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Cloning of a Hypothetical NiCoT gene of *Neurospora crassa*

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In the present investigation attempts have been made to clone the NiCoT gene of *N.crassa*. For this purpose, the RNA was isolated from *N. crassa* and it was converted into cDNA. The nct gene was amplified using the gene specific primers. The genomic and protein sequences of nct genes have traced out using the FCGS web page and the sequences have been reported. It has also been observed that the rate of transportation in *N.crassa* is enhanced by the action of NiCoT gene. The transmembrane domains of the nct gene were identified using the TMpred software.

Key words: Cloning, Nickel, Cobalt, NiCoT transporter gene, *Neurospora crassa*.

Many microbes are capable of sensing cellular nickel ion concentrations and taking up this nutrient via nickel-specific permeases or ATP-binding cassette-type transport systems. The metal ion is specifically incorporated into nickel-dependent enzymes, often via complex assembly processes requiring accessory proteins and additional non-protein components, in some cases accompanied by nucleotide triphosphate hydrolysis. To date, nine nickel-containing enzymes are known: urease, NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarbonylase/synthase, methyl coenzyme M reductase, certain superoxide dismutases, some glyoxylases, aci-reductone dioxygenase, and methylenediurease. Seven of these enzymes have been structurally characterized, revealing distinct metallocenter environments in each case. In contrast to the diverse roles of nickel in microbial metabolism, cobalt is mainly found in the corrin ring of coenzyme B12, a cofactor involved in methyl group transfer and in rearrangement reactions and plays a number of crucial roles in many biological functions (Degen *et al.,* 1999, Eitinger *et al.,* 2005, Eitinger and Mandrand - Berthelot., 2000. and Sajani and Mohan., 1998).

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Therefore, in the present paper attempts have been made to clone the hypothetical NiCoT gene of *N.crassa*.

MATERIALS AND METHODS

Fungal Culture

Wild type *Neurospora crassa* FGSC # 4200(a) was obtained from Fungal Genetics Stock Center (FGSC), Kansas City, USA. The obtained fungal cultures were grown in laboratory conditions. The media used for culturing fungi was basal medium containing Glucose, Ammonium nitrate, Ammonium tartarate, Potassium dihydrogen orthophosphate, Magnesium sulfate, Calcium chloride and Sodium chloride at a concentration of 2.0, 0.2, 0.1, 0.3, 0.05, 0.01 & 0.01 g/100ml respectively. In addition to this trace elements namely Zinc sulfate, Manganese sulfate, Copper sulfate, Ammonium molybdate, Ammonium ferric citrate, and Biotin at a concentration of 40.0, 40.0, 16.0, 4.0, 4.0 & 1.0 µg/100ml respectively. The cultures were incubated for 3 days at 28 ± 10 C. The pH of the medium was adjusted to 5.0. For the preparation of slants 3% agar was included in the medium. Metal ions were supplemented in the basal medium as sulfates.

Isolation of RNA from *Neurospora crassa*

For isolation of the RNA from *N.crassa* the following methodology was used:

- § Suspend three slants of *N.crassa* spores in sterile MQ and inoculate 200ml of this suspension to 10ml of basal media.
- § Harvest 24hrs mycelia and after squeezing transfer the mycelia to mortar.
- § Add liquid N2 and homogenate with pestle.
- § When liquid N2 dries add 800ml to 1000ml of trizol reagent.
- § Allow to stand this at room temperature and homogenize
- § Transfer 750ml of the clear homogenate to 1.5ml eppendrof tube and allow it to stand for 5min at room temperature.
- § Add 200ml of chloroform to 750ml of Tri reagent used.
- § Cover the samples tightly and shake vigorously for 15sec. Allow it to stand for 5min at room temperature
- § Spin at 12000g for 15min at room temperature.
- § Transfer the upper aqueous layer to a fresh tube.
- § Add 500ml of isopropanol and mix it well. Allow it to stand for 5min at room temperature
- § Spin at 12,000g for 10min. At this stage RNA is precipitated
- § Remove the supernatant and wash with 1ml of 75% ethyl alcohol. Mix well and spin at 7,500g for 5min.
- § Air dry the RNA pellet
- § Add 50ml of DEPC treated water to dissolve the pellet.
- § Keep the tubes at 60°C for 15min and load 5ml of the RNA on 2% agarose gel and quantitate the RNA at 260nm.

