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Highly potential compatible solute from halotolerant *Bacillus subtilis* MA04: Its functional and molecular characterization

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ABSTRACT

Glycine betaine (*N*, *N*, *N*-trimethylglycine) is an effective compatible solute, which maintains fluidity of membranes and protects the biological structure of the organisms under stress. In this study, glycine bataine biosynthesis genes; betaine aldehyde dehydrogenase (*GbsA*) and betaine alcohol dehydrogenase (*GbsB*) from halophilic *Bacillus subtilis* MA04 was heterologously expressed in *Escherichia coli* M15 (pREP4). The recombinant enzyme was purified by column chromatography using DEAE sepharose. The purified enzyme revealed a five-fold increase in the activity with choline as substrate and phenazine methosulfate as electron acceptor, compared to the control strain. The glycine betaine biosynthesis gene sequences reported in this study were diverse and appeared to be partially conserved with the GenBank reported sequences of many eubacteria, with subsequent amino acid changes in the translated proteins.

1. Introduction

Halophilic microorganisms are subjected to frequent fluctuations in the osmotic conditions of their habitat due to salinity of the soil. Halophilic bacteria must have the active mechanisms to compete successfully for their ecological niche (Csonka and Hanson, 1991; Lucht and Bremer, 1994). The more effective defence against these conditions is the accumulation of osmoprotectants, which can be amassed to high intracellular levels without disturbing essential functions of the cell (Boch et al., 1994). One of the most important osmoprotectants is glycine betaine (Kempf and Bremer, 1995). Synthesis of glycine betaine from choline is a two-step oxidation process with glycine betaine aldehyde as the intermediate (Lamark et al., 1991). Characterization of glycine betaine has been most intensively studied at both the molecular and biochemical levels in *Escherichia coli* (Lamark et al., 1996). Recently, bacterial glycine betaine synthesizing enzymes have become a major target in developing stress tolerant crop plants of economic interest. Previous studies reports the resistance towards salinity and low temperature in transgenic tobacco expressing the two *E. coli* genes *betA* and *betB* (Holmstrom et al., 2000), signifying the practical applications of choline dehydrogenase. In *E. coli*, choline dehydrogenase (*betA*) oxidizes choline to glycine betaine aldehyde and betaine aldehyde dehydrogenase (*betB*), converts glycine betaine aldehyde to the osmoprotectant glycine betaine. The choline-glycine betaine synthesis pathway is an important facet in *Bacillus subtilis* to cope with high osmotic stress (Lamark et al., 1991). However, the genetic and biochemical details governing choline uptake and glycine betaine synthesis have remained largely unknown. In this study, for the first instant we report the characterization, production and *in silico* structural analysis of *GbsA* and *GbsB* genes in *B. subtilis* MA04 isolated from the salt pan. Moreover, the sequence analysis of glycine betaine aldehyde dehydrogenase and glycine betaine alcohol dehydrogenase from *B. subtilis* MA04 displayed several base substitutions with that of reported sequences in GenBank, resulting in the altered amino acid sequences of the translated protein sequences.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacillus subtilis MA04 was isolated from the soil samples collected from the saltpan at Ennore, Tamil Nadu, India. Microbial identification and biochemical characterization of *B. subtilis* was carried out as described in Bergeys Manual of Systematic Bacteriology (Holt et al.,

Abbreviations: *GbsA*, betaine aldehyde dehydrogenase; *GbsB*, betaine alcohol dehydrogenase; DEAE, diethylaminoethyl; *X-gal*, 5-bromo-4-chloro-3-indolyl-beta-*D*-galacto-pyranoside; Ni-NTA, nickel-nitriloacetic acid column; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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1994) *E. coli* JM109 was used for cloning and *E. coli* M15 (pREP4) was used as expression host. The plasmids used for cloning and expression was pDrive and pQE30 (Qiagen, Germany) respectively.

2.2. PCR amplification of glycine betaine biosynthesis genes

Genomic DNA extraction from *B. subtilis* MA04 was performed by following the methodology of Ausubel et al. (Ausubel et al., 1994). Plasmid DNA was purified from the cells by alkali lysis method. Plasmids and PCR products were recovered from agarose gel using MinElute Gel purification Kit (Qiagen) by following the manufacturer's instructions. A pair of gene specific primers *GbsA*F (5′-ATGAGTCAAACATTATTC-3′), *GbsA*R (5′-TTATGAATTAAACCAGTT-3′) and *GbsB*F (5′- ATGCAGAAAT TCCACACA -3′), *GbsB*R (5′- TTATACCGTTTTAGGCGT -3′) were designed and used to amplify the *GbsA* and *GbsB* genes. PCR amplification was performed with a primary denaturation at 94 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 2 min at 72 °C. Additional extension was carried out for 5 min at 72 °C using high fidelity PCR enzyme mix (MBI Fermentas, USA). The PCR amplicons were analyzed on a 1.5% agarose gel along with a DNA molecular weight marker (MBI Fermentas) and documented in a gel documentation system (UVP BioSpectrum Imaging system, USA).

2.3. Cloning and sequencing

The PCR amplicons of *GbsA* and *GbsB* genes were purified using Qiagen PCR purification Kit and cloned into pDrive according to the manufacturer's instructions. The pDrive-*GbsA* and *GbsB* construct was transformed into *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (*rK-mk*+), e14–(*mcrA*–), *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB*+, *lac I*q, *lacZ*ΔM15). White colonies were selected for PCR amplification with vector primers M13f-M13r (MBI Fermentas) and the clones with the correct insert as judged by size were sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA).

