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Research Article

# Topological analysis of carbon flux during multi-stress adaptation in *Halomonas* sp. AAD12

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ABSTRACT

*Background:* Osmolytes with their effective stabilizing properties are accumulated as protectants not only against salinity but also against denaturing harsh environmental stresses such as freezing, drying, high temperatures, oxygen radicals and radiation. The present work seeks to understand how *Halomonas* sp. AAD12 cells redirect carbon flux specifically to replenish reactions for biomass and osmolyte synthesis under changing salinity and temperature. To accomplish this goal, a combined FBA–PCA approach has been utilized.
*Results:* Experimental data were collected to supply model constraints for FBA and for the verification of the model predictions, which were satisfactory. With restrictions on the various combinations of selected anaplerotic paths (reactions catalyzed by phosphoenolpyruvate carboxylase, pyruvate carboxylase or glyoxylate shunt), two major phenotypes were found. Moreover, under high salt concentrations, when the glucose uptake rate was over 1.1 mmoL $DCW^{-1}$ $h^{-1}$, an overflow metabolism that led to the synthesis of ethanol caused a slight change in both phenotypes.
*Conclusions:* The operation of the glyoxylate shunt as the major anaplerotic pathway and the degradation of 6-phosphogluconate through the Entner–Doudoroff Pathway were the major factors in causing a distinction between the observed phenotypes.



## 1. Introduction

Halophilic microorganisms demand high salt concentrations to grow. They evolved two major osmoadaptation mechanisms to cope with salinity: the salt-in-cytoplasm mechanism and the organic osmolyte mechanism [1]. In the first mechanism, enzymes and structural cell components adapt to the presence of molar concentrations of KCl, and the continued presence of high salt concentrations becomes a requirement for survival. In the second mechanism, however, osmolytes are accumulated to protect the proteins, nucleic acids, biomembranes and even the whole cells against denaturation, inactivation, and inhibition. The design of the cell's interior remains basically unaltered [2]. As a response to changing external salinity, these organisms adjust their cytoplasmic osmolyte pools by remodeling their metabolism to redirect carbon flux [3,4,5,6]. Interestingly, osmolytes have been reported to be beneficial not only as osmoregulatory solutes but also as protectants against the denaturing environmental stresses of harsh environments such as freezing, drying, high temperatures, oxygen radicals and even radiation [7,8,9,10]. They possess powerful stabilizing properties that are explained by the osmophobic effect [11].

Constraint-based metabolic network modeling (CBM) methods are powerful tools that can be used to elucidate the metabolic responses of microorganisms to environmental conditions. Among the many CBMs, optimality-based flux balance analysis (FBA) is a powerful tool to simulate and study the flux distributions of the cellular metabolism [12]. FBA results can be utilized for the interpretation and prediction of distinct patterns of metabolic pathway utilization in a phenotypic and functional context [13,14]. An FBA study can be enhanced by the integrated use of statistical analysis methods such as principle component analysis (PCA). As proposed by Liang et al. [15], a combined FBA–PCA approach can not only help gain insight into the cellular metabolism of the microorganism but also enable the identification of key reactions that affect phenotypic responses of the cells to environmental conditions.

The combined FBA–PCA approach involves running *in silico* experiments, with each experiment represented as a solution to an FBA problem. In the present work, for *Halomonas* sp. AAD12 cells, the effects of the environmental conditions, salinity and temperature, and the resulting phenotypic responses are introduced into the FBA problems via constraints on fluxes such as oxygen and glucose uptake and osmolyte synthesis rates, the values of which are acquired experimentally under relevant conditions. The optimality condition for

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all *in silico* experiments is maximal growth. The results of the *in silico* experiments (series of flux distributions) are then statistically analyzed using PCA to identify the key reactions that give rise to metabolic patterns. PCA is a statistical method that is commonly used for dimension reduction in high-dimensional data sets to identify the directions of largest variation [16].

## 2. Materials and methods

### 2.1. Bacterial strains and chemicals

The chemicals used were supplied by Merck AG (Darmstadt, Germany) and Sigma Chem. Ltd. (USA). The *Halomonas* sp. strain AAD12 was isolated from salt sediments in ponds found in the Çamalti Saltern area in Izmir (western Turkey) and identified as described previously [17,18]. The organism is deposited at our research laboratory at Marmara University, and its 16S rRNA sequence has been deposited in the NCBI database with the accession no. GU397429 [17]. No specific permissions were required for the collection of the samples because no endangered or protected species were involved during the field studies.

### 2.2. Calculation of the growth rate and glucose uptake rate

Cells were cultured at 180 rpm in 250 or 500 mL flasks containing 50 or 100 mL of M9 minimal medium, respectively. The growth medium was supplemented with 5 or 15% sodium chloride and 0.5% (w/v) glucose as the carbon source. Growth was achieved at both 37°C and 20°C. The growth was monitored spectrophotometrically at OD 600 nm. Values from triplicate measurements were averaged.