Synthesis of cDNA

The cDNA was synthesized using the Fermentas cDNA synthesis kit. The reaction mixture was prepared in a tube on ice Template RNA (total RNA) 10ng, Oligo (dT) primer (0.5µg/µl) 1µl, DEPC-Treated water 12µl.

Primer Designing

Primers for PCR are typically short sequences, around twenty nucleotides long. Primers bind to opposite strands of the DNA on either side of the target sequence, so that the section of DNA between, not outside of them, is copied. Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature of DNA duplex increases both with its length, and with increasing (G+C) content Primers can be designed manually or by using software like Primer 3.

Forward primer: 5' GCG GCC GCA TGG CCC GCC TCA AAC TCC C 3'

Reverse primer: 5' GGA TCC AAC AAT CCG TTC CGG AAC ACG TTC 3'

PCR (Polymerase Chain Reaction) Amplification

The total 50ml reaction mixer used for PCR amplification contains master mix (dNTPs, taq DNA polymerase and Mgcl2) 25ml, Template 1ml (100ng/ml), Primer F - 1ml (100pmoles/ml), Primer R-1ml (100pmoles/ml) and Milli Q 22ml. The above reaction mixture is kept in the thermocycler for 30 cycles and the conditions for nct amplification are Initial Denaturation 94°C for 5min, Denaturation 94°C for 1min, Annealing 60°C for1min 30 seconds, Extension 72°C for1 min and Final extension 72°C for 10 minutes. The PCR fragments obtained were separated on 1% agarose gel electrophoresis and purified by gel extraction procedure according to manufacturer's protocol using Qiaquick gel extraction kit.

TA cloning

TA Cloning is one of the most popular methods of cloning the amplified PCR product using Taq polymerases. TA cloning is brought about by the terminal transferase activity of certain type of DNA polymerase such as the Taq polymerase. This enzyme adds a single, 3'-A overhang to each end of the PCR product. As a result, the PCR product can be directly cloned into a linearized cloning vector that have single base 3'-T overhangs on each end. Such vectors are called T- vectors. The PCR product with "A" overhang, is mixed with this vector in high proportion. The complementary overhangs of a "T" vector and the PCR product hybridize. The result is a recombinant DNA, the recombination being brought about by DNA ligase.

Then prepare a bacterial competent cell by using DH5a- E.coli strain and grow overnight, Pick single colony and grow in 10 ml LB media for overnight under shaking conditions. Subculture into 50-100 ml LB media without antibiotic and allow to grow cells with vigorous shaking until an absorbance of 0.4-0.6 OD is achieved; on keep the cells in ice for 30min. Dispense the cells into four 50 ml sterile centrifuge tubes and spin cells at 6000 rpm at 4°c for 10 min. Discard the supernatant and wash the cells with 25 ml sterile, ice-cold 0.1M CaCl2 by gentle pipetting leave on ice for 30 min. Spin again at 6000 rpm for 10 min. Resuspend the pellet in 2ml sterile, ice-cold 0.1 M CaCl2 in 15% glycerol. Freeze the aliquots of cells (100ml) in sterile and labeled micro centrifuge tubes (at -80°c).

Transformation

For this purpose, freeze- thaw the competent cells by keeping in ice for 30min prior to the addition of Ligation mix (plasmid). Add 2-5ml of Ligation mix (plasmid) to the cells, mix by tapping and incubate in ice for 30min. Give heat shock to the cells at 420C for 3min. Immediately place them on ice for 5min then add 900ml of pre-warmed LB media and incubate at 370C for 1 hour. Spin down for 3mins at 6000rpm and resuspend the bacterial pellet in 100ml of LB broth by discarding the remaining media. Mix and spread the bacterial suspension on respective antibiotic resistance plate and incubate it at 370C for overnight.

