Transcriptional profiling of CRP-regulated genes in deep-sea bacterium *Shewanella piezotolerans* WP3

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**Abstract**

The cAMP receptor protein (CRP) is a conserved regulator in bacteria and involved in regulation of energy metabolism, such as glucose, galactose, and citrate (Green et al., 2014 [1]). As an important catabolite activator protein, it has been well characterized in model microorganism such as *Escherichia coli*. However, our understanding of the roles of CRP in deep-sea bacteria is rather limited. To indentify the function of CRP, we performed whole genome transcriptional profiling using a custom designed microarray which contains 95% open reading frames of *Shewanella piezotolerans* WP3, which was isolated from West Pacific sediment at a depth of 1914 m (Xiao et al., 2007 [2]; Wang et al., 2008 [3]). Here we describe the experimental procedures and methods in detail to reproduce the results (available at Gene Expression Omnibus database under GSE67731 and GSE67732) and provide resource to be employed for comparative analyses of CRP regulon and the regulatory network of anaerobic respiration in microorganisms which inhabited in different environments, and thus broaden our understanding of mechanism of bacteria against various environment stresses.

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**2. Experimental design, materials and methods**

2.1. Construction of *crp* gene deletion mutants

Two *crp* deletion mutants were constructed by the method as described previously [4]. First, the upstream and downstream fragments flanking both sides of the cAMP binding domain (D1) and DNA binding domain (D2) of *crp* gene were amplified with PCR primer pairs (Supplementary Table 1), respectively. These two fragments were used as templates in a second fusion PCR, resulting in a fragment with a deletion in the *crp* gene. Then, the PCR products were cloned into pRE112, yielding pRE112-*crpD1* and pRE112-*crpD2*, respectively. These plasmids were transformed into *Escherichia coli* WM3064 and then moved into *Shewanella piezotolerans* WP3 (hereafter referred to as WP3) by two-parent conjugation. The transconjugant was selected by chloramphenicol resistance and verified by PCR. The WP3 strains with pRE112-*crpD1* and pRE112-*crpD2* inserted into the chromosome were plated on 2216E agar medium supplemented with 10% sucrose. The successful *crp* deletion mutants were screened and confirmed by PCR (Table 1).

2.2. Bacterial culture conditions

For aerobic cultivation, the WP3 strains were cultured in modified 2216E marine medium (2216E) (5 g/l tryptone, 1 g/l yeast extract,
0.1 g/1 FePO₄, 34 g/l NaCl). The single clone of WP3 strains was inoculated into a 5 ml test tube, and then the culture was diluted 1000-fold in the same medium with shaking (220 rpm) at 20 °C. The culture was: For anaerobic cultivation, an oligotrophic medium (0.1 g/l tryptone, 0.2 g/l yeast extract, 34 g/l sodium chloride, 4.8 g/l HEPE, and 3.4 g/l sodium lactate) was dispensed into serum bottles gassed with O₂-free nitrogen. After the media were autoclaved, fumarate (20 mM) was added as electron acceptor. The growth of the WP3 strains was determined using turbidity measurements at 600 nm with a spectrophotometer (Nanodrop 2000c, Thermo Scientific, USA) according to the manufacturer's instructions as described previously [5,6]. The quality of RNA samples was determined by running a 1.0% TAE (Tris-Acetate-EDTA) agarose gel (Fig. 1B). The total RNA was treated with DNase I at 37 °C for 1 h to remove DNA contamination and the purity was checked by PCR amplification with RNA as template. The quantity and integrity of RNA was evaluated with a UV spectrophotometer (Nanodrop 2000c, Thermo Scientific). In general, the ratios of absorbance at 260 nm/280 nm (OD₂₆₀/₂₈₀) should be ~1.2 under the aerobic condition (Fig. 1A). The samples were centrifuged for 30 s at the maximal speed (16,000 × g). The cells were immediately frozen in liquid nitrogen for subsequent RNA extraction.

