Solvent-tolerance and trehalose accumulation by expression of *otsA* and *otsB* homologs in the response to toluene of *Pseudomonas sp.* BCNU 106 isolated from waste water

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Abstract

Pseudomonas sp. BCNU 106 accumulated approximately 4.12 mM trehalose after cultivation of 12 hr probably by the arising action of trehalose-6-phosphate synthase/ phosphatase. The cDNA clones of trehalose-6-phosphate synthase/ phosphatase were isolated from *Pseudomonas* sp. BCNU 106, and named as *PsTPS* and *PsTPP* (*Pseudomonas* sp. BCNU 106 trehalose-6-phosphate synthase/phosphatase). The two mRNA levels of trehalose-6-phosphate synthase/ phosphatase peaked at 12 hr after exposure to toluene, and thereafter were declined slightly. These results support an important role of trehalose accumulation by expressions of *Ps*TPS and *Ps*TPP in toluene-tolerance of *Pseudomonas* sp. BCNU 106.

Introduction

Organic solvents containing toluene are known to be extremely toxic to microbial cells, even at very low concentration of 0.1% (v/v), and to accumulate in and disrupt the bacterial cell membrane thus affecting the structural and functional integrity of the cell[1)]. Many compatible solutes including trehalose, betaine and proline proved to be effective stabilizers for the adaptative mechanisms, providing protection to high concentration of salt, high temperature, freeze-thawing and drying[2)]. Among them, trehalose synthesis occurred in yeast cells subjected to various stress treatments such as ethanol, dehydration or hyperosmotic shock[3)]. Intriguingly, trehalose content in a solvent- tolerant *Pseudomonas* strain significantly increased in response to organic

solvents[4]. However, gene expression levels for trehalose synthesis in solvent-tolerant bacteria have not been well determined after the exposure to solvents.Therefore, we are interested in elucidating the mechanisms of bacterial tolerance to toxic solvents and identifying genes involved in the trehalose synthesis in toluene- tolerant bacteria.

Materials and methods

2.1 Determination of intracellular trehalose and assay of trehalase activity Aliquots of 40 mg (wet weight) were harvested from the culture and washed twice. Trehalose was extract from the cells in 1 ml of boiling water and hydrolyzed to glucose using acid trehalase. The trehalase activity was determined using an assay condition containing 200㎕ of 200 mM trehalose in 100 mM sodium acetate (pH 5.6) and 50㎕ of cell-free extracts by incubating at 37℃ for 30 min. The reaction was stopped by heating at 100℃ for 5 min. One unit of trehalase is defined as the amount of enzyme which produces 2 mol glucose per min. Specific activity is expressed as mU/mg wet weight. Protein concentration was measured as described.

2.2. Cloning and nucleotide sequencing of *PsTPS* and *PsTPP* genes

The *PsTPS* and *PsTPP* genes were obtained from *Pseudomonas* sp. BCNU 106 genomic DNA by amplification by the *Taq* PCR with the following primers (*PsTPS* ; upstream primer: 5-AAC**ATG**AGTCGTTTAGTCGTAGTATCT-3; downstream primer: 5-TTG**CTA**CGCAAGCTTTGGAAAGGTAGC-3), and (*PsTPP*; upstream primer: 5-AAC**ATG**ACAGAACCGTTAACCGAAACC-3; downstream primer: 5-TTG**TTA**GATACTACGACTAAACGACTC-3), which contain the translation initiation and termination codons (bold) of the *E. coli OtsA* and *OtsB* genes, respectively. After a standard PCR of 30 cycles, aliquots were run on an agarose gel. Each fragment of accurate size was cloned into a *pGEM-T Easy* vector (Promega, USA) and detected by automatic DNA sequencer (ABI 373A; Applied Biosystems, USA). The nucleotide sequence analysis was performed with the National Center for Biotechnology Information BLAST server.

2.3. Reverse transcriptase (RT)-PCR analysis

For RT-PCR experiments, RNA was extracted using hot phenol method and purified RNA samples treated with DNase I of the two microgram were incubated with each downstream primers described as above and Superscript II reverse transcriptase (Life Technologies) at 42℃ for 60 min. 2$\mu\ell$ of this reaction mixture was used as the substrate for PCR (25 cycles of 94℃ for 2 min, 50℃ for 1 min, and 72℃ for 2 min in a 50$\mu\ell$ reaction mixture with 2 mM $MgCl_2$), using Amplitaq DNA polymerase.

Results and discussion

3.1. Accumulation of intracellular trehalose in toluene-treated *Pseudomonas* sp. BCNU 106

It is a solvent-tolerant strain able to grow in a culture medium containing high 50% (v/v) toluene. Interestingly, the strain was retarded the growth until 12 hr following 10%, 20%, 30% and 50% toluene additions and then, well -adapted to various concentrations of toluene (Fig. 1). After 20 hr from start point of the culture, the growth at high concentrations of toluene was more rapid than that at low concentrations of toluene. To further confirm the cell growth, cell dry weight was measured in the presence of 10% (v/v) toluene and without toluene. As shown in Fig. 2, toluene did not retard the growth of *Pseudomonas* sp. BCNU106. These results indicate that the strain is a novel solvent-tolerant bacteria which is able to grow with toluene as the sole carbon source. Parallel measurements of intracellular trehalose content revealed a very low basal level of the disaccharide in the non-treated cell lines, but after 10% (v/v) toluene treatment, these cells gradually accumulated a great amount of trehalose, which reached its highest level at 12 hr. Thereafter, the trehalose content declined slightly (Fig. 3). This accumulation of intracellular trehalose may be explained by the toluene-induced activations of the trehalose-6- phosphate synthase/phosphatase, which will be discussed below.

