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ORIGINAL PAPER

Regulation of expression of trehalose-6-phosphate synthase during cold shock in *Arthrobacter* strain A3

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Abstract Trehalose is a chemical chaperone known to protect a variety of organisms against cold stress. Members of the genus *Arthrobacter*, which belongs to the *Actinomycetales* group, exhibit strong resistance to stress conditions, but exactly how trehalose synthesis is regulated in conditions of cold stress is still unknown. Here, we report that *Arthrobacter* strain A3, which was isolated from the alpine permafrost, has only two trehalose synthesis pathways (OtsA/B and TreS), while other *Arthrobacter* spp. have three. Mutants and immunoblot analyses indicate that trehalose is mainly synthesized via OtsA at low temperatures in *Arthrobacter* strain A3. Therefore, we have focused on the regulation of OtsA expression during cold shock. The results indicated that both low temperature and accumulation of trehalose can inhibit OtsA expression. The elongation factor Tu, which binds to OtsA, stabilizes the expression of OtsA in the cold.

Keywords Trehalose · OtsA · *Arthrobacter* · Cold shock · EF-Tu

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X.-M. Chen and Y. Jiang contributed equally to this work.

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Introduction

Arthrobacter strains, which are most frequently isolated in soils, play important roles in biogeochemical cycles and decontamination (Frias et al. 2009; Hayashi et al. 1993; Igloi and Brandsch 2003; Negrete-Raymond et al. 2003; Nordin et al. 2005; Turnbull et al. 2001; Unell et al. 2008). They have high resistance to cold, heat, desiccation, ionizing radiation, oxygen radicals, and toxic chemicals (Boylen 1973; Labeda et al. 1976; Robinson et al. 1965; Zevenhuizen 1966). However, the mechanisms by which they survive in stress environments are still unknown. We have isolated *Arthrobacter* strain A3, a psychrotrophic bacterium, from the alpine permafrost of the Tianshan Mountains in China. Its optimal growth temperature is 20°C, but it can survive and grow at near-freezing temperatures as low as −4°C. This study focuses on the cold shock response and cold adaptation of this strain.

Trehalose (α-D-glucopyranosyl(1,1)-α-D-glucopyranoside) is a non-reducing disaccharide that has been reported to serve as a protectant against a variety of stress conditions including desiccation, dehydration, heat, cold, and oxidation (Elbein et al. 2003). It is known to be critical for cellular survival at near-freezing temperatures (Kandror

et al. 2002). At least four different pathways for trehalose biosynthesis have been reported, described as OtsA/B, TreY/Z, TreS, and TreT (Elbein et al. 2003; Qu et al. 2004). The most widely reported and best characterized pathway is OtsA/B, which depletes UDP-glucose and glucose-6-phosphate (Elbein et al. 2003). In response to many environmental stresses, rapid increase of the trehalose pool has been attributed to the increased expression of trehalose-6-phosphate synthase (OtsA, or TPS in *Arabidopsis thaliana*) (Cytryn et al. 2007; White-Ziegler et al. 2008). In addition to synthesizing trehalose-6-phosphate (T6P), OtsA may also control certain metabolic pathways or even affect growth in *Saccharomyces cerevisiae* (Noubhani et al. 2000). Through its involvement in glucose transport and kinase activities, OtsA may have a metabolite regulatory function that restricts glucose influx (Thevelein 1992). Furthermore, T6P, the product of OtsA, inhibits hexokinase activity (Blazquez et al. 1993) and effects post-translational redox activation of ADP-glucose pyrophosphorylase to regulate starch synthesis in *A. thaliana* (Kolbe et al. 2005). Taken together, these data have led to a temporal model where OtsA is believed to play an important role in cell metabolism which therefore must be tightly regulated. In turn, this implies that changing the OtsA content may result in not only alteration of trehalose levels, but also may impact many related metabolic pathways.

EF-Tu is a GTPase that plays a central role in bacterial protein synthesis by mediating the transport of aminoacyl-tRNA to the ribosomal A site (Miller and Weissbach 1977). Caldas et al. (1998) and Suzuki et al. (2007) showed that in addition to its role in translational elongation, EF-Tu has chaperone-like capacity to promote renaturation of denatured. It may also interact with the transcriptional apparatus as a positive regulator of RNA synthesis (Travers et al. 1970; Vijgenboom et al. 1988). In plants, EF-Tu may activate signal transduction and defense responses (Zipfel et al. 2006). This research focuses on the regulatory role of EF-Tu in *Arthrobacter* strain A3.

Previous work has not reported the regulation of trehalose and OtsA under cold stress in *Arthrobacter*. Here, we show that in *Arthrobacter* strain A3 there are two trehalose synthesis pathways and the intracellular OtsA level is a key factor which controls the trehalose level at low temperatures. The results indicated that both low temperature and accumulation of trehalose can inhibit OtsA expression. However, the elongation factor Tu, which can bind to OtsA, stabilizes the expression of OtsA under cold conditions.

Materials and methods

A complete description of recombinant proteins, primary antibodies, antibodies purification, mass spectrometry, and statistical analysis is available in the supplemental material.

