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Diversity of the ectoines biosynthesis genes in the salt tolerant *Streptomyces* and evidence for inductive effect of ectoines on their accumulation

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ABSTRACT

Streptomyces commonly produce ectoines as compatible solutes to prevent osmotic stresses. Fine structure of the genes producing ectoine (*ectC*) and hydroxyectoine (*ectD*) enzymes in *Streptomyces rimosus* C-2012 as a slightly halophilic bacterium is reported in this study. Deduced amino acid sequences of *ectC* and *ectD* genes from strain C-2012 and some other related species were compared and 72–90% and 13–81% identities were detected for *ectC* and *ectD*, respectively. High similarity of *ectC* between closely or distantly related *Streptomyces* to the strain C-2012 may indicate horizontal transfer of this gene. However, phylogenetic relationships of *ectD* were correlated with phylogenetic affiliation of the strains. It suggests that the ability of *Streptomyces* to produce hydroxyectoine has been the result of a vertical transfer event. HPLC analysis showed that strain C-2012 was able to produce ectoine and hydroxyectoine both in the presence and absence of external salinity (up to 0.45 M NaCl). Accordingly, reverse transcription quantitative PCR (RT-qPCR) showed that *ectABCD* operon in this strain is positively affected by salt. Also, inductive effect of the salt was increased when it was applied with 1 mM of ectoines. Transcription level of *ectC* was increased 2.7- and 2.9-fold in the medium supplied with salt and ectoine and salt and hydroxyectoine, respectively. The effect of salinity with or without ectoines was more on *ectD* transcription level than that of *ectC*. In *S. rimosus* under salt stress, ectoine and hydroxyectoine biosynthesis primarily depends on the stimulation of *ectABCD* operon transcription. However, drastic accumulation of ectoine and hydroxyectoine without increase in *ectC* and *ectD* transcripts was observed in the medium supplied with salt and ectoines and that suggest there might be additional posttranscriptional level of control. Increases in ratio of some intracellular free amino acids in salt stressed to unstressed conditions were observed in cells grown with ectoines. Our results suggest the possibility of a supplementary role of ectoines to improve structure and function of the cells in stressful environments as well as their important role as osmoprotectants.

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1. Introduction

Members of the *Streptomyces* genus are among the most important industrial microorganisms because of their economic importance as producers of antibiotics, enzymes and pharmacologically active agents (Bérdy 2005; Watve et al. 2001). *Streptomyces* cope with osmotic stress by accumulating or *de novo* synthesizing of low molecular weight, highly water-soluble organic solutes, so-called compatible solutes or osmolytes. Ectoine, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid (Galinski et al. 1985) and its hydroxylated derivative, 5-hydroxyectoine (Ingbar and Labidot 1988) are two of the most commonly found osmolytes in *Streptomyces*. As well as their osmoprotective function, these compatible solutes affect the stability and correct folding of proteins and protect biomolecules such as enzymes and nucleic acids under stress conditions (Harishchandra et al. 2010; Roychoudhury et al. 2012; Graf et al. 2008). To date, several diseases related to protein misfolding are introduced. Potential application of ectoines to inhibit misfolding of proteins highlighted the role of these components as therapeutic (Barth et al. 2000; Arora et al. 2004).

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Also, due to preventing whole cell damage and loss of viability (Pastor et al. 2010), ectoines have been target of cosmetics interest. The biosynthesis pathways for ectoine and hydroxyectoine have been elucidated in many microorganisms and usually occur in four enzymatic steps (Peters et al. 1990). Firstly, aspartate semialdehyde is converted into diaminobutyric acid by diaminobutyrate transaminase (EctB), which is subsequently acetylated to Nγ-acetyldiaminobutyrate by diaminobutyrate acetyl transferase (EctA). The cyclic condensation of this compound by ectoine synthase (EctC) leads to the formation of ectoine. Hydroxyectoine is synthesized via ectoine hydroxylation by ectoine hydroxylase (EctD protein) (Bursy et al. 2007; Prabhu et al. 2004). Ectoine and hydroxyectoine biosynthetic enzymes are encoded by an evolutionarily highly conserved gene cluster, *ectABCD* in the *Streptomyces* species (Bursy et al. 2008). Biosynthesis, uptake and accumulation of ectoines and also enzymatic properties of the purified ectoine hydroxylase, EctD in *Streptomyces coelicolor* A3(2) are previously reported (Bursy et al. 2008; Kol et al. 2010). These reports demonstrated that intracellular levels of ectoines have been increased in response to elevated salt concentrations. The osmoregulated expression of *ectABC* was also revealed in Gram-negative *Methylomicrobium alcaliphilum* (Reshetnikov et al. 2006) and *Chromohalobacter salexigens* (Calderon et al. 2004). However, little is known about the regulation of the ectoine biosynthetic pathway in *Streptomyces* and whether accumulation of ectoines is regulated at posttranscriptional or posttranslational level. However, little is known about the regulation of ectoine biosynthesis and/or accumulation in *Streptomyces.* In the present study, the molecular structure of the predicted *ectABCD* gene cluster in a salt tolerant Actinomycetes *Streptomyces rimosus* C-2012 was undertaken. In addition, attempts were made to reveal the regulatory effect of high salinity and exogenously provided ectoines on accumulation of intracellular ectoine, hydroxyectoine and amino acids in this strain.