Plasmid isolation was carried by transfering a single bacterial colony in to a 10ml of LB medium containing the 100mg/ml ampicillin. Incubate the culture overnight at 370C with vigorous shaking. Pour 3ml of the culture into a microfuge tube. Centrifuge at 10,000 rpm for 3 minutes. Decant the medium leaving the bacterial pellet as dry as possible. Resuspend the bacterial pellet in 100 µl of ice-cold solution-I (chilled resuspending solution) by vigorous vortexing. Keep it at room temperature for 5 minutes. Add 100µl of freshly prepared solution II (lysis buffer) to each bacterial suspension. Don't vortex and move to next step. Add 150µl of ice-cold solution III (Neutralising buffer), close the tube and disperse Solution III in the viscous bacterial lysate by inverting the tube several times. Allow it to stand for 5-10 minutes. Centrifuge the bacterial lysate at 13,600rpm for 10 minutes at 40C in microfuge. Transfer the supernatant to a fresh tube. To the supernatant add 1 ml of isopropanol and allow it to stand for 10min at room temperature. Centrifuge at 10,000rpm for 20 min and discard isopropanol. Wash the pellet with 1ml of 70% ethanol by centrifuge at 13,600rpm for 10minutes. Decant the 70% ethanol and air dry the pellet till the alcohol evaporates completely. Dissolve the DNA pellet in 50 µl of MQ or TE buffer. Store the DNA at -200C. The plasmid isolated above is kept for digestion using enzyme BamH? &Tango buffer.

RESULTS

The RNA was isolated from *Neurospora crassa* (Fig. 1). The RNA was converted into cDNA and the nct gene was amplified using the gene specific primers.

The genomic and protein sequences of nct gene have traced out using the FGCS web page and the sequences have been shown in (Fig.2 and Fig.3).

The coding sequences of nct gene (hypothetical NiCoT gene)

