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Investigation of compatible solutes synthesis and transport of *Virgibacillus halodenitrificans* PDB-F2 with complete genome analysis

Yang Zhou [a, 1], Zhi-Yuan Sun [a, 1], Hui Li [a, *], Chan-Juan Qian [a], Xiao Wu [a], Hong-Zhi Tang [b], Fawad Ali [b], Yong-Di Liu [a, **]

[a] State Environmental Protection Key Laboratory of Environmental Risk Assessment and Control on Chemical Process, School of Resource and Environmental Engineering, East China University of Science and Technology, Shanghai 200237, China
[b] State Key Laboratory of Microbial Metabolism & School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

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ABSTRACT

The salt-tolerant mechanism of compatible solutes in the different microorganisms is always a research hotspot, which can help us understand how organism endures the salty environment. *Virgibacillus halodenitrificans* PDB-F2 could survive in high salinity and degrade phenol, which is a good candidate for wastewater treatment. This study investigated the salt-tolerant mechanism of compatible solutes of this strain and got an insight into its genetic basis through genome sequencing and analyzing. The results found that *Virgibacillus halodenitrificans* PDB-F2 endured 12% (w/v) NaCl condition by synthesizing or uptaking ectoine, hydroxyectoine, trehalose, glutamic acid and betaine. Osmoprotective effects of exogenous compatible solutes on this strain were hydroxyectoine > ectoine > L-proline > trehalose > glutamate acid > betaine. Under osmotic shock, the strain had a higher preference for hydroxyectoine than ectoine, and the ectoine transport was stimulated at both levels of transport activity and transcription. The sequencing and analyzing of strain genome showed that this strain contained a circular chromosome (3,869,935 bp) and one plasmid (47,824 bp), revealing the genes related with synthesis and transport of above compatible solutes. This study provided further information on the understanding of salt-tolerant mechanism of *Virgibacillus halodenitrificans* PDB-F2 by compatible solutes.

1. Introduction

Microorganisms living in the salinity habitats have two strategies to adjust the intracellular osmolarity and avoid the water loss. One is the 'salt-in' strategy, where inorganic salt, such as KCl, is accumulated to provide the cellular osmotic pressure to counterbalance the external osmotic pressure; the other is the 'compatible solutes' strategy, where low-molecular-weight, highly soluble organic compounds are accumulated to provide cellular osmotic-balance without interfering the vital cellular activities even when they are at the high levels (Pflüger and Müller, 2004). The 'salt-in' strategy is adopted by the extremely halophilic *Halobacteria* and *Salinibacter rube* and the anaerobic moderately halophilic *Haloanaerobiales* (Pastor et al., 2010), and the 'compatible solutes' strategy is adopted by the majority of bacteria, especially by the moderate halophilic organisms (Pastor et al., 2010; Wang et al., 2014).

When encountering the high-osmolality stresses, bacteria usually synthesize or uptake varieties of compatible solutes from the external surroundings to acquire enough hydration of cytoplasm and adjust the cell turgor pressure (Kuhlmann et al., 2011; Ongagna-Yhombi et al., 2015), and then get used to the high-osmolality environment (He et al., 2017). Bacteria can adapt to the salty fluctuation quickly by adjusting the compositions and contents of the internal solute pool, which is called 'osmolyte switching' (Saum and Müller, 2007; Kuhlmann et al., 2008). Compatible solutes consist of varieties of organic compounds, including polyols, sugars, N-acetylated diamino acids, betaines, amino acids and derivatives (e.g. ectoine and hydroxyectoine) (Saiz-Jimenez and Laiz, 2000; Pastor et al., 2010; Huang et al., 2014). They act as osmoprotectant to stabilize metabolism enzymes

* Corresponding author.
** Corresponding author.
E-mail addresses: huili@ecust.edu.cn (H. Li), ydliu@ecust.edu.cn (Y.-D. Liu).
[1] Yang Zhou and Zhi-Yuan Sun contributed equally to this work.

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(Khatoon et al., 2014) and play a key role in the salt-tolerant mechanism of microorganisms. So far, numerous researches have been done to study the compatible solutes in different organisms and identify relevant biosynthesis and transport genes for compatible solutes, e.g. the researches on *Corynebacterium glutamicum* and *Lactococcus lactis* (Pflüger and Müller, 2004), *Sinorhizobium meliloti* (Mohamed et al., 2005), *Halomonas elongata* (Schwibbert et al., 2011), *Virgibacillus pantothenticus* (Kuhlmann et al., 2011) and *Vibrio parahaemolyticus* (Ongagna-Yhombi et al., 2015), etc. These studies are helpful to understand how organisms tolerate the high osmolality environment.

Previously, only limited researches have reported the compatible solutes in the germs of *Virgibacillus*, e.g. *Virgibacillus pantothenticus*, and they mainly focus on the ectoine and hydroxyectoine. *Virgibacillus halodenitrificans* PDB-F2, a gram-positive light-yellow rod-shaped moderately halophilic bacterium surviving in the 3–15% (w/v) NaCl salinity and degrading phenol, also have been investigated the ectoine and hydroxyectoine accumulation under high osmotic environment and the *ect*ABC and *ect*D genes with their transcription levels (Tao et al., 2016). However, it is still unclear that the osmoprotective effects of other compatible solutes (except ectoine and hydroxyectoine) on this strain, the transport characteristics of ectoine and hydroxyectoine under osmotic shock as well as the related genes involved in the synthesis and transport for compatible solutes, etc.