2. Materials and methods

2.1. Bacteria and growth conditions

S. rimosus strain C-2012 (Sadeghi et al. 2014), *S. griseus* DSM 40941, *S. griseoflavus* DSM 40456, *S. rimosus* DSM 40260, *S. cellulosae* DSM 40362 and *S. monomycini* DSM 41801 were propagated and maintained on MYA plates (containing 10 g/l malt extract, 4 g/l yeast extract, 4 g/l glucose and 18 g/l agar, adjusted to pH 7.2) at 29 °C. Spores of *Streptomyces* strains were suspended in sterile saline solution (0.9% NaCl) and adjusted to a concentration of 10^6 cfu/ml, 1 ml of the spore suspension was grown in (250 ml) Erlenmeyer flasks containing 50 ml MYB medium (MYA without agar) amended with 0–0.45 M NaCl in the absence of added compatible solutes or in the presence of either 1 mM ectoine (Fluka), 1 mM hydroxyectoine (Fluka) or an equimolar mixture of both ectoines. The Erlenmeyer flasks were incubated at 29 °C and 150 rpm in rotary shaker incubator. In all experiments, medium adjusted to pH 7.2 after salt addition. After 3 days, mycelia and spores were harvested by centrifugation and were lyophilized, then dry weight of the cells was determined. The temperature range and NaCl tolerance were determined in the MYA medium amended with 0.7–1.2–1.7 and 2.2 M NaCl, as described by Shirling and Gottlieb (1966), followed by incubation for 7 days at 29 or 45 °C.

2.2. Genomic DNA preparation and phylogenetic studies

Isolation of genomic DNA was performed according to the method of Tripathi and Rawal (1998). PCR amplification of a 2.85 kb DNA fragment containing almost-complete sequence of *ectABCD* gene cluster from strain C-2012, *S. cellulosae* and *S. monomycini* was carried out by using primers ectA-F (5′-ACCGTGGAGACCACCATCA-3′) and ectD-R (5′-ACTTCACTCCGGTGAAGTAG-3′). This primer pair was designed to amplify the *ectD*, *ectC*, *ectB* and 5′ end of the *ectA* genes based on the registered sequences. The PCR product was sequenced at Sequetech (Mountain View, California, United States). The sequences generated in this study were deposited in the GenBank database under the accession numbers: KC876560, KC876561, KC876562 (strain C-2012), KF435138, KF435140 (*S. monomycini*), KF435139, KF435141 (*S. cellulosae*) and KJ365307, KJ365308 (*S. griseoflavus*). Sequences of the genes were aligned manually with corresponding sequences of available *Streptomycetes* species drawn from the GenBank, EMBL and DDBJ databases by using BLAST (Altschul et al. 1997). Phylogenetic trees were performed based on the *ectC* and *ectD* sequences from strain C-2012 and other *Streptomyces* viz. *S. rimosus*, *S. griseus*, *S. griseoflavus*, *S. cellulosae* and *S. monomycini*. The software package MEGA 4 (Tamura et al. 2007) was used after multiple alignments of the sequence data with CLUSTAL W (Thompson et al. 1994). The trees were constructed using the neighbor-joining algorithm with MEGA (version 4) software (Tamura et al. 2007). Bootstrap analyses were used to evaluate the stability of relationships based on 1000 resampling.

2.3. Reverse transcription quantitative PCR (RT-qPCR)

Total RNAs of the each growth condition was extracted from the cells grown in MYB medium using Trizol reagent (Invitrogen). One microgram of each RNA sample was used for constructing cDNA after it was treated with RNase-free DNase I (Invitrogen) using an iScript cDNA Synthesis kit (BioRad). The transcription of *ectC* and *ectD* were analyzed by reverse transcriptase PCR (RT-PCR) using specific primers: ectC-F (5′-TTCAAGGAGATCGAAGGCAC-3′), ectC-R - (5′-GCGTACAGGATCGTCTCG-3′) and ectD-F (5′-ACGAGGCGCTGACGAAGAT-3′), ectD-R (5′-GCTGTTGAACACGATGAACAC-3′). Gene expression was assayed using the iCycleriQ, multicolor real-time PCR detection system (BioRad) and iQ SYBR Green Supermix kit (BioRad), according to the manufacturer's instructions. The following PCR profile was used: 3 min at 95 °C, (45 s at 95 °C, 45 s at 60 °C, 45 s at 72 °C) × 50, and 5 min at 72 °C followed by recording of a melting curve. The lack of primer-dimer or nonspecific product accumulation was checked by melt-curve analysis. The transcription of the 16S rRNA gene was used as an internal control with 16S-F (5′-GGGAGCGAACAGGATTAGATAC-3′) and 16S-R (5′-CCTTTGAGTTTTAGCCTTGCGG-3′) primers. Results were expressed as the normalized ratio of mRNA level of genes of interest (*ectC* and *ectD*) over internal control (16S rRNA gene). For estimating fold changes in gene expression, a Microsoft Office Excel 2007 sheet was planned by using relative standard curve method based on the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The induction fold resulted in three independent biological samples was averaged, and each reaction was performed twice for approval. Standard deviations and ranking with Duncan's multiple range test were used to show significant differences between transcript abundance in normal and salt treated samples at $p < 0.05$.

2.4. High performance liquid chromatography (HPLC) analysis of ectoine and hydroxyectoine from cell extracts

Bacterial cells were harvested by centrifugation and were lyophilized. The dry weight of the cells was determined, and 10 mg lyophilized cells were extracted with 570 μl of an extraction mixture (methanol/chloroform/water, 10:5:4 [v/v/v]) by vigorous shaking for 5 min. Equal volumes (170 μl) of chloroform and water were then added. The mixture was again shaken for 10 min; phase separation was enhanced by centrifugation in an Eppendorf tabletop centrifuge at 13,000 rpm for 30 min. The hydrophilic top layer

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