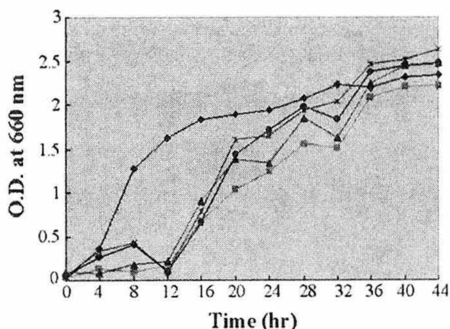

Fig. 1. Growth of *Pseudomonas* sp. BCNU 106 with various toluene concentrations.

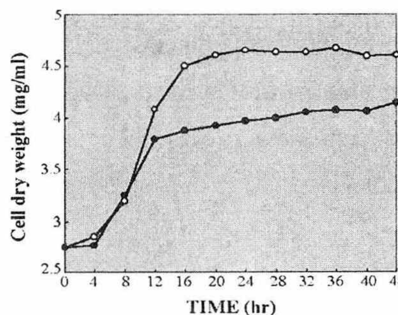

Fig. 2. Growth of *Pseudomonas* sp. BCNU 106 with 10% (v/v) toluene and without toluene for 44h.

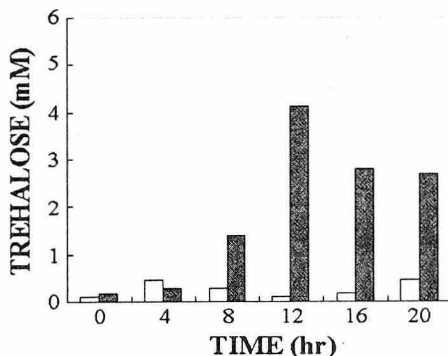

Fig. 3. The intracellular trehalose accumulation in toluene tolerant *Pseudomonas* sp. BC3.

3.2. Enzymatic assay of trehalase.

The trehalase activity was determined during batch culture supplemented with 10% (v/v) toluene and without toluene. The trehalase activity was practically unaffected in the toluene tolerant *Pseudomonas* sp. BCNU 106 with toluene when compared with variation of trehalose (Fig. 4).

.3.3. Isolation and identification of *PsTPS* and *PsTPP* genes

In *E. coli*, two enzymes, the trehalose-6-phosphate synthase/phosphatase encoded by *OtsA* (TPS activity) and *OtsB* (TPP activity) play a key role in the pathway for total trehalose accumulation [29]. Therfore, we isolated two genes involved in trehalose

synthesis from an organic solvent-tolerant bacteria *Pseudomonas* sp. BCNU 106 genomic DNA using the two sets of oligonucleotides to PCR amplify 1.5 kb and 0.8 kb regions of the strain that contained the putative *TPS/TPP* coding regions. Based on BLAST analysis, the predicted PsTPS and PsTPP amino acid sequences exhibited highly homologous to otsA (96%) and otsB (99%) of *E. coli*, respectively. It is assumed that the two strains have similarity in the gene arrangement because of the same gram-negative bacteria.

3.4. Expression pattern of *PsTPS* and *PsTPP* genes at the mRNA level in response to toluene

As shown in Fig. 5, A and B, the two mRNA levels were sustained at high levels for 4 to 12 hrs, and declined slowly by 24 hr. These results is in good agreement with data of trehalose concentrations shown in Fig. 3. Taken together, it has been shown that *Pseudomonas* sp. BCNU 106 strain is resistant to organic solvents by trehalose accumulation by expressions of *Ps*TPS and *Ps*TPP in one of the mechanisms of bacterial tolerance to toxic solvents.

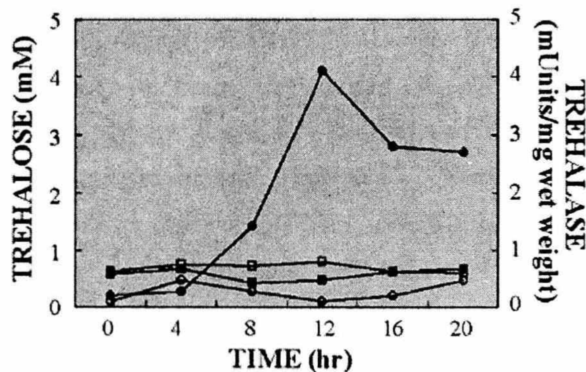

Fig. 4. Comparison of toluene effect on the level of trehalose content (circles) and trehalase activity (squares) in ***Pseudomonas* sp. BCNU** 106 cells.

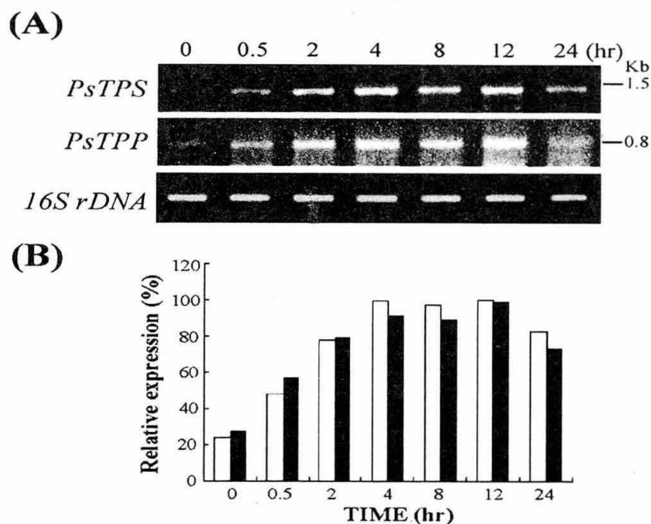

Fig. 5. Expression pattern of *Pseudomonas* sp. BCNU 106 *TPS* and *TPP* genes in response to toluene.

References

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