Cloning and Overexpression of a DhIFN-α1 Gene from the Halophilic Yeast of *Debaryomyces hansenii* into *Pichia methanolica* to Enhance its Tolerance to Salt and Temperature Stresses

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**ABSTRACT**

The interferon-α1 (IFN-α1) gene, a gene of type I interferons (IFNs), was cloned from *Debaryomyces hansenii* under 2.5 M of salt stress. DhIFN-α1 was constructed into a pMETB vector that carries the promoter P*α208*, which was induced by methanol. The expression cassette of P*α208*/DhIFN-α1/N5/His was excised from the vector and transformed *Pichia methanolica* through electroporation. The gene and expression of the transformant DhIFN-α1 was detected by PCR and dot blotting, respectively. The enhanced tolerance to salt and temperature stress of the transformant was verified by exposing it to 1.5 M of salt stress and a low and high temperature of 5 and 37°C, respectively. DhIFN-α1 has been successfully cloned and expressed in *P. methanolica*. This study confirmed that DhIFN-α1 might enhance the tolerance of organisms to salt and temperature stress.

**Key words:** *Debaryomyces hansenii*, DhIFN-α1, interferons, stress


**INTRODUCTION**

*Debaryomyces hansenii* is one of the most salt tolerant species of yeast and is usually found in salty environments such as the sea and in salty foods. Kushner categorized halophiles as slight halophiles (with optimal growth at 3% NaCl), moderate halophiles (optimal growth at 3%-15% NaCl) and extreme halophiles (optimal growth at 25% NaCl). Sodium chloride significantly improved the growth of *D. hansenii*, which could tolerate a salinity level of up to 24% (4.11 m) NaCl and was designed to be a halophilic yeast. Methylotrophic yeasts are used to express foreign proteins, especially *P. methanolica* possesses 2 genes (MOD1 and MOD2), which are induced by methanol; however, it is a salt-sensitive yeast compared to *D. hansenii*.

Interferon-α1 (IFN-α1) is a gene of type I interferons (IFNs), which are a group of cytokines possessing various biological functions including inhibition of proliferation, induction of differentiation, modulation of the immune system and inhibition of angiogenesis. IFNs function primarily as a first line of defense against viral infection by inducing the expression of genes that interfere with viral replication and act as natural killer cells. IFNs are usually used in the medical treatment of various illnesses including tumors, viral infections, autoimmune disorders and neurological syndromes. However, IFNs also appear to play an integral role in several autoimmune diseases. Kubo et al. reported that IFN-α, β, or γ combinations result in synergistic cell suppression.

In an experiment, Chao et al. inoculated *D. hansenii* under salt stress and then cloned the DhIFN-α1 gene from *D. hansenii* using cDNA forward subtractive hybridization. *P. methanolica* was transformed with a DhIFN-α1 gene to examine how DhIFN-α1 demonstrated stress tolerance. These results suggest that the stress tolerance of transformant *P. methanolica* was enhanced by the overexpression of the DhIFN-α1 gene.

**MATERIALS AND METHODS**

**Yeast strains and inoculations:** The halophilic yeast species, *Debaryomyces hansenii* strain BCRC No. 21947, isolated from Hsiho County, Taiwan, was obtained from the BCRC, Food Industry Research and Development Institute, Taiwan. The *Pichia methanolica* strain PMAD16
genotype ade-16 was obtained from (Invitrogen, USA). D. hansenii was inoculated at 24°C in a YM11 liquid medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% dextrose) and P. methanolica was inoculated at 28°C in a YPAD liquid medium (1% yeast extract, 2% peptone, 2% dextrose and 0.01% adenine).

