
|------------|--------|----------------|------------|--------|----------------|
| Ala (A)    | 12     | 11.9           | Arg (R)    | 4      | 4              |
| Asn (N)    | 3      | 3              | Asp (D)    | 4      | 4              |
| Cys (C)    | 0      | 0              | Gln (Q)    | 2      | 2              |
| Glu (E)    | 3      | 3              | Gly (G)    | 9      | 8.9            |
| His (H)    | 0      | 0              | Ile (I)    | 9      | 8.9            |
| Leu (L)    | 15     | 14.9           | Lys (K)    | 1      | 1              |
| Met (M)    | 5      | 5              | Phe (F)    | 7      | 6.9            |
| Pro (P)    | 2      | 2              | Ser (S)    | 1      | 1              |
| Thr (T)    | 9      | 8.9            | Trp (W)    | 0      | 0              |
| Tyr (Y)    | 6      | 5.9            | Val (V)    | 9      | 8.9            |

Fig-2: Analysis of hydrophobicity of *mnhB*

Protein cell localization and structure prediction

DNMAN software analysis showed that the length of the foreign DNA fragment of the recombinant plasmid was 433 bp, which contained two possible ORF1 and ORF2. It was predicted that 305 amino acids related to cation reverse transporter and 128 amino acids related to H⁺ / cation transporter were encoded respectively. It was preliminarily speculated that the protein was a membrane protein. Utilize http://www.cbs.dtu.dk/services/TMHMM/ Topoisoromeric analysis of ORF2 protein showed that there were two transmembrane domains (Fig. 3), indicating that ORF2 protein was a transmembrane protein located in the cell membrane. Using the website https://swissmodel.expasy.org/*mnhB* gene and its five related sequences (accession No_ 101843976.1、WP_082234107.1、WP_079529999.1、WP_128522668.1、WP_085029880.1). The results showed that the three-dimensional structures of the six proteins were very similar (Fig. 4). It is speculated that *mnhB* gene may have the function of sodium / hydrogen ion reverse transport.
Construction of mnhB phylogenetic tree

The phylogenetic tree of mnhB was constructed by using the "neighbor joining" method selected by mega5.1 software [7] (Fig. 5). mnhB and Na⁺/H⁺ retrotransporters on other protein β subunits gathered together and formed an independent branch. It is speculated that mnhB gene is probably a new member of Na⁺/H⁺ retrotransporter gene family on β subunit [8].

Activity determination

The reverse membrane was prepared by high pressure cell crusher JG-1A of Ningbo Xinzhi Biotechnology Co., Ltd. and low temperature and high speed centrifuge and low temperature and high speed centrifuge of Hitachi CRG Series. In the AO fluorescence quenching method, Hitachi F-7000 fluorescence spectrophotometer of Hitachi Ltd was used to determine the protein activity of mnhB. All the above were carried out with reference to the literature. In the reaction system, the transport activity of mnhB gene of Halobacillus Y5 to univalent positive ionons (Na⁺, Li⁺, K⁺) was measured. Therefore, we believed that the transport activity of mnhB for monovalent cations Na⁺, Li⁺ and K⁺ occurred but not those of /pUC18 (Fig.6).
CONCLUSION AND DISCUSSION

_Halobacillus_ is a Gram-positive moderately halophilic bacterium that can produce spores [9]. In prokaryotes, sodium/hydrogen antiporters play an important role in catalyzing the excretion of base cations such as Na⁺, Li⁺ or K⁺ in cells in exchange for external protons, so as to reduce the cytoplasmic concentration of toxic alkaline metal cations and maintain intracellular pH homeostasis [10, 11]. At present, the mnhB gene of bacillus halophilus has been preliminarily studied, but the Na⁺/H⁺ reverse transport function of mnhB gene has not been reported.

In this study, we first cloned _mnhB_ gene, and then transformed the ligation product into competent cells of E.coli KNabc by functional complementation method, which showed salt tolerance in high saline alkaline medium. Bioinformatics software was used to analyze the hydrophobicity and transmembrane structure of the protein. It was found that the protein was hydrophilic and had transmembrane structure. The _mnhB_ gene of _Halobacillus_ Y5 and 5 sequences of _Halobacillus sp. marseille-p3879, Halobacillus massiliensis_ [12], _Halobacillus humanensis_ [13], _Halobacillus litoralis_ [14] and _Halobacillus mangrovi_ [15] (accession No. WP_ 101843976.1, WP_082234107.1, WP_079529999.1, WP_128522668.1, WP_085029880.1) were similar, suggesting that _mnhB_ gene may have the function of sodium / hydrogen ion reverse transport. At the same time, DNA sequence homology analysis showed that the homology was 93 %. It is further confirmed that _mnhB_ gene is likely to be a new member of Na⁺/H⁺ antiporter gene family on β subunit [16]. In conclusion, using a large number of data from bioinformatics tools and bioinformatics database, and combined with the experimental data of this study, we put forward some guiding opinions on the Na⁺/H⁺ antiporter function of _mnhB_ gene, and laid a foundation for the further study of Na⁺/H⁺ antiporter gene family.

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