To calculate the glucose uptake rate in the presence of 5% NaCl at 37°C, 1 mL samples from actively growing cultures were taken at different time intervals during the exponential growth phase. Following centrifugation, the cell-free supernatants were analyzed by HPLC (Agilent Technologies, USA) to quantify the residual glucose in the growth media using an Agilent ZORBAX CARBOHYDRATE column. The values obtained were used to calculate the amount of glucose that was consumed. The glucose uptake rate was found by dividing the amount of glucose consumed per gram dry cell in a given period by the duration of that period.

### 2.3. Construction of the FBA model

FBA methodology was selected to investigate the metabolic network of *Halomonas* sp. AAD12. The input information required for FBA analysis comprises the metabolic network of the microorganism, glucose uptake rate, oxygen transfer rate, and osmolyte synthesis rates. The minimal network, which consisted of the central carbon metabolism and the pathways for the measured osmolytes, was based on the genome data of *Halomonas elongata* and on the information for *Chromohalobacter salexigens* in the KEGG database [2,19]. Reactions with no impact on the central biological topic being addressed have been excluded. The related 82 reactions were compiled in a stoichiometric matrix with a total of 88 metabolites [20]. Biomass formulation was based on the composition of *Escherichia coli* synthesized from 13 precursor metabolites with modifications to account for variations in the halophiles. The details of the biomass formulation for the high and low salt cases are given elsewhere [20].

The experimentally obtained values in this study for the glucose uptake rate were used as input constraints in flux analysis. The upper limits for the approximate oxygen uptake rates for the low and high salt cases were taken to be 2.675 and 1.175 mmoL $DCW^{-1}$ $h^{-1}$, respectively. These numbers were obtained from a previous study that was conducted in 500 mL fermentors at 37°C. Because there were no measurements at 20°C, the same upper limits were assumed for the low temperature cases. Experimental data for the amounts of the three osmolytes, ectoine, hydroxyectoine and proline, in *Halomonas* sp. AAD12 cytoplasm in the early exponential and mid-exponential phases, were obtained from Dilek Kazan (personal communication, unpublished data). One assumption that was made to complete the necessary input information for FBA was related to the consumption of glucose at various growth conditions: 50% of the initially available glucose was assumed to be consumed by the growing culture between the early exponential and mid-exponential phases. Because these time points also coincided with sampling for osmolyte measurements, this choice was a reasonable assumption. Moreover, HPLC measurements also confirmed that this assumption was valid for the case of growth in the presence of 5% NaCl at 37°C.

### 2.4. In silico experimentation

To elucidate the possible distinct patterns of *Halomonas* sp AAD12 metabolism, 5 case studies were devised. Each case study was designed to study the flux distributions when certain metabolic pathways of the minimal network were restricted. The first case study had no restrictions imposed, while case studies 2, 3 and 4 had restrictions on the anaploretic paths: reactions catalyzed by phosphoenolpyruvate carboxylase, pyruvate carboxylase or glyoxylate shunt, respectively. For case 5, the reactions that were catalyzed by both phosphoenolpyruvate carboxylase and pyruvate carboxylase were restricted.

The conditions for the *in silico* investigation were chosen in parallel to the conditions that the available experimental data were acquired: low and high salinity conditions each at 20°C and 37°C growth temperatures giving rise to a four-by-four experimental design space. The independent variable to be examined was the glucose uptake rate, which was varied between 0.1 and 1.5 mmoL $DCW^{-1}$ $h^{-1}$ by 0.1 increments resulting in a maximum of 75 *in silico* experiments for each case mentioned.

The flux distributions for 82 intracellular metabolic reactions for each set of experimentation were analyzed using PCA to identify the key reactions that govern the observed metabolic patterns. The *in silico* experimental results were inserted into a data matrix in which each column corresponds to a flux vector under a specific condition and each row corresponds to the fluxes of a specific reaction under different conditions.

## 3. Results

### 3.1. Growth and glucose consumption under different salinities and temperatures

Growth of *Halomonas* sp. AAD12 has been investigated at 20 and 37°C in the presence of 5 and 15% NaCl. The fastest growth was at 37°C in the presence of 5% NaCl, with a growth rate of 0.1216 $h^{-1}$ during the exponential phase. Exponential growth was approximately 20 h under these conditions. At a constant temperature, when the salinity increased to 15%, the maximum growth rate dropped to 0.0869 $h^{-1}$. Although the length of the exponential phase remained basically unchanged, the lag phase was approximately 3 times longer. Reducing the growth temperature to 20°C further repressed the

**Table 1**
Growth rate of *Halomonas* sp. AAD12 during the exponential phase under different conditions.

| NaCl concentration | 37°C | | 20°C | |
|---|---|---|---|---|
| | Experimental | Simulated result | Experimental | Simulated result |
| 5% | 0.12 $h^{-1}$ | 0.12 $h^{-1}$ | 0.06 $h^{-1}$ | 0.02 $h^{-1}$ |
| 15% | 0.09 $h^{-1}$ | 0.04 $h^{-1}$ | 0.03 $h^{-1}$ | 0.01 $h^{-1}$ |