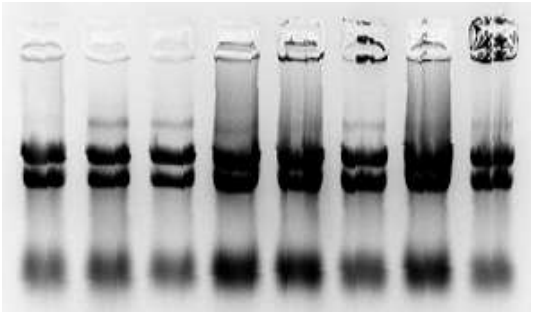


Fig.1. *Neurospora crassa* total RNA using TRI reagent

```
ATGGCCCGCCTCAAACTCCCCCACCTCCGGCTCCCACCACCCCTCTCCTACATCCCCCGCCCCGCCCTCCA
CCTCATCACCCTCCTCATCCTCATCAACTGCCTCGTCTGGGCCGGCGTCGGCATCACCCTGCACTACTTCC
CCAAGATGATCTCGCCCGCCGTGCTCTCCTACACCCTCGGCCTCCGCCACGCCCTCGACGCCGACCACATC
AGCGCCATCGACCTGATGACGCGCCGTCTGATTGCTTCCGGTCAGCGACCCGTAGCCGTGGGCACCTTTTT
CAGCTTGGGACACAGCACCGTTGTCATCATCACTTGCATCGTGGTAGCTGCGACGAGTGGAGCGCTAAGAG
ACAGGTTTGATGGGTTTCAGCGGGTGGGCGGCATCATTGGCACGTCTGTGAGCGCCGCCTTTTTGATTATC
TTGGGAGTGGGGAACGGGTGGGTGTTGTATAAGCTGGTGAAGCGGTTGCGGGTGGTGATGAAGGAGCAGAG
GGAGCGCAGAAGGAGGGTGGAGGAAGGAGAGGATTTGCGAGCCGAGGAGGAGCAGGAGGCCATGAATAACT
TGCAGTTGGAAGGGGCCGGGTTTCTGGCGAGGGTGTTTAGGAGGGTGTTTGCCATTGTGGATCGGCCGTGG
AAGATGTACCCGCTGGGTGTACTGTTTGGACTCGGGTTCGATACCAGTTCGGAGGTGGCCTTGCTGGGGAT
TGCGAGCATACAGGCAGTGCAGGGGACGAGCATCTGGTTGATTCTGATTTTTCCGATTTTGTTTACTGGTG
AGTTTCCACTTTGAAGAGTGTGTAAGAGTCAAGATGATTGCTAACGAGATGCTACGACAGCCGGCATGTGC
ATGCTCGACACGACCGACGGCGCCCTGATGATGGCCCTGTACACATCCAAGGCCTTCTCGAGAGACGTGGT
GGCCATCCTGTACTACTCGATTGTGCTTACCGGCATCACGGTCGTTGTGTCGGCCTTCATCGGCGTCATCC
AGATCCTGTCGCTTGCCTACAACGTGGCCGAGCCGACAGGTCGTTTCTGGGATGGGGTTGAAGCGATAGGC
GATCACTACGACGTTATTGGCGGATGCATCTGCGGCTTGTTTGTCGTTGTCGGTCTGGCGTCGGTCATCAT
CTATCGCCCCTGGAGGAAAAAGATGGAGGATCGGATGGATGCCATGAGCATATTGGATGTCGAAAGCCCGG
TCGCGTCGCCCGCTGCGGTTGCGGATGGTGCTATGAGCCCGTATGCCGATAGTCCGGTGGTCACGCCCTTG
GAACGTGTTCCGGAACGGATTGTTTGA
```

Fig. 2. Coding sequence (including introns)

```
ATGGCCCGCCTCAAACTCCCCCACCTCCGGCTCCCACCACCCCTCTCCTACATCCCCCGCCCCGCCCTCCA
CCTCATCACCCTCCTCATCCTCATCAACTGCCTCGTCTGGGCCGGCGTCGGCATCACCCTGCACTACTTCC
CCAAGATGATCTCGCCCGCCGTGCTCTCCTACACCCTCGGCCTCCGCCACGCCCTCGACGCCGACCACATC
AGCGCCATCGACCTGATGACGCGCCGTCTGATTGCTTCCGGTCAGCGACCCGTAGCCGTGGGCACCTTTTT
CAGCTTGGGACACAGCACCGTTGTCATCATCACTTGCATCGTGGTAGCTGCGACGAGTGGAGCGCTAAGAG
ACAGGTTTGATGGGTTTCAGCGGGTGGGCGGCATCATTGGCACGTCTGTGAGCGCCGCCTTTTTGATTATC
TTGGGAGTGGGGAACGGGTGGGTGTTGTATAAGCTGGTGAAGCGGTTGCGGGTGGTGATGAAGGAGCAGAG
GGAGCGCAGAAGGAGGGTGGAGGAAGGAGAGGATTTGCGAGCCGAGGAGGAGCAGGAGGCCATGAATAACT
TGCAGTTGGAAGGGGCCGGGTTTCTGGCGAGGGTGTTTAGGAGGGTGTTTGCCATTGTGGATCGGCCGTGG
AAGATGTACCCGCTGGGTGTACTGTTTGGACTCGGGTTCGATACCAGTTCGGAGGTGGCCTTGCTGGGGAT
TGCGAGCATACAGGCAGTGCAGGGGACGAGCATCTGGTTGATTCTGATTTTTCCGATTTTGTTTACTGCCG
GCATGTGCATGCTCGACACGACCGACGGCGCCCTGATGATGGCCCTGTACACATCCAAGGCCTTCTCGAGA
GACGTGGTGGCCATCCTGTACTACTCGATTGTGCTTACCGGCATCACGGTCGTTGTGTCGGCCTTCATCGG
CGTCATCCAGATCCTGTCGCTTGCCTACAACGTGGCCGAGCCGACAGGTCGTTTCTGGGATGGGGTTGAAG
CGATAGGCGATCACTACGACGTTATTGGCGGATGCATCTGCGGCTTGTTTGTCGTTGTCGGTCTGGCGTCG
GTCATCATCTATCGCCCCTGGAGGAAAAAGATGGAGGATCGGATGGATGCCATGAGCATATTGGATGTCGA
AAGCCCGGTCGCGTCGCCCGCTGCGGTTGCGGATGGTGCTATGAGCCCGTATGCCGATAGTCCGGTGGTCA
CGCCCTTGGAACGTGTTCCGGAACGGATTGTTTGA
```

