Biochemical and Biophysical Research Communications 470 (2016) 331–335

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of an interaction between EI and a histidine kinase-response regulator hybrid protein in *Gluconobacter oxydans*

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ARTICLE INFO

Article history:
Received 23 December 2015
Accepted 8 January 2016
Available online 11 January 2016

Keywords:
Gluconobacter oxydans
Phosphoenolpyruvate: carbohydrate phosphotransferase system
Yeast two-hybrid assay
GST pull-down
Bimolecular fluorescence complementation
Two-component system

ABSTRACT

Gluconobacter oxydans may contain an incomplete phosphoenolpyruvate: carbohydrate phosphotransferase system consisting of three components - EI, HPr and EIIA, while the function of individual members of the system remains unknown. In this research, a specific interaction between EI and a histidine kinase-response regulator hybrid protein was screened by yeast two-hybrid assay, and the interaction was further identified with GST pull-down assay and bimolecular fluorescence complementation assay *in vitro* and *in vivo*, respectively. As the histidine kinase-response regulator hybrid protein serves as a member of two-component system in *G. oxydans*, its interaction with EI implied that PTS may play certain roles in bacteria under stress.

1. Introduction

The phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) is recognized as a transport system in bacteria that catalyzes the uptake of numerous carbohydrates and their simultaneous conversion into respective phosphoesters. After its first discovery in *Escherichia coli*, PTS was also found in many other bacterial species [1]. PTS has two general soluble components, enzyme I (EI) and HPr, and a membrane-bound sugar specific permease (enzymes II) [2].

EI of the PTS is a highly conserved bacterial protein which has only minimal similarity to animal proteins [3,4]. The protein is composed of an N-terminal protein binding domain (EI-N) and a C-terminal PEP-binding domain (EI-C). EI-N contains a histidine residue which transfers phosphoryl from PEP to HPr. The three-dimensional crystal structure of EI-N from *E. coli* has been elucidated, and its mode of interaction with HPr has also been characterized by NMR spectroscopy [5,6]. EI-N consists of an HPr-binding α-helical subdomain and a α/β subdomain that bears the phosphorylatable histidine [7,8].

In addition to effecting the catalysis of sugar uptake, the bacterial PTS regulates various physiological processes, including the repression or induction of certain genes expression upon exposure of cells to glucose [9].

Gluconobacter oxydans is one of the most frequently used microorganisms in industrial biotechnology. Its unique capacity to incompletely oxidize polyol substrates has resulted in numerous production processes for the synthesis of compounds such as vitamin C, (keto) gluconic acids, dihydroxyacetone and vinegar. *G. oxydans* is also a suitable host to produce bio-polymers and transglucosylating enzymes. The very recent sequencing of the *G. oxydans* genome has had a major impact on the understanding of its metabolism and respiratory chain [10].

The availability of the genome sequence from *G. oxydans* 621H allowed the identification of several PTS related genes [11]. These genes are clustered in the genome, where GOX0812, GOX0813, and GOX0814 encode putative EI, HPr and EIIA, respectively, while the permease and EIIB of the EII complex have not been identified, so *G. oxydans* may contain an incomplete PTS [12,13].

As revealed by DNA microarray, oxygen limitation leaded to a significant increase in mRNA levels of the putative PTS proteins in *G. oxydans* [14]. Transcriptome analysis also showed that the mRNA levels of the putative PTS proteins increased drastically in *G. oxydans* during its late stage of growth on glucose [15]. Although such data implied that the PTS proteins might play important physiological roles in *G. oxydans* for its survival under stresses, the

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http://dx.doi.org/10.1016/j.bbrc.2016.01.052
0006-291X/

biochemical properties of these proteins remain unknown.

In this study, we identified a specific interaction between EI and a histidine kinase-response regulator hybrid protein (HK) in *G. oxydans*. As the HK serves as a member of bacterial two-component system, our discovery suggested a possible association between PTS and the bacterial two-component system.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

G. oxydans 621H was obtained from NWBI and maintained at 30 °C in a medium containing 20.0 g/l yeast extract, 80.0 g/l sorbitol, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4$ and 0.1 g/l L-Glutamine. *E. coli* strains DH5α, BL21 (DE3) and Trans B (DE3) were cultivated in Luria-Bertani medium with appropriate antibiotics at different temperatures. Y2HGold™ yeast strain was purchased from Clontech and cultured at 30 °C in YPD medium (5 g/l yeast extract, 10 g/l peptone, 10 g/l dextrose). pGBKT7 and pGADT7 from Clontech were used to construct the bait plasmid and the prey library for Y2H assay, respectively. pGEX-4T-2 was used to construct the expression vector for fusion protein GST-EI, while pET series plasmids were used to construct the expression vectors for HK and various fusion proteins for BiFC assay.

