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Article

In-silico analysis and screening of trehalose biosynthesis pathways in Antarctica lichen-associated *Variovorax* sp. PAMC28711

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Abstract: Trehalose is a naturally occurring disaccharide that is abundantly present in nature. Its presence in cells as several effects, especially because it acts as a natural defender of proteins and membranes. It is utilized in food, cosmetics, and medications because of its excellent water retention properties. Following its esterification with fatty acids of different chain lengths, trehalose has also been shown to have anti-bacterial, anti-biofilm, and anti-inflammatory properties. Trehalose plays a structural part in the bacterial cell wall's adaptive reactions to stressors including osmotic variations high temperature. Additionally, it was proposed that when bacterial cells are exposed to harsh environment challenges such as heat, cold, desiccation, or reactive oxygen species, these organisms biosynthesize high concentrations of both intra- and extracellular trehalose to help them to survive. Therefore, we analyzed *Variovorax* sp. PAMC28711 isolated from Antarctica, which was predicted by bioinformatics tools (RAST, Prokka, KEGG, CGView Server, and MetaCyc) to predict the enzymes involved in the different pathways of trehalose production. Furthermore, TLC analysis was carried out to characterize various trehalose biosynthesis pathways. According to the findings, it was revealed that our polar bacteria *Variovorax* sp. PAMC28711 have potential to produce trehalose through three (Trehalose-6 phosphate synthase/trehalose-6-phosphat phosphatase, Trehalase synthase, and Maltooligosyl-trehalose synthase/maltooligosyl-trehalose trehalohydrolase) pathways. We anticipate that this strain could be of potential use for manufacturing trehalose on the industrial scale.

Keywords: Trehalose, Lichen-associated, Metabolic pathways, Antarctica, *Variovorax* species

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1. Introduction

Trehalose is a non-reducing disaccharide that is synthesized when two glucose molecules (α-D-glucopyranosyl-α-D-glucopyranoside) are linked by α, α-1-glycosidic linkage [1]. Normally, cells produce most of their trehalose under glucose limitation when there is very high flux of gluconeogenic energy from several carbon sources. However, trehalose is also synthesized when glucose is continuously used in a constantly unproductive manner to balance glycolysis; this process is triggered by various (occasionally external) factors, but will all reflect a "starvation-state", which is far less well-known [2, 3].

There are various biosynthetic processes that can be used to synthesize trehalose. The most prominent pathway is the TPS/TPP biosynthetic pathway, also known as OtsBA. Two enzymes known as trehlose-6 phosphate synthase (TPS/OtsA) and trehalose-6-phosphate phosphatase (TPP/OtsB) convert glucose-6 phosphate and UDP-glucose to trehalose. Maltooliosyl-trehalose synthase/maltooligosyl-trehalose trehalohydrolase (TreY/TZ) is another pathway for trehalose synthesis. In this pathway, an isoamylase hydrolyzes the 1,6-glucosidic bonds in glycogen or other polysaccharide like starch to form maltodextrin. Then employing an intermolecular transglucosylase mechanism, a maltooligosyl-trehalose synthase (MTS) catalyzes the transformation of maltodextrin into maltooligosyl-trehalose by creating an 1,1-glucosidic bond. Maltooligosyl-trehalose trehalohydrolase

(MTH), a third enzyme, hydrolyzes the product to produce trehalose and a maltodextrin that is two glucosyl residues shorter [4].

Trehalose with different physical properties (low hygroscopicity, high freezing point, high glass transition temperature, greater stability, and high-water retention capacity), chemical and functional properties (low carcinogenicity and less sweetness (45% that of sucrose)) are found great application in the food industry and cosmetic industry. Therefore, we have studied trehalose biosynthesis pathway in cold-adapted lichen associated bacteria *Variovorax* sp. PAMC28711 to produce trehalose. Trehalose is typically extracted from yeast cells. However, due to tis low yield and high cost, this technology is inappropriate for industrial manufacturing. In some instances, trehalose has been manufactured industrially in Japan using microbial enzymes. Since starch is a very cheap substrate, using enzymes to make trehalose from it offers financial benefits because it could reduce production costs. The results indicate that our cold-adapted *Variovorax* sp. PAMC28711 possess the trehalose-producing genes necessary for survival in a harsh environment like Antarctica. The identification of bacteria strains that can manufacture trehalose and the characterization of their metabolic pathways may also be an advantage from the results of this study.