Plasmid construction, cultivation, and electroporation

The pART2 plasmid, a vector for gene expression in *Arthrobacter*, was kindly donated by Cristinel Sandu (The Rockefeller University, NY, USA) (Sandu et al. 2005). Plasmids and strains used in this study are listed in supplemental material (Table S1), and oligonucleotide primers are shown in Table S2. *Arthrobacter* strain A3 was grown at 20°C in LB medium. Plasmid electroporation was carried out as described previously (Gartemann and Eichenlaub 2001; Zhang et al. 2011) except that cells were harvested and incubated at 20°C with lysozyme for 1 h. Then the cells were washed three times with deionized water/10% glycerol (vol/vol) followed by transformation using the following parameters: capacity 25 mF, voltage 2.5 kV/cm, resistance 800 Ω. After electroporation, cells were incubated in SB medium for 6 h at 20°C before plating on appropriate selective media.

Cloning of genes

Degenerate primers were designed based on the conserved sequences of *otsA*, *treS*, *glgX*, and *tuf*. Based on the sequences of PCR fragments, the flanking sequences were amplified by Tail-PCR (Liu et al. 2005). Likely ORFs were identified by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). All primers used for DNA cloning are shown in Table S2.

Construction of knock-out mutant

The *otsA* knock-out mutant was constructed as described previously (Murphy et al. 2005). Approximately 1 kb flanking sequences of *otsA* was amplified using the primers listed in Table S2. The construct was designed to remove the entire coding region of the *otsA* gene. Fragments were cloned on either side of the kanamycin resistance cassette, transformed into *Arthrobacter* strain A3 and selected on LB plates containing 140 μg/ml kanamycin. Recombination was confirmed by PCR and sequencing.

Measurement of trehalose

Trehalase (TreA) was expressed and purified from *Escherichia coli.* Trehalose was measured by assaying the release of free glucose following incubation of samples with TreA. Trehalose content was estimated as described (De Smet et al. 2000) with a few minor modifications. Briefly, 10 μl samples were added to 10 μl 1 M NaH_2PO_4, pH 6.0, 80 μl water and 1 μl recombinant trehalase in microtiter-plate

wells and incubated at 37°C for 1 h. Glucose was then measured by adding 100 µl 50 mM NaH_2PO_4, pH 6.0, containing 0.75 mg/ml *o*-dianisidine, 2 U glucose oxidase, and 0.1 U peroxidase (all from Sigma). After 30 min, 134 µl H_2SO_4 (12 N) was added to every sample. After 5 min, color development was measured at 540 nm (Spectramax m2^e, Molecular Devices).

Western blot, coimmunoprecipitation, and immunoprecipitation assay

Western blot analyses were performed as described previously (Yang et al. 2005). Protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane with cold transfer buffer [25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3] for 1 h at 200 mA. Then the membrane was blocked in TBST blocking solution [10 mM Tris, pH 7.4, 0.9% (w/v) NaCl, up to 0.1% Tween-20, 5% skimmed milk powder] for 1 h at 37°C and then hybridized with primary antibody solution (the primary antibody was first diluted in the blocking solution) for 1 h at 37°C. After washing 5 times with TBST, the membrane was hybridized with secondary antibody solution for 1 h at 37°C. The membrane was washed 5 times with TBST, and the signal was detected using a chemiluminescent detection kit (Invitrogen).

Coimmunoprecipitation (coIP) was carried out as previously described (Simpson 2003). Cells were harvested and lysed by ultrasonic disruption in coIP buffer (containing 1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris and 2 mM EDTA pH 7.4) at 0°C. Cell lysates (2 mg protein) were incubated with the purified antibodies (15 µg) at 4°C with agitation for 4 h. The reactions were then incubated with Sepharose-protein A (Pharmacia) at 4°C with agitation for 1 h. Precipitated complexes were subjected to SDS-PAGE and visualized with Coomassie blue.

The immunoprecipitation (IP) assay was carried out using the purified fusion proteins hybridized in coIP buffer at 25°C. After 1 h, purified antibodies were added, and the solution was agitated for another 1 h at 25°C. Then the reactions were incubated with Sepharose-protein A (Pharmacia) at 4°C with agitation for 1 h. Precipitated complexes were subjected to SDS-PAGE and immunoblot.

Nucleotide sequence accession numbers

The DNA sequences of the four genes described in this study have been deposited in GenBank under accession numbers GQ_331049 (*otsA*), GQ_331050 (*tuf*), GQ_331051 (*treS*), GQ_331052 (*glgX*), and DQ173036 (16S rRNA).

Bacteria strain accession numbers

The *Arthrobacter* strain A3 has been deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number 1.8987.

Results

Characterization of *Arthrobacter* strain A3

We have isolated a psychrotrophic bacterium from the alpine permafrost of the Tianshan Mountains in China. It forms long and short rods during the early stages of growth and cocci during the stationary phase. This bacterium is gram positive, oxidase negative, and catalase positive. By comparing the sequence of its 16S rRNA and total cellular fatty acid content (Table S3) with other bacteria, it was identified as a member of the genus *Arthrobacter* and designated strain A3 (the wild-type strain is named Ar0001). The optimal growth temperature and the maximum growth temperature of this bacterium is 20 and 30°C, respectively. It can grow at near-freezing temperatures as low as −4°C. Therefore, it is an ideal strain to study the cold adaption mechanisms of *Arthrobacter*.