2.2. Construction of the genomic library of G. oxydans

The prey library of *G. oxydans* was constructed referring to In-fusion SMARTer Directional cDNA Library Construction Kit User Manual (Clontech). *G. oxydans* 621H genomic DNA was extracted by TIANamp Bacteria DNA Kit (Tiangen) and cut equably with ultrasonication. DNA fragments ranging from 0.8 kb to 3 kb were collected and ligated with adapters (Table 1) after end polishing. The resulting fragments with adapters were inserted into pGADT7 (Clontech), downstream to *GAL4* DNA-binding domain, by In-fusion Recombination Kit (TaKaRa). After transformation into *E. coli* DH5α and subsequent plating on medium containing 100 μg/ml ampicillin, the library was evaluated as described in the user's manual. The library plasmids were extracted by Plasmid Maxi Kit (Omega) and stored at −80 °C for further experiments.

2.3. Yeast two-hybrid (Y2H) assay

Yeast two-hybrid assay was carried out with Matchmaker™ Gold Y2H system (Clontech) according to the user's manual. The DNA fragment encoding EI was amplified by PCR using *G. oxydans* 621H genome as a template and primer pair A (Table 2). The resulting DNA was digested and ligated with pGBKT7 digested with the same enzymes to construct the bait plasmid pGBKT7-EI. pGBKT7-EI was transformed into Y2HGold yeast and plated on SD/-Trp minimal agar medium cultured at 30 °C for 2 d. The grown colonies were collected to prepare the competent Y2HGold yeast harboring pGBKT7-EI. After transformation with the prey library of *G. oxydans*, the Y2HGold yeast was plated on SD/-Trp/-Leu/-His/X-α-gal/Aba minimal agar medium cultured at 30 °C for 5 d. The grown blue colonies were picked for streak cultivation on fresh SD/-Trp/-Leu/-His/X-α-gal/Aba minimal agar medium plates cultured at 30 °C for 2 d, and the newly grown blue colonies were picked for sequence analysis.

2.4. Interaction between EI and HK in yeast

The sequence encoding full-length HK was amplified by PCR using *G. oxydans* 621H genome as a template and primer pair B (Table 2). The resultant DNA was digested and ligated with pGADT7 digested with the same enzymes to construct pGADT7-GOX0645. pGADT7-GOX0645 was transformed into the competent Y2HGold yeast harboring pGBKT7-EI, plated on SD/-Trp/-Leu/-His/X-α-gal/Aba minimal agar medium, and cultured at 30 °C for 3 d to detect blue colony formation.

2.5. Protein expression and purification

The sequence encoding EI was amplified by PCR using *G. oxydans* 621H genome as a template and primer pair C (Table 2). The DNA fragment was digested and ligated with pGEX-4T-2 digested with the same enzymes to construct pGEX-4T-2-EI. The sequence encoding HK was amplified by PCR using *G. oxydans* 621H genome as a template and primers pair D (Table 2). The DNA fragment was digested and ligated with pET28a digested with the same enzymes to construct pET28a-HK. The recombinant plasmids were transformed in *E. coli* BL21 (DE3) separately, and the transformants were cultured at 37 °C until OD_{600} reached 0.6. Protein expression was induced by adding 0.05 mM IPTG and cultured for 15 h at 18 °C. GST-EI fusion protein was purified with a 5 ml GSTrap™ FF column (GE Healthcare Life Science, USA) according to manufacturer's instructions. HK was purified with a 5 ml Ni-NTA column (GE Healthcare Life Science, USA) as recommended. The protein purity was determined by SDS PAGE, and the concentration was determined by Bradford assay [16].

2.6. GST pull-down assay

GST pull-down assay was conducted as described [17]. 1 mg GST-EI in 2 ml equilibration buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) was incubated with 2 ml glutathione-agarose suspension (Sigma) with gentle mixing for 1 h at 4 °C. The precipitate was collected by centrifugation with 10,000 g for 10 min at 4 °C and incubated with 1 mg HK in the same buffer with gentle mixing for 1 h at 4 °C. The beads were washed three times with 2 ml equilibration buffer and the proteins bound was eluted with 1 ml elution buffer (10 mM reduced glutathione, 50 mM Tris−HCl, pH 9.0) by gentle mixing for 15 min three times. The supernatants before and after elution were analyzed by SDS PAGE.

2.7. Bimolecular fluorescence complementation (BiFC) assay

Both pET21a and pET28a were digested with *Sal*I and *Xho*I. The DNA encoding MN (mCherry 1–159) and MC (mCherry 160–237) were amplified by PCR with primer pairs E_2, F_1, G_1, H and I (Table 2), respectively. The DNA encoding EI and HK were amplified by PCR using *G. oxydans* 621H genome as a template and primer pairs E_1, F_2

Table 1
Adapters used in genomic library construction.

Adapter	Sequence
Forward	5′ -GATTACGCTCATATGGCCATGGACAACTTTGTACAAAAAAGTTGG- 3′ 3′ - GAGTATACCGGTACCTGTTGAAACATGTTTTTTCAACC- 5′
Reverse	5′ -TGCAGCTCGAGCTCGATGGATACAACTTTGTACAAGAAAGTTGGGT- 3′ 3′ - GCTACCTATGTTGAAACATGTTCTTTCAACCCA- 5′