Cloning and decorating DhIFN-α1: To screen differentially up-regulated genes, subtractive hybridization was performed using a PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA). D. hansenii was inoculated in a YM11 medium overnight; after adding NaCl to the medium for a final concentration of 2.5 M of NaCl, the total RNA was extracted after 24 min. The fragment of the DhIFN-α1 gene from a subtraction cDNA library was cloned with the rapid amplification of 5′- and 3′-cDNA ends (5′- and 3′-RACE) using the Gene Racer TM Kit (Invitrogen, USA) by following manufacturer instructions. The sequence of the fragment was queried for similarities through the NCBI database by using BLASTX sequence comparison software (http://www.ncbi.nlm.nih.gov/BLAST). The gene of Ifna 1 (Mus musculus interferon alpha 1, GI: 117168292) encoding the protein of the interferon α1 precursor (NP_034632) had the highest similarity by using the queried sequence (the DhIFN-α1 gene). Comparing the cDNA sequence of the Ifna 1 gene and the fragment of DhIFN-α1 gene showed that Ifna 1 has an additional 24 bp at the 5′ end cDNA sequence, which encodes a portion of the signal peptide based on the Target P 1.1 Server prediction (www.cbs.dtu.dk/services/TargetP). DhIFN-α1 was competed using a single peptide sequence and DhIFN-α1 was cloned with PCR using the forward primer of IFN-α1/F and the reverse primer of EcoRI for the \textit{\texttt{*}} decorated region

\texttt{IFN-α1/F: 5′-AGGAATTCTAATTGGCTAGGCTCTGTGCTTTCCGTGATGTT-3′}
\texttt{EcoRI for the \textit{\texttt{*}} decorated region}

\texttt{IFN-α1/R: 5′-ATGGATCTTTTCTCTCTCCCTCAGTCTCTCCAG-3′}

\texttt{BamHI:} PCR was performed under the following cycling conditions: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 1.5 min, 65°C for 1.5 min and 72°C for 1.5 min, followed by 72°C for 10 min and 4°C hold. The presence of the DhIFN-α1 gene was demonstrated through the PCR amplification of a 567 bp fragment by using the described forward and reverse primer. The PCR products were analyzed by using electrophoresis in a 1% agarose gel. The DNA fragment was cloned into a pGEM-T Easy vector (Promega, USA) for sequencing.

Construction of DhIFN-α1 in pMETB expression vector: To examine the DhIFN-α1 functions further, the pMETB vector was transformed using the P. methanolica Expression Kit (Invitrogen, USA). The entire DhIFN-α1 gene was first amplified by performing PCR and using the overexpressed 5′ primer, which introduced an EcoRI site in front of the starting ATG codon and the overexpressed 3′ primer, which introduced a BamHI site before the stop codon. The PCR-amplified product was cloned into the pGEM-T Easy vector to produce DhIFN-α1/pGEM-T. The DNA was transformed into E. coli. After digestion of DhIFN-α1/pGEM-T with EcoRI and BamHI, a 567-bp fragment was gel-purified from agarose by using a Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) and was ligated to a similarly digested pMETB expression vector. The DhIFN-α1/pMETB fragment was further verified by sequencing.

Overexpression of DhIFN-α1 in P. methanolica: The DNA fragment of the P.\textsubscript{wad}/DHIFN-α1/V5/His expression cassette was produced by \textit{\texttt{Pst I}} digesting the pMETB expression vector and was purified by the Wizard SV Gel and PCR Clean-Up System. The purified DNA fragments of the P.\textsubscript{wad}/DHIFN-α1/V5/His expression cassette were added to the P. methanolica electrocompetent cells and then a voltage intensity of 25 \textit{\texttt{μF}}×0.75 kV (Gene Pulser II Electroporation System, BIO-RAD, USA) was applied through an electro-cuvette. All the cells were then spread on YPAD agar plates containing 1.0 M of NaCl to screen out the transformants of \textit{\texttt{P. methanolica}}. A portion of absolute methanol was added to the plates to obtain a final concentration of 0.5%. Methanol was added to the plates every day until the colonies grew. First, the transformants were verified using PCR with AUG1 primers by following manufacturer instructions (\textit{\texttt{P. methanolica}} Expression Kit K1780-01, Invitrogen, USA). PCR analysis was then performed under the following cycling conditions: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 45 sec, 60°C for 30 sec, 72°C for 1.5 min, followed by 72°C for 10 min and 4°C hold. The PCR amplification of an 875 bp fragment was performed using the forward primer: 5′-CAATTATACATTTTATATTTAACG-3′ and reverse primer: 5′-GAAGAGAACCATTAGTGGC-3′. The PCR products were analyzed using electrophoresis in 1% agarose gel.