The decrease of trehalose level corresponds to a reduction of intracellular OtsA in the cold

In *E. coli* and *S. cerevisiae*, trehalose levels are almost undetectable during exponential growth but increase rapidly and dramatically under near-freezing temperature shock (Kandror et al. 2002; Tai et al. 2007). In *Arthrobacter* strain A3, we observed that the trehalose level also increased when it was subjected to a 4°C cold shock. The maximum level was reached at around 2.5 h then decreased rapidly after 3 h (Fig. 1a). However, trehalose levels were almost constant at 20°C (Fig. 1b).

Previous bioinformatic analysis demonstrated the presence of three potential pathways for trehalose biosynthesis in different *Arthrobacter* species (Mongodin et al. 2006). To identify their relative contribution to trehalose synthesis in the cold, we cloned the genes of enzymes involved in these pathways. However, we were unable to identify the TreY/Z pathway in the genome of *Arthrobacter* strain A3 (Fig. S1a), and the respective genes are absent in the region of the genome of strain A3 that is otherwise syntenic with the region containing these genes in sequenced *Arthrobacter* genomes (Mongodin et al. 2006). In addition, we determined the conserved regions of the amino acid sequences of TreY and TreZ through sequence alignment and chemosynthesized these regions for antibody preparation (Table S4). However,

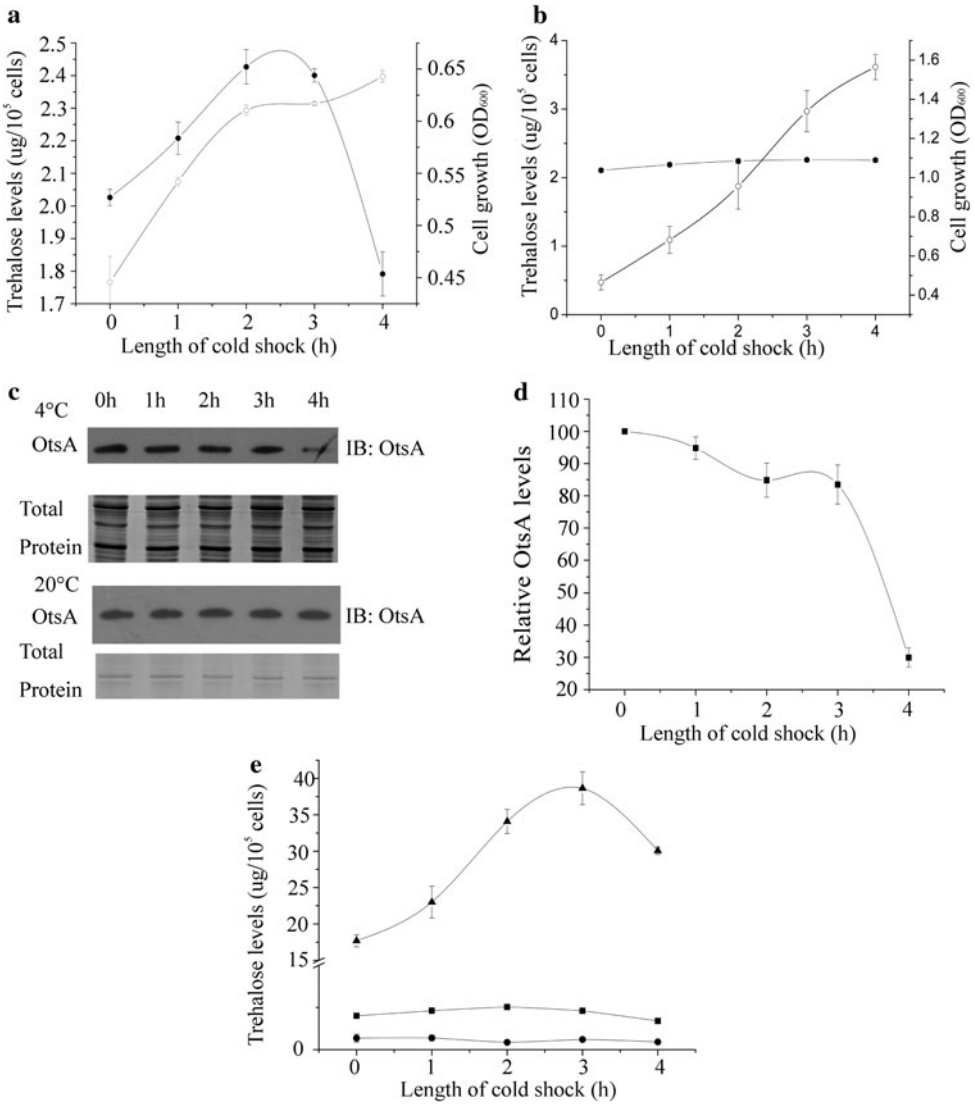

Fig. 1 Changes in trehalose levels correspond to OtsA levels during cold shock. **a** Strain Ar0001 was grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C and then subjected to 4°C cold shock. Trehalose content, represented by *black circles*, was measured as described in "Materials and methods" and normalized to cell counts. Cell growth, represented by *white circles*, was monitored by optical density at 600 nm (Smart Spec Plus, Bio-Rad, 10 mm cuvette). **b** Strain Ar0001 was grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C. Trehalose content is represented by *black squares*. Cell growth is represented by *white squares*. **c** Immunoblot analyses were carried out with anti-OtsA antibody and normalized to total proteins. **d** Graphical representation of OtsA protein levels at 4°C cold shock, normalized to total proteins. **e** The levels of trehalose in wild-type strain (Ar0001/pART2), OtsA overexpressing strain (Ar0001/pART2-*otsA*), and Δ*otsA* strain (Ar0002) are represented by *black squares*, *black triangles*, and *black circles*, respectively. Results shown are representative of three independent experiments. *Error bars* show standard errors of the mean (SEM). $P < 0.05$

