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# Characterization of inducible cold-active β-glucosidases from the psychrotolerant bacterium *Shewanella* sp. G5 isolated from a sub-Antarctic ecosystem

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ABSTRACT

The psychrotolerant bacterium *Shewanella* sp. G5 was used to study differential protein expression on glucose and cellobiose as carbon sources in cold-adapted conditions. This strain was able to growth at 4 °C, but reached the maximal specific growth rate at 37 °C, exhibiting similar growing rates values with glucose (μ: 0.4 $h^{-1}$) and cellobiose (μ: 0.48 $h^{-1}$). However, it grew at 15 °C approximately in 30 h, with specific growing rates of 0.25 and 0.19 $h^{-1}$ for cellobiose and glucose, respectively. Thus, this temperature was used to provide conditions related to the environment where the organism was originally isolated, the intestinal content of *Munida subrrugosa* in the Beagle Channel, Fire Land, Argentina. Cellobiose was reported as a carbon source more frequently available in marine environments close to shore, and its degradation requires the enzyme β-glucosidase. Therefore, this enzymatic activity was used as a marker of cellobiose catabolism. Zymogram analysis showed the presence of cold-adapted β-glucosidase activity bands in the cell wall as well as in the cytoplasm cell fractions. Two-dimensional gel electrophoresis of the whole protein pattern of *Shewanella* sp. G5 revealed 59 and 55 different spots induced by cellobiose and glucose, respectively. Identification of the quantitatively more relevant proteins suggested that different master regulation schemes are involved in response to glucose and cellobiose carbon sources. Both, physiological and proteomic analyses could show that *Shewanella* sp. G5 re-organizes its metabolism in response to low temperature (15 °C) with significant differences in the presence of these two carbon sources.



## 1. Introduction

In nature, as well as during industrial processes, bacteria are exposed to changing physico-chemical environmental parameters which may impair their growth or survival [1]. One such environmental condition rarely applied in laboratory studies is the induction of cold-responsive genes. Cold regions have been colonized by psychrophiles (0–15 °C) and psychrotolerant (15–20 °C) microorganisms which have adapted to the strong effect of low temperature on biochemical reactions that enable cell survival in cold environments [2]. A cold-active enzyme tends to have reduced activation energy at low temperature, leading to high catalytic efficiency, which may possibly be attributed to an enhanced local or overall flexibility of the protein structure [3,4]. Two glycosyl hydrolases, or β-glucosidases (EF141823 and DQ136044), were isolated from a psychrotolerant *Shewanella* sp. G5. These cold-active β-glucosidases may be of interest for biotechnology, e.g. in food processing at low temperatures and in a broad pH range [5].

The identification of proteins and protein expression patterns under different growth conditions (e.g., carbon source or temperature change) can be studied by proteome analyses which are greatly enhanced if genome sequence data are available [6]. For the genus *Shewanella*, the *S. oneidensis* MR-1 (NC_004347) genome is available with 4467 predicted genes; 1623 of which are annotated as hypothetical (36%) [7]. This assignment is given to genes that have not been characterized and whose functions cannot be deduced from simple sequence comparisons [8]. Using a closely related organism, the available genome data can be exploited to identify proteins expressed under specific conditions. Thus, it is possible to investigate a cold-tolerant relative like *Shewanella* sp. G5 for protein expression under environmentally pertinent conditions. Those approaches are now available for comprehensive measurements of gene and protein expression, a valuable tool since the lack of knowledge of the function in the protein expression in the genome limits the ability to take full advantage of capabili-

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ties for advancing in the compressive of its biology. Even, these genes encode several intact proteins, which is expresses in different growth conditions in a bacterium. Nonetheless, every new approach in the expression of proteins provides results in hundreds of new hypothetical genes [8]. To highlight variations in gene expression, proteome analysis of soluble and whole cell proteins represents a suitable tool. Two-dimensional gel electrophoresis (2D), which introduction of immobilized pH gradients (IPGs) for isoelectric focusing (IEF) couples in the first and SDS-PAGE in the second dimension.

It is the method of choice for resolving complex protein mixtures and reveals even very small changes in protein expression patterns [9]. It allows for quantitative and qualitative separation of complex protein mixtures typically found in cellular extracts of living cells form organisms. Both methods IEF and SDS-PAGE are critically affected by the solubility of proteins prior to electrophoresis. Proteins can only be analyzed by 2D if they are kept in solution or solubilized during the entire process [10].