To determine the growth of the transformant \textit{\texttt{P. methanolica}} in NaCl and under temperature stress, a single colony of the wild type \textit{\texttt{P. methanolica}} or the transformant was first inoculated in the YPAD liquid
medium overnight at 28°C. An aliquot of 1 mL of each cell culture was transferred to 30 mL of the YPAD liquid medium at 28°C and was shaken until the O.D. 600 had reached 1. The cell culture was then diluted to an O.D. 600 = 0.3 with a fresh YPAD liquid medium supplement. For the drop test under both salt or temperature stress, portions of the 3 μL serial dilution (1x, 10x and 100x) were spotted onto the agar plates, which contained final concentrations of 0.5, 1.0, 1.15, 1.3, 1.5 and 1.8 M of NaCl, respectively for the temperature stress test, the inoculations were 5, 8, 28, 35, 37 and 40°C, respectively. Because the expression of the insertion gene was induced by the methanol-inducible AUG1 promoter, the plates were with/without MeOH by adding absolute methanol to a final concentration of 0.5%.

Verifying the expression of the DhIFN-α1/V5/His fusion protein: Wild type P. methanolica and the transformant were streaked onto the MD plates and then a single colony of wild type P. methanolica and transformant was transferred into the BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5} % biotin and 2% Dextrose) for overnight inoculation at 28°C with shaking. To verify the expression of the fusion protein of DhIFN-α1/V5/His, the cell pellets were harvested by centrifugation and then resuspended in the BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 0.5% methanol and 4×10^{-3} % biotin); absolute methanol was added to the medium with a final concentration of 0.5% every 24 h for 5 day to induce fusion protein expression. The secreted protein was analyzed using dot blotting. Protein was extracted from individual transformants by using the Total Protein Miniprep Purification Kit (GMBiolab Co., Ltd. USA). Thirty micrograms of protein from the transformants and the wild type were loaded onto the PVDF membrane for immunostaining and the PVDF membrane was incubated with the first antibody of mouse anti-V5 (NOS, 37-750), Invitrogen, USA) and the second antibody of AP-conjugated rabbit anti-mouse IgG (No. 81-6522, Invitrogen, USA). Finally, BCIP/NBT phoshase (ZYMED, Invitrogen, USA) was added to the PVDF membrane until spots appeared.

RESULTS
Cloning and characteristics of DhIFN-α1: In this study, PCR was successfully employed to clone a fragment of the DhIFN-α1 gene from the subtraction cDNA library of D. hansenii, which was induced by 2.5 M of NaCl for 24 min and inoculated in a YM medium. The DNA fragment of the PCR product was sequenced with a 567 bp open reading frame (Fig. 1a) and it encoded a deduced protein of 189 amino acid residues (Fig. 1b), the sequence of which was a homolog of the gene of Ilf1 (GI: 117168292) of M. musculus. Furthermore, the deduced protein sequence was compared with those of related proteins from the EMBL database by using the EMBOSS alignment program; the deduced protein had a 99% similarity to the M. musculus interferon alpha-1 precursor (NP_034632) and therefore, was named DhIFN-α1.

Overexpression of DhIFN-α1 in P. methanolica: After transferring the P. walgae/DhIFN-α1/V5/His expression cassette into P. methanolica, the transformed products were spread onto YPAD agar plates that contained 1 M of NaCl to screen out the successful transformants of P. methanolica that had gained a salt tolerance ability. The DNAs extracted from the transformants of P. methanolica were used as the templates for the PCR reactions with the AUG1 primers. The transformants presented an 875 bp fragment of the P. walgae/DhIFN-α1/V5/His expression cassette (Fig. 2) and DNA from the wild type P. methanolica disappeared from the DNA fragment. The results confirmed that the expression cassette DNA fragment was integrated into the transformed P. methanolica genome.