In this study, we further investigated the salt-tolerant mechanism of compatible solutes of *V. halodenitrificans* PDB-F2, and the genome of strain was sequenced and analyzed to identify synthesis and transport genes of compatible solutes in *V. halodenitrificans* PDB-F2, which was useful to further understand the salt-tolerant mechanism of *Virgibacillus halodenitrificans.*

2. Materials and methods

2.1. Strain and cultured media

V. halodenitrificans PDB-F2 was isolated in our laboratory and kept in China Center for Type Culture Collection (numbering CCTCC AB 2016002). The media were Luria-Bertani (peptone, 10 gl^{-1}; yeast extract, 5 gl^{-1}; NaCl, 8 gl^{-1}) and M63 medium (KH_2PO_4, 13.6 gl^{-1}; $(NH_4)_2SO_4$, 2 gl^{-1}; $MgSO_4{\cdot}7H_2O$, 0.25 gl^{-1}; $FeSO_4{\cdot}7H_2O$, 0.5 mgl^{-1}; glucose, 5 gl^{-1}; yeast extract, 0.1 gl^{-1}; vitamin solution, 1 ml l^{-1}). The pH of medium was adjusted to 7.5 with KOH (4.2 gl^{-1}). The M63 medium was used for transport investigation because its components were sample and did not interfere the results of HPLC, and Luria-Bertani was used for cultivating cells to extract the genomic DNA because the strain grew very well and fast in Luria-Bertani medium. The salinity of medium was adjusted by adding different amounts of NaCl, and calculated as % (w/v) NaCl. OD_{600} was used to monitor cell growth by BioTek *Elx 808*. The strain was cultivated aerobically at 30 °C and 150 rpm on a rotary shaker.

2.2. NMR analysis of intracellular compatible solutes

V. halodenitrificans PDB-F2 was cultured in M63 medium with 12% (w/v) NaCl for 48 h and then was extracted the intracellular compatible solutes to take the ^{1}H-NMR analysis (Tao et al., 2016). The detection was conducted at 296 K with the BRUKER AVANCE III 400 operating at 400 MHz by an inverse multinuclear probe head fitted with gradient along the z-axis.

2.3. HPLC analysis

The analysis of ectoine and hydroxyectoine were performed by the LC-20A (SHIMADZU) using an Inertsil-HILIC, 4.6 × 150 mm (3 μm) column (SHIMADZU) at 40 °C and determined at 210 nm by a UV detector. The mobile phases were acetonitrile/0.3% (w/v) $NH_4H_2PO_4$ (82:18, v/v) at 0.8 ml min^{-1}. The standard ectoine and hydroxyectoine (Sigma-Aldrich) were determined to get the retention times. Every sample was filtered by 0.22 μm filters before injected into HPLC and the injection volume was 10 μL.

2.4. Osmotic shock experiments

For the osmotic shock experiments, the strain was cultured in M63 medium with 8% (w/v) NaCl to OD_{600} = 0.6 and harvested at 2000 g, 20 min at room temperature, washed with the fresh and isosmotic M63 medium for three times, then resuspended in the M63 medium with 12% (w/v) NaCl and 1 mM ectoine or 1 mM hydroxyectoine, or in the M63 medium with 3% (w/v) NaCl to investigate the transport processes. For ectoine transport in response to the sudden salinity increase experiments, the cells were cultured in M63 medium with 8% (w/v) NaCl to OD_{600} = 0.6, and then subjected to cultures containing 0.5 mM ectoine and extra NaCl (the final NaCl concentration was 12%, w/v) with or without 100 μg ml^{-1} chloramphenicol. 1 ml samples were taken out at different time points, and the cells were pelleted by centrifugation (2000 g, 5 min) at room temperature and washed with distilled water, lyophilized for 24 h and measured the cell dry weight (CDW). The supernatant was filtered with 0.22 μm-pore-size filters and determined by HPLC. The transport quantities of ectoine and hydroxyectoine were calculated by using their cumulative consumption or accumulation in medium.

2.5. DNA extraction, genome sequencing and analyzing

The cells were grown in the liquid Luria-Bertani medium at 30 °C, 150 rpm overnight. The genomic DNA was extracted by a Genomic DNA Purification Kit (Promega) referring to its instructions. The genome of *V. halodenitrificans* PDB-F2 was determined by the Pacific Biosciences RS II platform and the Illumina MiSeq platform at Personal Biotechnology Company (Shanghai, China) to ensure the accuracy and efficiency of the sequencing results. After obtaining the complete sequence, the open reading frames (ORFs) were predicted by Glimmer 3.0 and function annotations were done to analyze the genome. Function annotations were based on the Gene Ontology (GO) annotation by using BLAST2GO with the default settings, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) annotation by using the blast software against eggNOG (V4) database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation by using the KEGG automatic annotation server (KAAS) against KEGG database.

3. Results and discussion

3.1. Compatible solutes synthesized by V. halodenitrificans PDB-F2 in 12% (w/v) NaCl

The intracellular extracts were analyzed by ^{1}H-NMR and revealed that this strain synthesized ectoine, hydroxyectoine, trehalose, glutamic acid and glycine betaine in 12% NaCl environment (as shown in Fig. 1), which was similar to the result of compatible solutes synthesized by this strain in M63 medium with 10% (w/v) NaCl (Tao et al., 2016). This suggested that ectoine, hydroxyectoine, trehalose, glutamic acid and glycine betaine were the major compatible solutes synthesized by *V. halodenitrificans* PDB-F2 in high osmotic environment. However, comparing with the ^{1}H-NMR of compatible solutes synthesized under 10% NaCl, Fig. 1 showed some new responding peaks and the responding