Fig. 3. Coding sequence (exons only)

Translated sequence: 414 aa

MARLKLPHLRLPPPLSYIPRPALHLITLLILINCLVWAGVGITLHYFPKMISPAVLSYTLGLRHALDADHISAIDLMTRRL
IASGQRPVAVGTFFSLGHSTVVIITCIVVAATSGALRDRFDGFQRVGGIIGTSVSAAFLIILGVGNGWVLYKLVKRLRVVM
QGTSIWLILIFPILFTAGMCMLDTTDGALMMALYTSKAFSRDVVAILYYSIVLTGITVVVSAFIGVIQILSLAYNVAEPTG
RFWDGVEAIGDHYDVIGGCICGLFVVVGLASVIIYRPWRKKMEDRMDAMSILDVESPVASPAAVADGAMSPYADSPVVTPL
ERVPERIV

Fig. 4. Amino acid sequence of nct gene

The nucleotide and predicted amino acid sequence of Nct (Fig. 4). The potential TATA box, CAAT box and polyadenylation sequences are noted. The ORF extends from +1 to +1305, interrupted by an intron from +788 to +840 (lowercase). The deduced amino acids are given below the sequence of the coding region. The arrow between residues 38 and 39 indicates a predicted signal cleavage site (Fig. 5).

```
   1 - ATGGCCCGCCTCAAACTCCCCCACCTCCGGCTCCCACCACCCCTCTCCTACATCCCCCGC - 60
   1 - M  A  R  L  K  L  P  H  L  R  L  P  P  P  P  L  S  Y  I  P  R  - 20

  61 - CCCGCCCTCCACCTCATCACCCTCCTCATCCTCATCAACTGCCTCGTCTGGGCCGGCGTC - 120
  21 - P  A  L  H  L  I  T  L  L  I  L  I  N  C  L  V  W  A  G  V  - 40

 121 - GGCATCACCCTGCACTACTTCCCCAAGATGATCTCGCCCGCCGTGCTCTCCTACACCCTC - 180
  41 - G  I  T  L  H  Y  F  P  K  M  I  S  P  A  V  L  S  Y  T  L  - 60

 181 - GGCCTCCGCCACGCCCTCGACGCCGACCACATCAGCGCCATCGACCTGATGACGCGCCGT - 240
  61 - G  L  R  H  A  L  D  A  D  H  I  S  A  I  D  L  M  T  R  R  - 80

 241 - CTGATTGCTTCCGGTCAGCGACCCGTAGCCGTGGGCACCTTTTTCAGCTTGGGACACAGC - 300
  81 - L  I  A  S  G  Q  R  P  V  A  V  G  T  F  F  S  L  G  H  S  - 100

 301 - ACCGTTGTCATCATCACTTGCATCGTGGTAGCTGCGACGAGTGGAGCGCTAAGAGACAGG - 360
 101 - T  V  V  I  I  T  C  I  V  V  A  A  T  S  G  A  L  R  D  R  - 120

 361 - TTTGATGGGTTTCAGCGGGTGGGCGGCATCATTGGCACGTCTGTGAGCGCCGCCTTTTTG - 420
 121 - F  D  G  F  Q  R  V  G  G  I  I  G  T  S  V  S  A  A  F  L  - 140

 421 - ATTATCTTGGGAGTGGGGAACGGGTGGGTGTTGTATAAGCTGGTGAAGCGGTTGCGGGTG - 480