2. Materials and Methods

In this study, we used in silico analysis to predict the trehalose biosynthesis pathways and associated genes. Furthermore, after making a prediction, we carried out a screening of trehalose biosynthetic pathways utilized by our Antarctica isolate *Variovorax* sp. PAMC28711.

2.1. Prediction of trehalose biosynthesis pathways in Variovorax sp. PAMC28711

We retrieved a complete genome information of *Variovorax* sp. PAMC28711 from the National Center for Biotechnology Information (NCBI) genome database (https://www.ncbi.nlm.nih.gov/) for the trehalose biosynthesis pathway study. The GenBank accession number of *Variovorax* sp. PAMC28711 is NZ_CP014517.1 [5]. The KEGG pathway database (http://www.kegg.jp/ or http://www.jp/kegg) [6] and MetaCyc database (http://metacyc.org) [7] were used to predict trehalose biosynthesis pathways in the complete genome of *Variovorax* sp. PAMC28711.

2.2. Screening of trehalose biosynthesis pathways in Variovorax sp. PAMC28711

As a growth medium, Reasoner's 2A (R2A) and Luria-Bertani (LB) media were used. The addition of 2% (w/v) glucose provided the bacteria with the stress. The strain PAMC28711 was cultivated in several substrates containing 2% (w/v) maltose, starch, and maltodextrin that were added individually to LB media and were grown at a temperature of 25 ^{0}C with 200 rpm agitation for 72 h to determine the various trehalose biosynthetic routes. Centrifugation was used to separate the cells at 25 ^{0}C and 12,500 rpm. Cells are permeabilized with 50% methanol and extracted for 30 min on ice. The mixture was mixed vigorously and centrifuged at 12,500 rpm for 5 min. The supernatant was assayed for trehalose by thin-layered chromatography on a silica gel plate with a solvent system consisting of the n-butanol/ethanol/water (5:3:2, v/v/v), and spots were visualized by spraying with 20% sulfuric acid and 5% ethanol followed by heating at 130 ^{0}C as described by Nguyen et al. [8].

3. Results and discussion

According to Tribelli and Lopez [9] Brininger et al. [10], both psychrophilic and psychotropic species have special adaptations that improve an enzyme's capacity to be active in a cold environment while still maintaining metabolism and growth. They utilize special cold-adapted proteins and cold active enzymes, which are catalytically effective even at low temperatures, to maintain their cell cycle to survive in harsh environments. The growth of our cold-adapted strain PAMC28711 was found to psychographs and was able to thrive from psychrophilic temperature (15 ^{0}C) to mesophilic temperature (37 ^{0}C). It possesses the genes necessary for living in a cold climate. The genes associated with a low temperature are highlighted in Table 1.

Table 1. Genes associated with a low temperature lifestyle identified in *Variovorax* sp. PAMC28711

Temperature	15-37°C
Response to low temperature	
Membrane fluidity	Fatty acid desaturase
Compatible solutes and antifreeze proteins	

Trehalose biosynthesis[a]	TS; TreY; TreZ; TPS; TPP
Glycine betaine[b]	OpuD
Oxidative stress and low-temperature response	
Cold shock response[c]	GyrA; Dead; PpiC; RecA; RbfA; Pnp; NusA; ClpB
Oxidative stress[d]	Grx; Ahp; SOD; Prx; BtuE

[a]TS, trehalose synthase; TreY, maltooligosyl-trehalose synthase; TreZ, maltooligosyl-trehalose trehalohydrolase; TPS, trehalose 6-phosphat synthase; and TPP, trehalose 6-phosphate phosphatase. [b]OpUD, Glycine betaine transporter; [c]GyrA, DNA gyrase; Dead, DEAD-box RNA helicase; PpiC, peptidyl-prolyl cis-trans isomerase; RecA, DNA strand exchange and recombination protein; RbfA, ribosome binding factor; Pnp, polyribonucleotide nucleotidytransferase; NusA, Transcription termination factor; and ClpB, ATP-dependent chaperone. [d]Grx, glutaredoxin; Ahp, alkyl hydroperoxide reductase; SOD, superoxide dismutase; Prx, peroxiredoxin; and BtuE, glutathione peroxidase.