using immunoblot analyses, we were unable to detect TreY and TreZ in strain A3 (Fig. S1c). As cold shock progressed, OtsA levels diminished (Fig. 1c, d) although total protein content was proportional to cell density (data not shown). Notably, the observed decrease in OtsA levels was consistent with the reduction of trehalose levels. The OtsA levels without cold shock were constant (Fig. 1c). The levels of TreS did not change at all under conditions of 4°C cold shock (Fig. S1b).

To confirm the relationship between OtsA and trehalose, we constructed an *otsA* deletion mutant (Ar0002) and an OtsA overexpression strain of *Arthrobacter* strain A3 (Ar0001/pART2-*otsA*). The Δ*otsA* strain had significantly lower trehalose levels than the wild-type strain at all times prior to and during cold shock. However, the OtsA overexpression strain had higher trehalose levels during cold shock (Fig. 1e). These results suggested that the decrease of trehalose under conditions of low temperature is specifically related to the decrease in OtsA levels.

We also analyzed the enzyme activity of OtsA. This indicated that the catalytic activity of OtsA at 4°C was 1.2-fold greater than that at 20°C, which may explain the increase of trehalose in the first 2–3 h of cold shock. All together, these data indicated that OtsA was the main factor responsible for regulating trehalose content in *Arthrobacter* strain A3.

Repression of synthesis of OtsA by cold

It was not known whether the decrease of OtsA level was caused by its accelerated degradation or its decelerated synthesis at low temperature. A strain that constitutively expressed OtsA-His_8 was subjected to immunoblot for analysis of the degradation of OtsA. If cold could accelerate

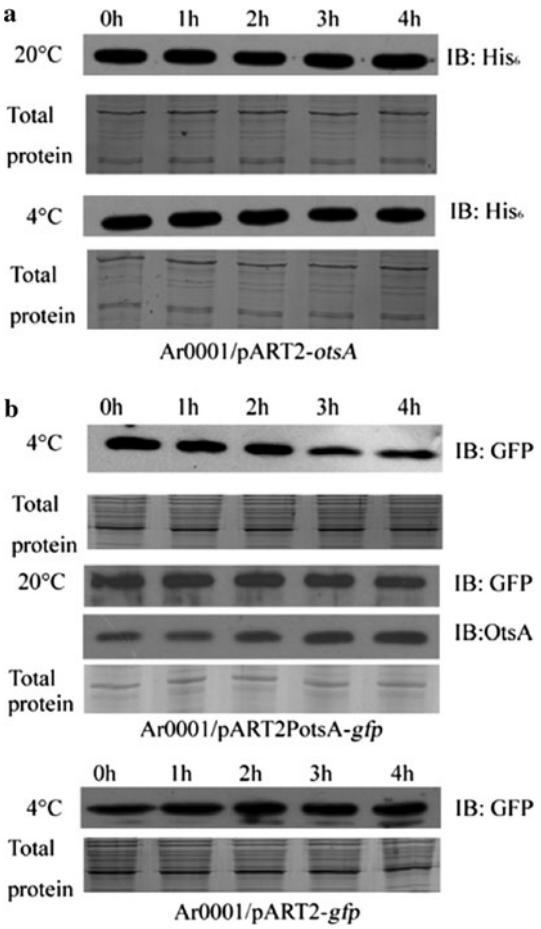

Fig. 2 Cold repressed OtsA synthesis. **a** Ar0001/pART2-*otsA* strain was grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C, and then half of the cells were subjected to 4°C cold shock. Immunoblot analyses were carried out respectively with anti-His_6 antibody and normalized to total proteins. **b** Strains Ar0001/pART2-*gfp* and Ar0001/pART2P_{otsA}-*gfp* grew to logarithmic phase (OD_{600} 0.5–0.8) at 20°C and then subjected to 4°C cold shock. Immunoblot analyses were carried out with anti-GFP and anti-OtsA antibodies and normalized to total proteins. Results shown are representative of three independent experiments