The function of DhIFN-α1 was further tested by overexpressing the gene using methanol and subjecting P. methanolica to salt stress. The growth of the wild type was not obviously different from the transformant when they were inoculated in the YPAD media content of 0.5, 1, 1.15 and 1.3 M of NaCl with and without methanol. When the NaCl concentration of the YPAD medium was increased to 1.5 M, the growth of the transformants was obviously better than that of the wild type, especially in the presence of methanol in the medium. The wild type and transformants appeared tolerant to 1.8 M of NaCl stress, both with and without methanol (Fig. 3). However, the transformant was able to maintain a better growth rate under high salt conditions, especially in the presence of methanol, which induced the overexpression of DhIFN-α1.

Furthermore, in a growth test under temperature stress, the wild type was not obviously different from the transformant, although it was inoculated in the YPAD liquid media at 8, 28 and 35°C, both with and without methanol. When the temperature of the inoculated YPAD medium was decreased or increased to 5 or 37°C, respectively, the growth of the transformants was obviously better than that of the wild type, especially in the presence of methanol (Fig. 4). The wild type and transformant appeared tolerant to 46°C stress both with and without the addition of methanol. The results indicated that the overexpression of DhIFN-α1 in the transformant of P. methanolica conferred an enhanced temperature tolerance, which enabled the transformant to grow at higher and lower temperatures.
Expression of DhiIFN-α1/V5/His fusion protein: The white colonies on the MD plate were chosen to verify the Mut<sup>+</sup> (methanol usage plus) and Mut<sup>−</sup> (methanol usage slow) transformant colonies of P. methanolica. The transformants were selected to assay the fusion protein expression using dot blotting with an anti-V5 antibody. The transformants were then confirmed as having good expression of the fusion protein that had a V5 epitope in its C-terminal region, resulting in a spot appearing in the dot blotting (Fig. 5). The results confirmed that the methanol-inducible AUG1 promoter drove the expression of the DhiIFN-α1/V5/His fusion protein by using methanol.

DISCUSSION

D. hansenii has the unusual ability to grow under high NaCl concentrations<sup>5</sup>. Prista <em>et al.</em> demonstrated that the genetic material from <em>D. hansenii</em> caused a rise in salt tolerance. In this study, the DhiIFN-α1 gene was cloned from <em>D. hansenii</em> and P. methanolica was transformed through electroporation. The transformants of <em>P. methanolica</em> were obtained by screening these colonies on plates containing 1 M of NaCl. Successful transformants were confirmed to gain the DNA fragment of the <em>P<sub>AUG1</sub>/DhiIFN-α1/V5/His</em> expression cassette through PCR and the successful expression of the fusion protein of DhiIFN-α1/V5/His. Overexpression of
Fig. 2: Confirmation of the constructed DhIFN-α1/pMETB using PCR with AUG1 primer, W: Wild type and T: Transformant

Fig. 3: Growth of wild type (W) and transformant (T) of *P. methanolicus* of serial dilutions suspensions were spotted on YPAD medium containing NaCl and MeOH, (a) 0.5 M NaCl, (b) 0.5 M NaCl+MeOH, (c) 1 M NaCl, (d) 1 M NaCl+MeOH, (e) 1.15 M NaCl, (f) 1.15 M NaCl+MeOH, (g) 1.3 M NaCl, (h) 1.3 M NaCl+MeOH, (i) 1.5 M NaCl, (j) 1.5 M NaCl+MeOH, (k) 1.8 M NaCl, (l) 1.8 M NaCl+MeOH