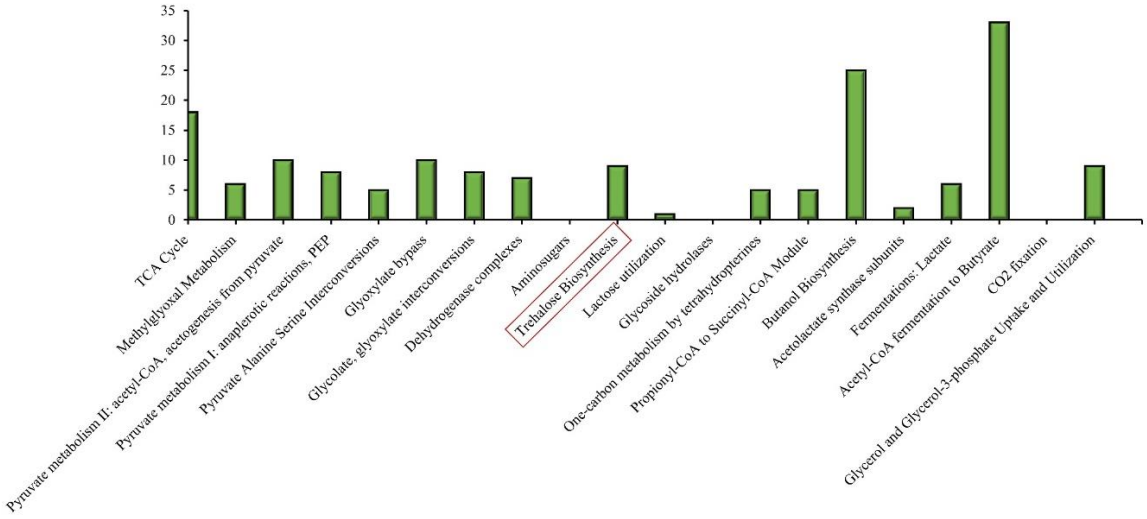
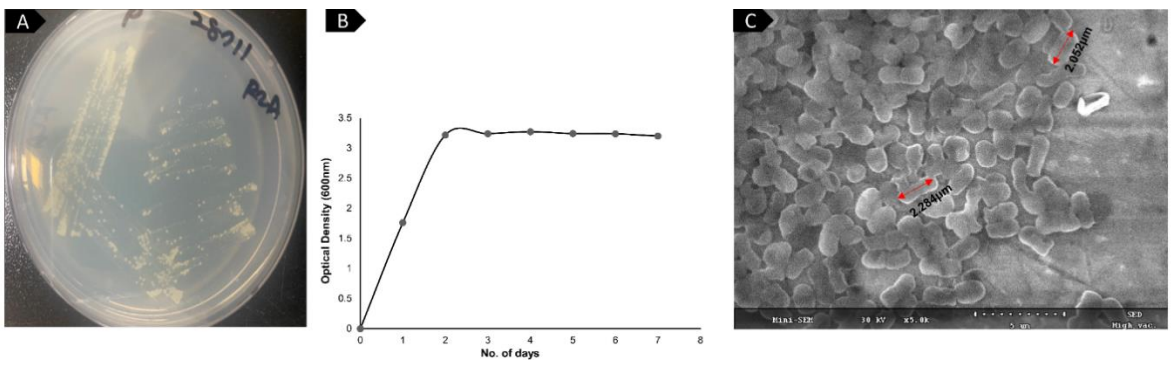


Figure 1. Carbohydrate metabolism subsystems distribution in *Variovorax* sp. PAMC28711 (based on RAST annotation server).

Variovroax sp. PAMC28711 is composed of circular chromosome of 4,320,000 bp with GC content of 66%. The RAST annotation identified that the strain PAMC28711 possesses 196 carbohydrates (11.6%) including trehalose biosynthesis pathways (Fig. 1). The colonies of strain PAMC28711 showed yellow, slimy, and shiny on R2A media (Fig. 2A) when grown at 15 ^{0}C for 7 days. Growth was monitored at 6000 nm by using spectrophotometer (Fig. 2B) and found to be rod shaped with the size of ~2 μm (Fig. 2C).