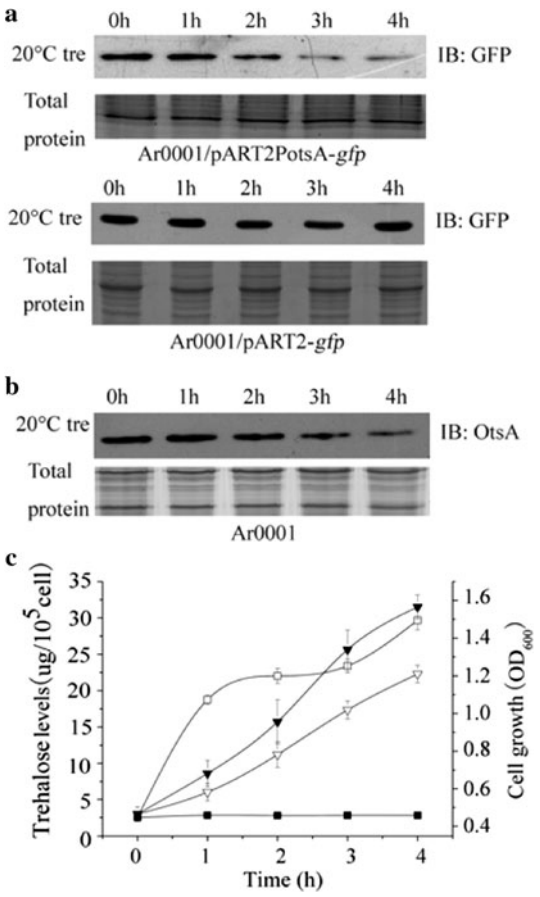

Fig. 3 Accumulation of trehalose represses OtsA expression. **a** Strains Ar0001/pART2-*gfp* and Ar0001/pART2P_{otsA}-*gfp* were grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C, and then 100 mM trehalose was added to the growth medium. Immunoblot analyses were carried out with anti-GFP antibody and normalized to total proteins. **b** Strain Ar0001 was grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C, and then 100 mM trehalose was added to the growth medium. Immunoblot analyses were carried out with anti-OtsA antibody and normalized to total proteins. **c** Trehalose levels in cells with and without 100 mM trehalose incubation are represented by *white squares* and *black squares*, respectively. Cell growth with and without 100 mM trehalose incubation at 20°C is represented by *white triangles* and *black triangles*, respectively. Results shown are representative of three independent experiments. *Error bars* show standard errors of the mean (SEM). $P < 0.05$

OtsA degradation, the OtsA-His_8 levels would decrease at 4°C compared with those at 20°C. However, as shown in Fig. 2a, cold did not accelerate the degradation of OtsA.

To determine whether the synthesis of OtsA decreased in the cold, we constructed strains Ar0001/pART2P_{otsA}-*gfp* and Ar0001/pART2-*gfp* (used for control). GFP levels, which were under the control of the *otsA* promoter, decreased during 4°C cold shock, but were constant at 20°C. The levels of OtsA followed the same trend as the GFP levels (Fig. 2b). Trehalose levels exhibited no significant changes at 20°C as well (Fig. 3c). However, cold had no negative effect on the constitutive promoter (Fig. 2b). Together, these results suggested that cold reduces the rate of transcription of the gene under the control of the *otsA* promoter.

Accumulation of trehalose repressed OtsA expression

In yeast, during heat shock, trehalose can act as an activator of Hsf 1, which is a transcription factor, by mediating phosphorylation (Conlin and Nelson 2007). To determine the effect of a high level of trehalose on OtsA expression, we incubated strain Ar0001/pART2P_{otsA}-*gfp* with 100 mM trehalose added to the growth medium at 20°C. Figure 3c indicated that trehalose accumulated in the cells, and the increased level was similar to that in OtsA overexpressing strain (Fig. 1d). Cell growth was not significantly affected by this level of trehalose (Fig. 3c), and consequently, it is