DhIFN-α1/V5/H5s in the transformant was caused by the *P. wortmanni* promoter under the influence of methanol. The fusion protein detected by dot blotting using an anti-V5 antibody was secreted by the transformant of *P. methanolicus*, which demonstrated a higher stress tolerance than the wild type did; hence, we concluded...
Fig. 4: Growth of wild type (W) and transformant (T) of *P. methanolica* in serial dilutions suspensions were spotted on YPAD medium with and without MeOH under 5, 8, 28, 35, 37 and 40°C temperature stress. (a) 5°C, (b) 5°C+MeOH, (c) 8°C, (d) 8°C+MeOH, (e) 28°C, (f) 28°C+MeOH, (g) 35°C, (h) 35°C+MeOH, (i) 37°C, (j) 37°C+MeOH, (k) 40°C, (l) 40°C+MeOH

Fig. 5: Dot blot analysis of DhiIFN-α1/V5/His fusion protein detected by anti-V5 antibody extracted from transformants (T) of *P. methanolica* and wild type (W)

that the DhiIFN-α1 protein might enhance the stress tolerance of yeast.

The growth test for the wild type and transformants of *P. methanolica* exposed them to salt or temperature stress (Fig. 3 and 4). The drop tests for growth showed that all *P. methanolica* transformants were more tolerant to salt and temperature stress than the wild type were, especially in the presence of methanol, which is an inducer that drives the overexpression of DhiIFN-α1. However, the DhiIFN-α1 gene cloned from the halophilic yeast of *D. hansenii* demonstrated that overexpression of DhiIFN-α1 in the transformants of *P. methanolica* enhanced its salt or temperature tolerance, enabling the transformant to grow under salt and temperature stress conditions. Most studies have shown that IFNs have great potential as drugs in the treatment of various virus-infected diseases, such as hepatitis C and severe acute respiratory syndrome (SARS). Most nucleated cells secrete one or more type I IFNs in response to viral infection. Type I IFNs then induce viral protective responses in neighboring non-infected cells. Therefore, IFN-α1, a member of the IFN family, is best known for its antiviral activity. In addition, Belarain *et al.* demonstrated that the stabilization of hrIFN-α is highly dependent on the salt species and its ionic strength. Therefore, it is remarkable that DhiIFN-α1 is one of the major up-regulated genes under salinity stress. However, salt stress in DhiIFN-α1 has not yet been studied. Our experimental results showed that the salt stress-induced expression of DhiIFN-α1 in *D. hansenii* provided the transformants of *P. methanolica* with an enhanced salt tolerance.

To test if the overexpression of DhiIFN-α1 enhanced stress tolerance, the overexpression of DhiIFN-α1 in the transformants of *P. methanolica* were inoculated at various temperatures (Fig. 4) in the presence of methanol. The results showed that the transformant of *P. methanolica* was considerably more tolerant to temperature stress at 5 and 37°C than the wild type was, based on the results of the drop test. Sharma and Kalonia reported that the tertiary structure of hrIFN-α2a was significantly degraded with temperatures that increased from 15-50°C. Huang *et al.* showed that the expression of hrIFN-α2a molecules were attractive and increased with temperature; however, the compact conformation of the protein became looser at higher and lower temperatures during bioprocessing. Perhaps most significantly, DhiIFN-α and HSP-70 both
accumulated in the cells of *D. hansenii* when they were exposed to salt stress for only 24 min (data not shown); thus, IFN-α and HSP-70 might play a protective role in responding to stress because Heat Shock Proteins (HSPs) are a group of proteins that are rapidly induced when cells are exposed to stress. Therefore, by rapidly inducing the expression of a wide array of genes in response to cell protein-denaturing stress, IFN-α might be one of the associated genes with high-level expression under salt stress conditions. However, the DhIFN-α1 gene related to salt stress in yeast has yet to be studied.

CONCLUSION

In this experiment, the DhIFN-α1 gene was cloned from the extremely halophilic yeast *D. hansenii* under salt stress exposure. The transformed yeast of the *P. methanolicum* overexpression provided DhIFN-α1 with a substantial tolerance to salt and temperature. We conclude that the overexpression of the DhIFN-α1 gene in *P. methanolicum* might enhance its stress tolerance.

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REFERENCES