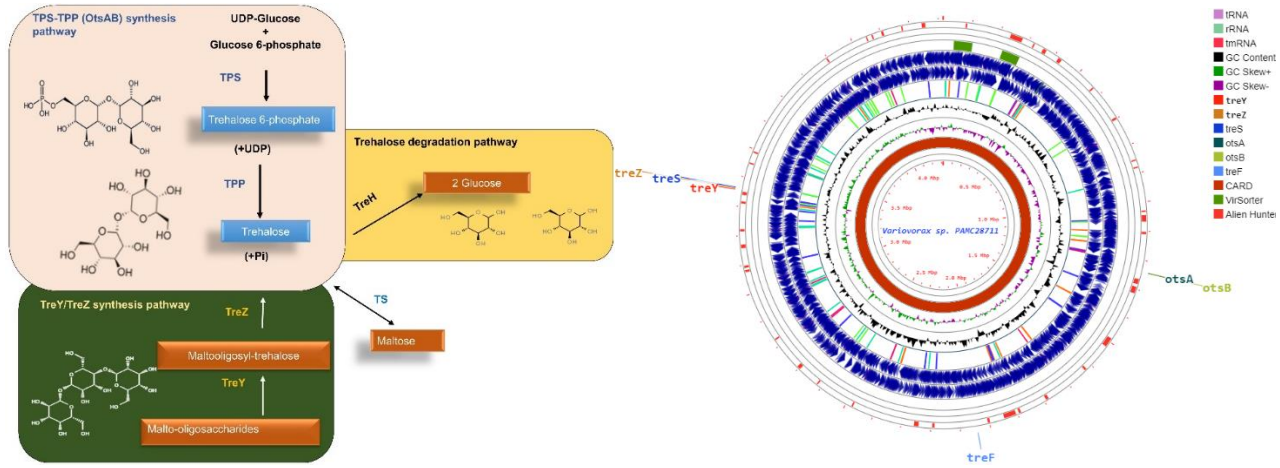


(**a**) (**b**) (**c**)

Figure 2. (**a**) Morphology colony of *Variovorax* sp. PAMC28711 in R2A agar; (**b**) Microbial growth curve of *Variovorax* sp. PAMC28711; (**c**) Microscopic observation of *Variovorax* sp. scanning electron microscope (SEM), magnification at 5000X.

There are several carbohydrate metabolism pathways found in *Variovorax* sp. PAMC28711. One of them is trehalose metabolic pathways. Trehalose can be used as an alternative carbon source in microorganisms [11]. The TPS/TPP biosynthetic pathways, also known as OtsBA, are the most common pathway for trehalose biosynthesis in bacteria. This pathway utilizes two enzymes, trehalose-6-phosphate synthase, and trehalose-6-phosphate phosphatase, to convert glucose-6-phosphate and UDP glucose into trehalose (as shown in Fig. 3). Alternatively, the TreYZ pathway can produce trehalose from maltooligosaccharides using maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose trehalohydrolas (TreZ). Another pathway, the TreS biosynthetic pathway involves a single trehalose synthase (TreS) enzyme that can reversibly convert between trehalose and maltose. Both the TreP and TreT biosynthetic pathways are also reversible, and the TreP pathway utilizes a trehalose phosphorylase enzyme (TreP). ADP-glucose and glucose are converted into trehalose through the TreT route by the enzyme trehalose glycosyl-transferring synthase (TreT). Bacteria have all these trehalose production mechanisms [12].

On the prediction of trehalose biosynthesis pathways in our Antarctica isolate *Variovorax* sp. PAMC28711, it showed tow TPS/TPP (enzymes such as trehalose phosphate synthase and trehalose-6-phosphate phosphatase) pathway and two TreY/TreZ (enzymes such as maltooligosyl-trehalose synthase and maltooligosyl-trehalose trehaldohydrolas) pathway enzymes, TS (trehalose synthase) pathway enzyme (Fig. 3 & Table 2) [13]. Similarly, it has been reported by Delorge and his colleagues that cold-induced trehalose biosynthesis genes (*otsA* and *otsB*) were present in *Escherichia coli* [14]. Thus, it gives assurance that under stressful conditions, extreme microbes are capable of synthesizing trehalose.



(**a**) (**b**)

Figure 3. (**a**) The three routes for trehalose biosynthesis observed in *Variovorax* sp. PAMC28711; (**b**) A map created with the use of the CGView server (https://cgview.ca/), showing the complete genome. The Prokka annotation and CDS are represented by the blue arrows, while the outermost rings are VirSorter and Alien Hunter, respectively. The black represents GC content, while the green and magenta plots, respectively, represent CG skew+ and -. The metabolic pathways for trehalose in *Variovroax* sp. PAMC28711 have been identified.

Table 2. Distribution of the trehalose biosynthesis genes extracted from RAST and MetaCyc annotation database and its gene clusters in *Variovorax* sp. PAMC28711. The forward arrows indicate the genes in the positive strand, while the backward arrows indicate the genes in the negative strand.