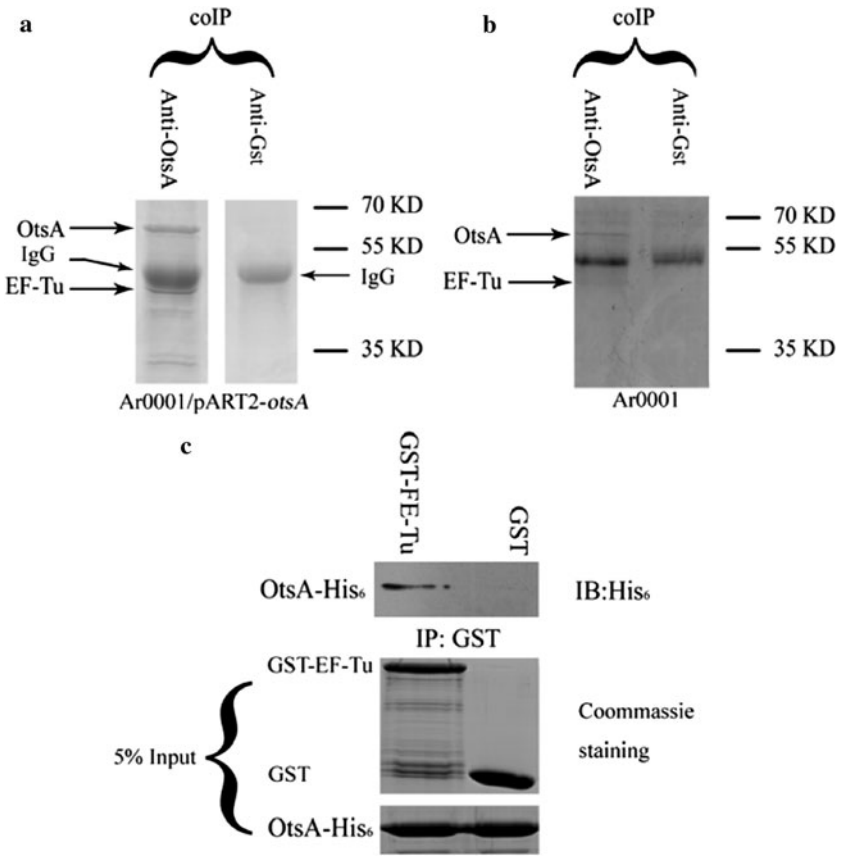

Fig. 4 Identification of EF-Tu as an OtsA-associated protein. **a** OtsA complex was coimmunoprecipitated with anti-OtsA-coupled Sepharose from Ar0001/pART2-*otsA* strain. Anti-GST-coupled Sepharose was the control. The eluted proteins were subjected to SDS-PAGE and stained with Coomassie blue. Proteins were identified by mass spectrometry. **b** Strain Ar0001 was subjected to coIP with anti-OtsA antibody. Anti-GST antibody was used for the control. The eluted proteins were subjected to SDS-PAGE and stained with Coomassie blue. **c** His_6-fused OtsA, GST-fused EF-Tu, and GST were individually generated and purified from *E. coli* (BL21/pET28-*otsA*, BL21/pGEX4T3-*tuf*, and BL21/pGEX4T3). The OtsA-His_6 was incubated with GST and GST-EF-Tu, respectively, and then subjected to IP with anti-GST antibody as described in "Materials and methods". The proteins were diluted to 5% and subjected to SDS-PAGE. Immunoblot analyses were carried out with anti-His_6 antibody. Results shown in **b** and **c** are representative of three independent experiments

unlikely to affect the overall metabolic activity of *Arthrobacter* strain A3.

Immunoblot results indicated that GFP levels decreased under the control of the *otsA* promoter in the presence of a high level of trehalose (Fig. 3a). Next we tested the OtsA content of the wild-type strain which was incubated with 100 mM trehalose at 20°C. Immunoblots revealed that OtsA levels decreased in the presence of a high level of trehalose (Fig. 3b), but the OtsA levels were constant in the absence of trehalose (Fig. 1c). These results imply that the accumulation of trehalose in *Arthrobacter* strain A3 can repress OtsA expression.

OtsA binds to elongation factor Tu

We identified proteins associated with OtsA. Both the wild-type strain and OtsA overexpression strain produced the same protein bands which coimmunoprecipitated with anti-OtsA antibody (Fig. 4a, b). From mass spectrometry analyses (Fig. S3), EF-Tu was identified and chosen for further research.

To confirm the interaction between OtsA and EF-Tu, GST-EF-Tu, GST and OtsA-His_6 were prepared for IP. The GST-fused EF-Tu and GST control were each incubated with the His_6-fused OtsA and then immunoprecipitated with anti-GST antibody and detected by anti-His_6 IB (Fig. 4c). The results indicated that OtsA actually bound to EF-Tu, and the interaction is direct.

Cold enhances the binding interaction of OtsA and EF-Tu

The wild-type strain was incubated at either 20 or 4°C for 1 h and subjected to OtsA IP. As shown in Fig. 5a, the abundance of EF-Tu binding to OtsA at 20°C was less than 4°C. Figure 5b revealed that the binding reaction was enhanced by cold in vitro. These results implied that low temperature facilitated the interaction between OtsA and EF-Tu.

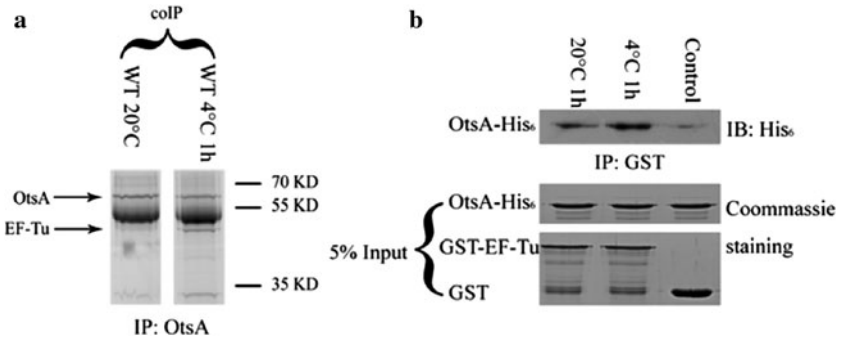

Fig. 5 Cold enhances the binding interaction of OtsA and EF-Tu. **a** Strain Ar0001, incubated at 20 and 4°C, respectively, was normalized to cell counts and subjected to coIP with anti-OtsA antibody. The eluted proteins were subjected to SDS-PAGE and stained with Coomassie blue. **b** His_6-fused OtsA was incubated with GST-fused EF-Tu at 20 and 4°C, respectively, and then subjected to IP with anti-GST antibody (normalized to protein content). OtsA-His_6 and GST incubation was used for the control. The proteins were diluted to 5% and subjected to SDS-PAGE. Immunoblot analyses were carried out with anti-His_6 antibody. Results shown in **a** and **b** are representative of three independent experiments