### 2.3. RNA isolation

Total RNA was isolated from the WP3 cultures with TRI reagent-RNA/DNA/protein isolation kit (Molecular research center, Cincinnati, USA) according to the manufacturer’s instructions as described previously [5,6]. The quality of RNA samples was determined by running a 1.0% TAE (Tris-Acetate-EDTA) agarose gel (Fig. 1B). The total RNA was treated with DNase I at 37 °C for 1 h to remove DNA contamination and the purity was checked by PCR amplification with RNA as template. The quantity and integrity of RNA was evaluated with a UV spectrophotometer (Nanodrop 2000c, Thermo Scientific). In general, the ratios of absorbance at 260 nm/280 nm > 2 and 260 nm/280 nm ≈ 2.2 indicate that the RNA is pure and could be used for the follow-up microarray analysis.

### 2.4. WP3 custom microarray designing

PCR primers for 4744 of the 4945 predicted ORFs in the WP3 genome (excluding 200 CDSs shorter than 150 bp) were designed using Primer 2.4. WP3 custom microarray designing is pure and could be used for the follow-up microarray analysis.

### 2.5. Preparation of fluorescent dye-labeled DNA and hybridizations

The total RNAs were reverse transcribed with Superscript II (Invitrogen, Carlsbad, USA) and the cDNAs were labeled with Cy3 and Cy5 by using a Klenow enzyme (Takara Bio Inc., Shiga, Japan) according to the manufacturers' instructions. Labeled cDNA was purified with a PCR purification kit (Macherey-Nagel, Düren, Germany) and resuspended in elution buffer. The labeling efficiency was evaluated with a UV spectrophotometer (Nanodrop 2000c, Thermo Scientific), and the fluorescence value should be > 150 pmol. Labeled controls and test samples were quantitatively adjusted based on the efficiency of the Cy-dye incorporation and mixed with 30 μl of hybridization solution (50% formamide, 1 × hybridization buffer; Amersham Biosciences). The DNA in hybridization solution was denatured at 95 °C for 3 min prior to loading onto a microarray. The arrays were hybridized overnight at 42 °C and washed with 2 consecutive solutions (0.2% SDS, 0.2× SSC for 5 min at 42 °C, and 0.2× SSC for 5 min at RT). The microarray slides were hybridized with cDNA prepared from 3 biological replicate samples. As a measure of

### Table 1

Bacterial strains used in microarray study.

| Strain | Description | Source |
|--------|-------------|--------|
| WP3   | Wild-type strain of Shewanella piezotolerans WP3, GenBank accession number CP000472 | Lab stock |
| WP3ΔcrpD1 | WP3, deletion mutant of cAMP binding domain of crp gene | This study |
| WP3ΔcrpD2 | WP3, deletion mutant of DNA binding domain of crp gene | This study |

Fig. 1. (A) Growth curve of WP3ΔcrpD1 and WP3ΔcrpD2 at 20 °C. The assays were performed in 2216E medium and oligotrophic medium, respectively. The average values and standard deviations displayed by the error bars resulted from three replicates. (B) Electrophoresis of total RNA of WP3 wild-type strain and crp gene mutants. Lane 1: WP3, lane 2: WP3ΔcrpD1, and lane 3: WP3ΔcrpD2.
technical replication, the dye-swap experiment was performed on each sample so that a total of 6 data points were available for every ORF on the microarrays.

2.6. Image acquisition, data processing and validation

A LuxScan 10 K scanner and microarray scanner 2.3 software (CapitalBio, Beijing, China) were used for the array image acquisition. We quantified the signal intensities of individual spots from the 24-bit Tiff images using SpotData Pro 2.2 (CapitalBio, Beijing, China). The linear normalization method was used for data analysis, based on the expression levels of WP3 housekeeping genes in combination with the yeast external controls. The normalized data were log-transformed and loaded into MAANOVA under R environment for multiple testings, by fitting a mixed-effects ANOVA model [7]. Microarray spots with $P$ values $< 0.001$ in the F-test were regarded as differentially expressed genes (DEGs) (Fig. 2). In addition, all of the DEGs were confirmed with Significance Analysis of Microarrays (SAM) software [8].

3. Discussion

Here we describe the data of differentially expressed genes in two crp gene deletion mutants compared to wild-type strain using our custom designed genome-wide $S$. piezotolerans array. The $Shewanella$ species are well-known for their versatile respiration ability and widely distributed in aquatic environment including deep-sea [2,3,9,10]. Meanwhile, the CRP protein is the key transcriptional regulator of anaerobic respiration [1,11]. Thus further investigations are required to clear the function of CRP and adaptation mechanism of bacterium in the extreme deep-sea environment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gdata.2015.04.019.

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