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Research paper

Metabolomic characterization of halophilic bacterial isolates reveals strains synthesizing rare diaminoacids under salt stress

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A B S T R A C T

Metabolomics-based approaches to study stress responses in bacteria have received much attention in recent years. In the present study, a metabolomic analysis of the representative halophilic bacterial isolates (*Halomonas hydrothermalis* VITP9, *Bacillus aquimaris* VITP4, *Planococcus maritimus* VITP21 and *Virgibacillus dokdonensis* VITP14) from a saltern region in India was performed using nuclear magnetic resonance spectroscopy. Chemometric analysis of ^{1}H NMR spectra revealed salt-dependent increase in the levels of metabolites, mainly from the aspartate and glutamate family, that are directed from the glycolytic pathway, pentose phosphate pathway and citric acid cycle. The composition of the metabolites was found to be different with respect to the species and the type of growth medium. Analysis of the two dimensional NMR data revealed accumulation of two rare diaminoacids, Nε-acetyl-α-lysine and Nδ-acetylornithine (by VITP21 and VITP4 strains respectively) apart from other well known solutes such as ectoine, proline, glutamate and glycine betaine. Metabolite profiles of strains capable of synthesizing Nε-acetyl-α-lysine and Nδ-acetylornithine suggested their biosynthesis from lysine and ornithine using aspartate and glutamate as their precursors, respectively. Further, the cells in moderate salinity (5% w/v NaCl) showed an increase in growth rate along with increase in the levels of nucleotides, whereas at higher salinity (10% w/v NaCl), the levels of aromatic and hydrophobic metabolites dropped, accompanied with a decrease in growth rate, rightly suggesting that at any salt-stress condition provided, cellular homeostasis was favored over growth.

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1. Introduction

Bacterial life can be found over the whole range of environmental conditions because of their ability to survive and proliferate under a variety of extreme conditions. Halophiles and halotolerant bacteria living in habitats of high ionic strength like marine water, salt lakes, brines, salterns, saline soils and salted foods have to cope up with the osmotic stress. Extensive physiological analyses of these bacterial species have revealed two mechanisms by which these bacterial species achieve osmotic equilibrium: [a] the 'high salt-in' strategy which involves accumulation of equimolar concentrations of inorganic ions in the cytoplasm and [b] the 'low salt-organic-solutes-in' strategy which involves the accumulation of certain highly water-soluble, low molecular weight organic compounds termed as osmolytes or compatible solutes [1]. These solutes are useful as stabilizers of biomolecules and whole cells against various stress conditions such as high temperature, freezing and desiccation. The ability to produce and/or accumulate high concentrations of these compounds makes moderate halophiles useful for the biotechnological production of these solutes [2]. The bacterial flora of saline environments is diverse and constitutes a heterogeneous group which has evolved from different genera [3], in which the capability to grow at high salt concentrations is a secondary adaptation process. Investigation of the metabolic response of these bacteria under high salinity would help to identify various compatible solutes and also to unravel the common mechanisms of halo-adaptation. Apart from the osmotic protection, the protective role of the osmolytes against freezing, desiccation and other denaturing conditions have been well documented with a great commercial use of solutes like glycine betaine and ectoine, which are already in the market [4]. The notable protective effects on the protein stability, solubility and folding under denaturing conditions have led these solutes to be termed as 'chemical chaperones' [5]. This unique strategy of defense has led these organisms to produce novel molecules as compatible solutes with great biotechnological potential.

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Metabolomics provides an approach and opportunity to explore the metabolic effects of environmental conditions on biological systems [6]. It gives a snapshot of the metabolic status of the cells in a particular physiological condition and also aids in the identification of metabolites that are perturbed in response to the stress factor under study. Such studies leading to the metabolomic characterization of the stress response in bacteria have received much attention in recent years [7–9]. High resolution ^{1}H NMR is a robust spectroscopic method which provides broad coverage of metabolites and is widely used to build metabolic profiles in diverse studies [10].

In an attempt to study salt-stress response of bacteria, the present study was aimed at investigating the salt-stress associated metabolic changes in the halophilic representatives of the bacterial isolates from a coastal saltern (pool of evaporated sea water prepared for extracting salt) of Kumta coast, Karnataka, India. The changes in the levels of metabolites of central metabolic pathways and the compatible solutes accumulated with respect to different growth media supplemented with various concentrations of salt were determined using NMR based metabolomic approach. Multivariate statistical analysis and metabolic pathway analysis were utilized to deduce a unified metabolic response exhibited by the bacterial strains in response to salt-stress.