EF-Tu prevents the observed decrease in the expression of OtsA under cold stress

As mentioned above, EF-Tu can interact with the transcriptional apparatus as a positive regulator of RNA synthesis in addition to the conventional role in polypeptide elongation (Travers et al. 1970; Vijgenboom et al. 1988). To determine if EF-Tu could regulate OtsA levels, we constructed strain Ar0001/pART2-tuf P_{otsA}-*gfp* which constitutively expressed EF-Tu and regulated GFP via the *otsA* promoter and strain Ar0001/pART2-tuf P-*gfp* (used for the control) which constitutively expressed EF-Tu and had a constitutive promoter to direct GFP. Whether the constitutively expressed EF-Tu had influence on OtsA expression during cold shock was judged from the GFP levels. After incubation at 4°C, the levels of GFP under control of the *otsA* promoter were not diminished (Fig. 6a). Comparing with Fig. 2b, which illustrates that levels of GFP under control of the *otsA* promoter decrease in the cold, it indicated that excess EF-Tu prevents the reduction of the GFP levels when the latter's expression is under control of the *otsA* promoter in the cold. To further confirm this result, strain Ar0001/pART2-*tuf* which constitutively expresses EF-Tu was shocked at 4°C and subjected to OtsA IB. Again, we observed that OtsA abundance did not reduce (Fig. 6b), compared to the wild-type strain in which EF-Tu is expressed from its native promoter (Fig. 1c). These results imply that constitutive EF-Tu expression can prevent the observed decrease in the expression of OtsA under cold stress.

To determine if the presence of OtsA can act on EF-Tu to stabilize the OtsA expression in the cold, we analyzed GFP levels of strains Ar0002/pART2-tuf P_{otsA}-*gfp* P-*bla* and Ar0001/pART2-tuf P_{otsA}-*gfp* P-*bla*. The results indicated that the GFP levels in the mutants were much lower than those in the control even without cold shock (Fig. 6c). Moreover, the GFP levels remained low in the Δ*otsA* strain during 4°C cold shock (Fig. 6d). By comparison, the GFP levels in the control strain were high. These data indicated that the role of EF-Tu on OtsA expression in the cold requires the presence of OtsA. We speculate that this is related to the intracellular abundance of the EF-Tu-OtsA complex at low temperatures.

Discussion

Escherichia coli and *Saccharomyces cerevisiae* accumulate trehalose dramatically during cold shock, but at the optimum growth temperature their trehalose levels are very low. However, *Arthrobacter* strain A3 has an appreciable intracellular concentration of trehalose at its optimum growth temperature. As a psychrotrophic bacterium, *Arthrobacter* strain A3 contains cold-adapted enzymes that have high catalytic efficiencies due to increasing conformational flexibility at the expense of stability (D'Amico et al. 2003). These cold-adapted proteins need a certain level of trehalose to protect against denaturation at 20°C. Previous studies have shown that trehalose is also an indispensable substrate for cell wall synthesis in *Actinobacteria* (Tzvetkov et al. 2003; Woodruff et al. 2004). Therefore, *Arthrobacter* strain A3 needs a certain level of trehalose at its optimum growth temperature.

During cold shock, *Arthrobacter* strain A3 exhibits only an initial minor increase in trehalose level compared with *E. coli* where it may be elevated by up to 8-fold and *S. cerevisiae* where it may be elevated by up to 50-fold (Kandror et al. 2002, 2004). This phenomenon indicates that *Arthrobacter* strain A3 is not very dependent on trehalose for resisting cold stress. We speculate that this is probably because *Arthrobacter* strain A3 is better at adapting to the cold than *E. coli* or *S. cerevisiae.*