The combination of 2D gel electrophoresis and mass spectrometry was used to study the differential expression of genes from the psychrotolerant *S. oneidensis*, identifying proteins by similarity to those annotated from the known genome sequence [11]. The proteomic changes of *Shewanella* sp. G5 were analyzed for cultures grown at low temperature in the presence of two alternative carbon sources, cellobiose or glucose. The enzyme β-glucosidase was used as a marker to indicate proteome re-arrangement and to elucidate environmentally relevant expression profiles.

## 2. Materials and methods

### 2.1. Cultivation

*Shewanella* sp. G5 isolated from the benthonic organism *Munida subrrugosa* collected on the coast of the Beagle Channel, Ushuaia, Argentina [5], was cultivated in 1 l flasks on an orbital shaker (250 rev/min), containing 300 ml liquid Luria-Bertani medium with 10 g/l cellobiose (LBC) or glucose (LBG) at 15 °C.

### 2.2. Basic characterization

The media were inoculated with 100 μl of culture; the growth was followed by absorbance in a Beckman (DU®640) spectrophotometer until an optical density ($OD_{540}$) of 0.8 at 540 nm. Specific growths were evaluated in LBC and LBG at different temperatures (15, 20, 25 and 37 °C). Absorbance values were transformed to $\log_e$ (absorbance), so specific growth rate values were calculated by fitting the growth curves with the equation described by Nerbrink et al. [12].

The *Shewanella* sp. G5 tested were grown in LBC at 15 °C and APIs (BioMérieux) strips were set up for this strain. Duplicate API ZYM, API 20E, API 20NE and API Coryne identification strips were set up as instructed by the manual and incubated at 15 °C for 1 h.

### 2.3. Protein extraction of Shewanella sp. G5

Sub-cellular protein fractions were obtained by differential centrifugation Kong et al. [13]. After obtaining proteins in the supernatant (15 min at 33,000 × *g*), cells were suspended in 5 ml of distilled water and disrupted by French Press (SLM Instruments) at 25,000 psi. Cell debris was removed (15 min at 30,000 × *g*), and cytoplasmic proteins were separated by ultra-centrifugation at 144,000 × *g* into soluble cytoplasmic proteins (SCP) and membrane proteins (MP) fractions.

### 2.4. β-Glucosidase enzyme activity

β-Glucosidase activity was assayed with 0.1 M *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) by incubation at 37 °C for 1 h using 0.1 M potassium phosphate buffer at pH 8 [5]. Different pH conditions were used by adjusting the phosphate buffer to pH 6–8, and cellular fractions were assayed after resuspending the pellets in 0.1 M potassium phosphate buffer pH 8. Absorbance of *p*-nitrophenol released during the reaction was monitored by spectrophotometer at 420 nm. One enzyme unit was calculated using the extinction coefficient of *p*-nitrophenol ($\varepsilon_{420\,nm} = 1.6 \times 10^4\ M^{-1}\ cm^{-1}$) and was *defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate per min under the experimental conditions*. Enzymatic activity was expressed as specific activity (U/mg); all analyses were performed in triplicate.

### 2.5. Zymogram assay

Native PAGE from a modified procedure of Long An et al. [14] was carried out to determine isoenzymes pattern of β-glucosidases, using a Bio-Rad Mini-Protein 3 Cell electrophoresis unit (Biorad). Protein identification was performed in 0.75 mm gel in a vertical slab unit, the separating gel containing acrylamide and bisacrylamide, 10 and 0.5%, respectively. The cellular fractions previously obtained were mixed with buffer (0.35 M Tris–HCl pH 6.8, 10% glycerol, 0.0002% bromophenol blue and 0.6 M dithiothreitol, DTT), and electrophoresis was carried out at 100 V until the tracking dye migrated to the bottom of the gel at room temperature. The gel was then washed twice in 0.1 M potassium phosphate buffer pH 7 and incubated in 1 mM of 4-methylumbelliferyl-β-D-glucopyranoside (MUG) for 30 min at room temperature. Fluorescent bands indicative of β-glucosidase activity were observed under UV light, visualized and captured using an Image Analyzer Gel Doc (Biorad). After washing the gel three times with buffer potassium phosphate, Coomassie brilliant blue staining was applied to visualize the protein bands [15].