2.4. Expression of recombinant glycine betaine biosynthesis genes

The recombinant plasmid was double digested with restriction enzymes, *Bam*HI & *Xba*I for *GbsA* and *Xba*I & *Hind*III for *GbsB* (MBI Fermentas) and purified by Perfectprep Gel Cleanup Kit (Eppendorf, Germany). The purified *GbsA* and *GbsB* genes were sub-cloned into pQE30 expression vector, which had previously been digested and purified. The resulting recombinant expression vector pQE30-*GbsA* and *GbsB* cassette was transformed into *E. coli* M15 (pREP4). A single colony of the recombinant clone was inoculated into 5 mL of Luria–Bertani (LB) broth containing 100 μg mL^{-1} of ampicillin and 25 μg mL^{-1} of kanamycin, and incubated overnight at 37 °C. 2·5 mL of the overnight culture was transferred into 50 mL of LB broth containing the corresponding antibiotics and incubated at 37 °C, until OD_{600} value reached 0.6. Isopropyl-β-*D*-thiogalactoside (IPTG, MBI Fermentas) was then added to the culture broth at the final concentration of 1.5 mM and was continuously incubated at 37 °C for 4 h. The induced bacterial cells were harvested by centrifugation and resuspended in 1× SDS-PAGE sample buffer and lysed in boiling water bath for 3 min. The cells were centrifuged at 14,000 *g* for 5 min and the supernatant was checked for expression of soluble proteins. The expression of the target proteins were analyzed by SDS-PAGE. The SDS-PAGE was performed as described previously by Laemmli (1970) along with the protein ladder (Sigma, USA) for the estimation of molecular mass.

2.5. Cell extraction and enzyme purification

The IPTG induced *E. coli* M15 (pREP4) cells with pQE30-*GbsA* and *GbsB* cassette was harvested by centrifugation at 10,000 *g* for 10 min and were resuspended with 30 mM Tris HCl (pH 7.5), 60 mM NaCl, 2 mM phenylmethylsulfonyl fluoride [PMSF] (Sigma) and 2.5 mM dithiothreitol [DTT] (Sigma). The cells were subjected to sonication for 5 min in 1 min pulses with discontinuous cooling in ice, using SonicMan multiwell sonicator (Matrical, Spokane, USA). The cell debris was removed by centrifugation at 12,000 *g* at 4 °C for 15 min.

The enzyme purification was performed as described previously (Gadda and Wilkins, 2003) with slight modifications. Briefly, the supernatant was subjected to 50% ammonium sulphate saturation and incubated on ice for 30 min, and centrifuged at 10,000 *g* for 10 min. The resulting pellet was suspended in 3.0 mL of 1.5 mM EDTA, 20 mM potassium phosphate (pH 7.0) and centrifuged at 10,000 *g* for 10 min. Later, the supernatant was transferred into a HiPrep 16/10 DEAE Fast Flow column (Amersham Pharmacia Biotech Inc., USA) equilibrated with 1.5 mM EDTA and 20 mM potassium phosphate (pH 7.0). Equilibrated buffer solution was also used as mobile phase at a flow rate of 1.0 mL/min. The elution was suspended in the mixture of 2.5 M sorbitol, 25% glycerol, 20 mM potassium phosphate (pH 7.0) and stored at −20 °C.

2.6. Enzyme assay

The concentration of dehydrogenase was determined by the method of Bradford Bradford, (1976) by using bovine serum albumin as the standard. The oxidase activity of the enzyme was measured with 15 mM choline (Sigma) as the substrate in 50 mM potassium phosphate (pH 7.0), by monitoring the rate of oxygen consumption (Gadda and Wilkins, 2003). The dehydrogenase activity of the enzyme was measured with 2 mM phenazine methosulfate (Sigma) as the primary electron acceptor using a coupled assay in which the enzymatically reduced phenazine methosulfate is spontaneously reoxidized by molecular oxygen. One unit of enzymatic activity corresponds to the conversion of a micromole of oxygen per minute.

2.7. In silico sequence analysis

The nucleotide sequences obtained were compared against database sequences using BLAST provided by NCBI (http://www.ncbi.nlm.nih.gov) and were aligned and clustered using CLUSTAL-X version 1.81 program (Thompson et al., 1997). The output alignments were imported into the GeneDoc program (http://www.psc.edu/biomed/genedoc/) and BioEdit version 7.05 program (www.mbio.ncsu.edu/BioEdit/) to calculate the percent identities among the nucleotide and amino acid sequences. The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (http://www.expasy.org/tools/protparam). The secondary structure prediction was performed using the PSIRED server (http://www.bioinf.cs.ucl.ac.uk/psipred). The nucleotide sequences generated in this study has been deposited in NCBI GenBank under the accession numbers JQ307203 and JQ307204.

3. Results

3.1. Cloning and sequence analysis of glycine betaine biosynthesis genes

The betaine aldehyde dehydrogenase gene (*GbsA*) and betaine alcohol dehydrogenase gene (*GbsB*) is encoded by polynucleotides of 1473 bp and 1182 bp respectively and the polypeptides of 490 and 393 amino acids with the calculated molecular masses of 53,665 and 42,386 Da., based on the *in silico* estimates. After PCR amplification, the purified products were cloned into pDrive. The recombinant transformants with *GbsA* and *GbsB* genes were also confirmed by double digestion with the restriction enzymes.

The *GbsA* and *GbsB* sequences from *B. subtilis* MA04 was compared with the reported nucleotide and amino acid sequences of other eubacteria *viz. Bacillus pumilus* (GenBank accession no. CP000813), *Bacillus licheniformis* (AE017333), *Bacillus licheniformis* (CP0000023), *Bacillus amyloliquefaciens* (CP0005601), using Clustal W program. *In silico* nucleotide sequences analysis of *GbsA* and *GbsB* genes revealed a