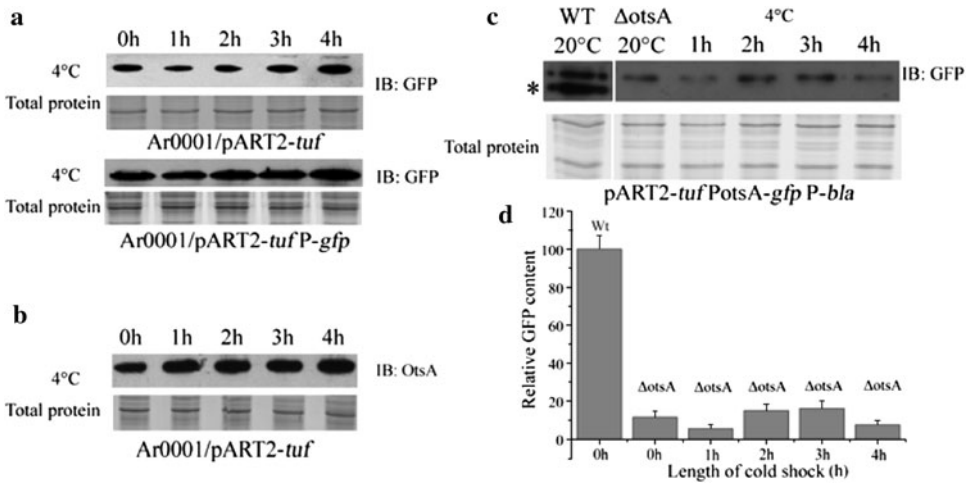

Fig. 6 EF-Tu has a positive effect on OtsA expression in cold in the presence of OtsA. **a** Strains Ar0001/pART2-*tuf* P_{otsA}-*gfp* and Ar0001/pART2-*tuf* P-*gfp* were grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C and then subjected to 4°C cold shock. Immunoblot analyses were carried out with anti-GFP antibody and normalized to total proteins. **b** Strain Ar0001/pART2-*tuf* was grown to logarithmic phase (OD_{600} 0.5–0.8) at 20°C and then subjected to 4°C cold shock. Immunoblot analyses were carried out with anti-OtsA antibody and normalized to total proteins. **c** Strains Ar0002/pART2-*tuf* P_{otsA}-*gfp* P-*bla* and Ar0001/pART2-*tuf* P_{otsA}-*gfp* P-*bla* were treated as described in **a**. The *asterisk* denotes a degraded band. **d** Graphical representation of GFP protein levels from **c**, normalized to total proteins. Results shown are representative of three independent experiments. *Error bars* show standard errors of the mean (SEM). $P < 0.05$

Figure S4a showed that the intracellular trehalose level at logarithmic phase was half of that at stationary phase. This result is similar to differences observed during growth of yeast (Gadd et al. 1987) and indicates that *Arthrobacter* strain A3 accumulates trehalose to protect cells when growth slows down. Therefore, the decrease of trehalose after 3–4-h cold shock is contrary to what occurs in stationary phase. Trehalose is a protectant at the beginning of the cold shock, but can also have negative effects on metabolism subsequently if its concentration remains elevated (Kandror et al. 2002). Moreover, trehalose is considered as an energy storehouse (Elbein et al. 2003). Thus, we speculate that the subsequent decrease of trehalose during cold shock may be due to the energetic utilization of trehalose for maintaining normal metabolism in *Arthrobacter* strain A3. Additionally, the division rate of *Arthrobacter* strain A3 reduces in the cold, and consequently, there is a decrease in the amount of trehalose required for cell wall synthesis.

Maruta et al. (1996) have reported that *Arthrobacter* sp. strain Q36 uses the TreY/Z pathway to synthesize trehalose. Other *Arthrobacter* strains whose genomes have been reported all have three trehalose synthesis pathways (Mongodin et al. 2006). However, *Arthrobacter* strain A3 does not have the TreY/Z pathway. The synthesis of trehalose at low temperature is dependent on the OtsA/B pathway. From the point of view of energetics, 1 mol of trehalose synthesized via the TreY/Z pathway consumes 2 mol of ADP-glucose (for glycogen synthesis). However, 1 mol of trehalose produced via the OtsA/B pathway is achieved from 1 mol Glc-6-P and 1 mol UDP-Glc (Tzvetkov et al. 2003). Therefore, energetically, the OtsA/B pathway is a better choice for cell growth of *Arthrobacter* strain A3 at low temperature.

Previous studies have reported that σ^S is an inducer of *otsA*/*B* of *E. coli* under cold and osmotic stress (Hengge-Aronis et al. 1991; Kaasen et al. 1992; Kandror et al. 2002). *Arthrobacter aurescens* strain TC1 contains 17 σ factors belonging to the σ^{70} family but does not contain the σ^S factor (Mongodin et al. 2006). Thus, the factors which regulate OtsA levels are still unknown. Here, we show that accumulation of trehalose in *Arthrobacter* strain A3 cells has a negative effect on OtsA expression (Fig. 3). It has been previously reported that trehalose has effects on the normal functions of some proteins in addition to protecting them against denaturation. In *Arthrobacter* strain A3, further studies need to be done to better understand if trehalose is involved with σ factors in regulation of *otsA* under conditions of cold stress.

EF-Tu is a multifunction protein. In addition to functioning in translational elongation, it also interacts with the transcriptional apparatus as a positive regulator of RNA synthesis (Travers et al. 1970; Vijgenboom et al. 1988). Here, we show that EF-Tu can prevent a decrease in the expression of OtsA under cold stress. Moreover, as EF-Tu can directly interact with OtsA, we speculate that the interaction between EF-Tu and OtsA plays a role in regulating OtsA expression at low temperature. This can explain the differences in trehalose levels in the wild-type *Arthrobacter* strain A3 and the mutant constitutively expressing OtsA during cold shock. Although low

temperature represses the *otsA* promoter, the enhanced interaction between OtsA and EF-Tu can stabilize OtsA expression at the beginning of cold shock. OtsA catalyzes conversion of UDP-Glc and Glc-6-P to produce UDP and Tre-6-P. In organisms, Glc-6-P is an important substrate for glycolysis. The intracellular concentration of Glc-6-P is high in bacteria and saturating for catalysis by OtsA (Varela et al. 2003). Therefore, the essential factor for OtsA catalysis is its affinity for UDP-Glc. Previous studies have indicated that the affinity for UDP-Glc of OtsA in *Arthrobacter* strain increases during cold shock (Jiang et al. 2010). Thus, trehalose levels increase in the first 2–3 h of cold shock even though there is no corresponding increase in the abundance of OtsA. After 3 h of cold shock, the accumulated trehalose represses *otsA* expression, so that subsequently trehalose levels decrease. The mechanisms of this aspect of regulation of *otsA* expression will be a topic of future studies.

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