2. Materials and methods

2.1. Bacterial strains

Isolates were obtained from a saltern region in Kumta, Arabian Sea Coast of India, enriched in Zobell marine broth and plated on the Zobell agar plate. Based on the species richness and differences in colony and cell morphology, four different major bacterial strains, VITP9, VITP4, VITP21 and VITP14, were isolated and characterized by 16S rRNA sequencing followed by phylogenetic analysis and identified to be *Halomonas hydrothermalis*, *Bacillus aquimaris*, *Planococcus maritimus* and *Virgibacillus dokdonensis* [11–14]. The NCBI GenBank identity numbers are FJ743438, FJ687490, HQ929427 and HQ829429 respectively. The strain *Halomonas* sp VITP9 is a gram negative bacteria belonging to the gamma subclass of Proteobacteria whereas the strains *Bacillus* sp VITP4, *Planococcus* sp VITP21 and *Virgibacillus* sp VITP14 respectively, are gram positive bacteria under the Bacilli subclass of Firmicutes.

2.2. Growth conditions

Microbial cultivation was carried out in 100 ml batch cultures with minimal medium (M9) containing 12.8 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 1 g $NaNO_3$, 4 g glucose, 0.24 g $MgSO_4$ and 0.01 g $CaCl_2$ in 1000 ml and complex medium (LB) 5 g yeast extract, 10 g peptone in 1000 ml. The media was supplemented with 0%, 5% and 10% w/v NaCl and was cultured at 37 °C with continuous shaking at 120 rpm. Bacterial growth was inferred from the optical density at 600 nm.

2.3. Sampling steps

Two biological replicates of cultures were harvested at logarithmic phase and the cells were collected by centrifugation and washed with cold NaCl solution isosmotic to the growth medium. The intracellular metabolites were extracted with 80% v/v cold methanol at room temperature for 30 min by vigorous stirring and this extraction process was repeated twice. The supernatants were pooled, the solvent was evaporated and the dried residue was dissolved in appropriate solvents (Milli-Q water for biochemical analysis or 100% D_2O for NMR spectroscopy). Intracellular extracts obtained from the culture samples of 400 ml (optical density at 600 nm of 0.5) or an equivalent thereof to obtain an identical cell mass were dissolved in 1 ml of D_2O for metabolomic analysis.

2.4. NMR spectroscopy

One-dimensional NMR spectra were acquired (at 300 K) on Avance 700 MHz Bruker NMR spectrometer (TIFR, Mumbai, India), using 5 mm triple resonance Broad Band Inverse probe using presaturation for water suppression and CPMG pulse sequence for suppression of signals from macromolecules, using a spectral width of 14,005 Hz, acquisition time of 3.1 s, recycle delay of 4 s and 128 transients. For quantification of osmolytes, dried methanolic extracts of cells grown at 0%, 5% and 10% w/v NaCl were dissolved in 100% deuterated water with 0.5 mM of sodium-3-(trimethylsilyl) tetradeuteriopropionate (TSP) which served as the internal standard. Chemical shifts and peak integrals for the metabolites were measured relative to TSP. Two dimensional TOCSY and COSY experiments (IICT, Hyderabad, India) using Avance 300 MHz Bruker spectrometer with a spectral width of 6188 Hz, 1 s relaxation delay and 2 transients.

2.5. Metabolome data analysis

Metabolomic data processing and statistical analysis was performed for the ^{1}H NMR spectra by following the workflow of MetaboAnalyst 2.0, a web-based comprehensive tool suite for metabolomic data analysis (http://www.metaboanalyst.ca). Accordingly, the NMR spectra were normalized using the intensity of the TSP signal and NMR peak list of different groups were uploaded. The peaks were aligned across all samples and grouped based on the ppm values using a moving window of 0.03 ppm and a step of 0.015 ppm. The peaks were aligned, within the same group, to their median chemical shifts and the missing values were replaced by a value of low signal intensity (half of the minimum positive values detected in the data). In order to improve the overall data consistency, row-wise normalization by median was selected and the data was mean-centered and divided by the standard deviation of each variable [15]. The data was found to follow normal distribution after data processing and normalization. The data so generated was used for two exploratory multivariate analysis techniques, unsupervised Principal Component Analysis (PCA) and supervised Partial Least Square-Discriminant Analysis (PLS-DA) for selecting important features (NMR peaks) from the metabolomic data sets. PCA was used to examine the intrinsic variation in the dataset and to detect outliers using 2D score plot. PLS-DA was used to view the class discrimination using score plot and the important NMR signals contributing to the variation were obtained from the loading plot values. The performance of PLS-DA model was measured using two parameters, R^2 and Q^2 and cross-validated by permutation test [16,17]. R^2 is defined as the proportion of variance in the data explained by the model. It signifies total explained variation in the data and indicates goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model. It explains the extent of separation between the classes and indicates predictability of the model. In case of an ideal model, R^2 and Q^2 should reach 1.

2.6. Assignment of NMR signals and targeted profiling

The important NMR resonances selected using PLS-DA were putatively assigned to specific metabolites by searching three publicly available databases of NMR spectra of metabolites: [a] Human Metabolome Data Base (HMDB), [b] Biological Magnetic Resonance Bank (BMRB) and [c] Madison Metabolomics Consortium Database

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