### 2.6. Two-dimensional electrophoresis

The cytosolic proteins were precipitated (20% trichloracetic acid, 50% acetone, 20 mM dithiothreitol, DTT) for 30 min at 20 °C, incubated for 2 h at 4 °C and centrifuged at 11,000 rpm. After a two-step acetone wash the pellet was dried (Speed Vac) and redissolved in rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). After ultracentrifugation at 75,000 × *g*, proteins [16] were quantified measured used for each IEF strip in the rehydration buffer [17].

#### 2.6.1. First dimension (IEF)

First dimension separation of proteins by isoelectric points was conduced with IPG Immobiline DryStrip pH 3–10 non-linear (NL) 18 cm (Amersham Biosciences). IEF strips were rehydrated for 12 h at 20 °C with 450 ml rehydration buffer containing 500 mg proteins and 3 ml 4% bromophenol blue for each sample. IEF was conducted in the IPGphor system (Amersham Biosciences) using the followed steps: S1 300 V (15 min), S2 500 V (30 min), S3 1000 V (1 h), S4 3000 V (1 h) and S5 8000 V (7 h). Afterward, the strip was equilibrated with 6 M Urea; 4% SDS (w/v); 30% glycerol (v/v); 1% DTT (w/v) and 3.3% bromophenol blue (w/v) at room temperature (RT) for 15 min. Subsequently, the strip was re-equilibrated with the same solution except for the addition of 4% iodoacetamide at RT for 15 min [17].

#### 2.6.2. Second dimension

The second dimension SDS-PAGE (Ettan DALT*six* Large Vertical System, Amersham Biosciences) was performed with strips sealed with 0.8% agarose in the top 1.5 mm of a 26 cm × 20 cm vertical 10% PAGE in a SE-600 system (Hoefer SE600). Electrophoresis was performed in the presence of 181.66 g Tris–HCl pH 8.8, 30 g glycine and 4 g SDS with constant voltage (600 V) followed by constant amperage (400 mA/gel) at 5 °C for 16 h or until the bromophenol blue reached the bottom of the gel. Afterwards, gels were rinsed with distilled water for 5 min and fixed overnight in 10 ml phosphoric acid 85%, 20 ml methanol and 79 ml distilled water. The gels were stained with Coomassie brilliant blue (Roti-Blue 20%, Roth) for 12 h at RT and then discolored with glycine and methanol for 24 h [17].

#### 2.6.3. In-gel digestion and mass spectrometry

In the present study, we used thiourea and urea as chaotropes combined with CHAPS (sulfobetine detergents) and DTT (reducing agent) to the solubilization of protein [11]. Protein spots digestion was performed in-gel [17]. Briefly, the excised spots were washed, air-dried and digested with 0.02 μg modified trypsin per spot in 50 mM ammonium bicarbonate at 37 °C overnight. Peptides were extracted with 60% (v/v) acetonitrile/0.1% (v/v) formic acid in water, thereafter dried in a SpeedVac (Thermo Savant, USA) and dissolved in 10 μl of 0.1% (v/v) formic acid.

One μl of the digested sample was added to an equal volume of matrix solution for electron spray ionization Mass Spectroscopy (ESI-MS; LCQ Deca XP, Thermo; [18] or MALDI TOF (matrix-assisted laser desorption ionization time of flight) [19]. For identification of peptides, the *S. oneidensis* MR-1 (NC_004347) genome (NCBI database) was used with Sequest 3.1 [7,17].

## 3. Result and discussion

### 3.1. Physiological characteristics

Significant differences were observed in the specific growth rate μ ($h^{-1}$), evaluated in LB medium with cellobiose and glucose as carbon sources. The strain presented higher μ values with cellobiose at all temperatures assayed. Nevertheless, the maxima specific growth rate in both media was reached at 37 °C (Fig. 1).

The bacterium *Shewanella gelidimarina*, which was isolated from Antarctic sea, has an optimal growth temperature at 17 °C. The related species *S. benthica* has an optimal growth temperature at 8 °C and the bacterium *Polaromonas vacuolata* a still lower (4 °C)