Gene	Feature ID	Length (bp)	Function	Gene cluster
otsB	fig|1795631.8.peg.1307	1389	α,α-trehalose-phosphate synthase [UDP-forming] (EC 2.4.1.15)	
otsA	fig|1795631.8.peg.1306	753	Trehalose-6-phosphate phosphatase (EC 3.1.3.12)	
treS	fig|1795631.8.peg.3315	3354	Trehalose synthase (EC 5.4.99.16)	
treY	fig|1795631.8.peg.3313	1830	Malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141)	
treZ	fig|1795631.8.peg.3312	4059	Malto-oligosyltrehalose synthase (EC 5.4.99.15)	

The strain PAMC28711 was cultivated under sugar stress condition to determine its ability to produce trehalose. The appropriate cell was analyzed after strain had been cultured in stress media. To determine which trehalose production pathways were presented in the strain, the culture was treated with the appropriate substrates (glucose, starch, maltose, and maltodextrin). The intracellular produced trehalose was examined by TLC. As shown in Fig. 4, the spot corresponding to glucose, sucrose, maltose, starch, maltodextrin, and trehalose on the TLC plates were detected from the samples subjected to respective substrates treatment, suggesting that the cell extract contained trehalose. Since, it was confirmed that strain PAMC28711 has potential to produce trehalose through three (OtsBA, TreY/Z, and TS) pathways. Natural microorganisms could utilize a wide range of nutrients and have a high capacity for environmental adaptation. One strategy used by microbial cells to deal with nutritional deficiencies is the intracellular storage of carbohydrates for usage as carbon and energy sources [15].

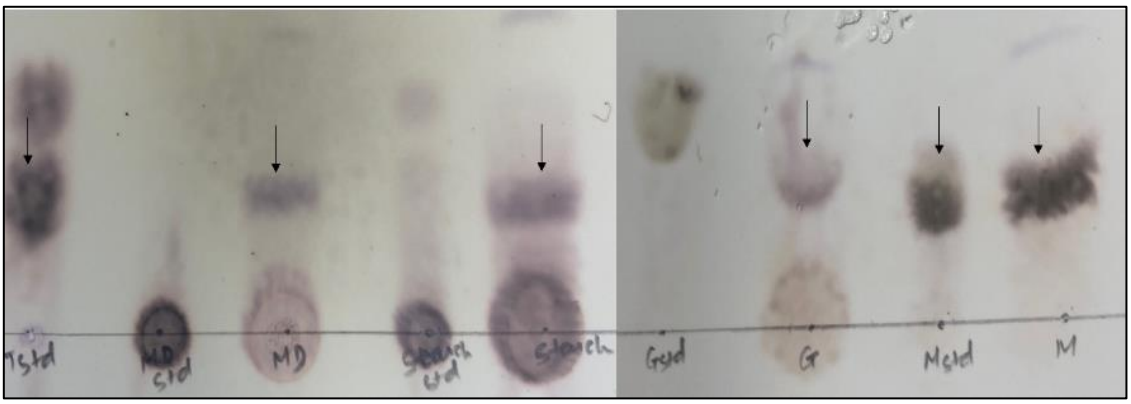


Figure 4. Qualitative analysis of trehalose-producing from strain PAMC28711. Tstd, trehalose standard; MD std, maltodextrin standard; starch std, starch standard; G std, glucose standard; M std, maltose standard; different sugar added (MD, maltodextrin; G, glucose; M, maltose). The arrowhead indicates the respective sample spots match with the standards.

4. Conclusions

In this study, we reported that cold adapted lichen associated *Variovorax* sp. PAMC28711 has potential to produce trehalose via three pathways (OtsBA, TreY/Z, and TS). We anticipate that the exploration of trehalose biosynthesis pathways in *Variovarax* sp. PAMC28711 revealed regarding the role of trehalose production at low temperatures and can be employed for biotechnological, industrial, and pharmaceutical applications.

Author Contributions: Conceptualization, P.S. and J.K.; methodology, P.S.; software, P.S.; validation, P.S., J.K. and T.-J.Oh.; formal analysis, P.S.; investigation, P.S.; resources, T.-J.Oh.; data curation, P.S.; writing—original draft preparation, P.S.; writing—review and editing, P.S.; visualization, P.S.; supervision, T.-J.Oh.; project administration, T.-J.Oh.; funding acquisition, T.-J.Oh.

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Conflicts of Interest: The authors declare no conflict of interest.

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