 141 - I  I  L  G  V  G  N  G  W  V  L  Y  K  L  V  K  R  L  R  V  - 160

 481 - GTGATGAAGGAGCAGAGGGAGCGCAGAAGGAGGGTGGAGGAAGGAGAGGATTTGCGAGCC - 540
 161 - V  M  K  E  Q  R  E  R  R  R  R  V  E  E  G  E  D  L  R  A  - 180

 541 - GAGGAGGAGCAGGAGGCCATGAATAACTTGCAGTTGGAAGGGGCCGGGTTTCTGGCGAGG - 600
 181 - E  E  E  Q  E  A  M  N  N  L  Q  L  E  G  A  G  F  L  A  R  - 200

 601 - GTGTTTAGGAGGGTGTTTGCCATTGTGGATCGGCCGTGGAAGATGTACCCGCTGGGTGTA - 660
 201 - V  F  R  R  V  F  A  I  V  D  R  P  W  K  M  Y  P  L  G  V  - 220

 661 - CTGTTTGGACTCGGGTTCGATACCAGTTCGGAGGTGGCCTTGCTGGGGATTGCGAGCATA - 720
 221 - L  F  G  L  G  F  D  T  S  S  E  V  A  L  L  G  I  A  S  I  - 240

 721 - CAGGCAGTGCAGGGGACGAGCATCTGGTTGATTCTGATTTTTCCGATTTTGTTTACT*ggt* - 780
 241 - Q  A  V  Q  G  T  S  I  W  L  I  L  I  F  P  I  L  F  T     - 259

 781 - *gagtttccactttgaagagtgtgtaagagtcaagatgattgctaacgagatgctacgaca* - 840
     -                                                               -

 841 - GCCGGCATGTGCATGCTCGACACGACCGACGGCGCCCTGATGATGGCCCTGTACACATCC - 900
 260 - A  G  M  C  M  L  D  T  T  D  G  A  L  M  M  A  L  Y  T  S  - 279

 901 - AAGGCCTTCTCGAGAGACGTGGTGGCCATCCTGTACTACTCGATTGTGCTTACCGGCATC - 960
 280 - K  A  F  S  R  D  V  V  A  I  L  Y  Y  S  I  V  L  T  G  I  - 299

 961 - ACGGTCGTTGTGTCGGCCTTCATCGGCGTCATCCAGATCCTGTCGCTTGCCTACAACGTG - 102
 300 - T  V  V  V  S  A  F  I  G  V  I  Q  I  L  S  L  A  Y  N  V  - 319

1021 - GCCGAGCCGACAGGTCGTTTCTGGGATGGGGTTGAAGCGATAGGCGATCACTACGACGTT - 108
 320 - A  E  P  T  G  R  F  W  D  G  V  E  A  I  G  D  H  Y  D  V  - 339

1081 - ATTGGCGGATGCATCTGCGGCTTGTTTGTCGTTGTCGGTCTGGCGTCGGTCATCATCTAT - 114
 340 - I  G  G  C  I  C  G  L  F  V  V  V  G  L  A  S  V  I  I  Y  - 359

1141 - CGCCCCTGGAGGAAAAAGATGGAGGATCGGATGGATGCCATGAGCATATTGGATGTCGAA - 120
 360 - R  P  W  R  K  K  M  E  D  R  M  D  A  M  S  I  L  D  V  E  - 379

1201 - AGCCCGGTCGCGTCGCCCGCTGCGGTTGCGGATGGTGCTATGAGCCCGTATGCCGATAGT - 126
 380 - S  P  V  A  S  P  A  A  V  A  D  G  A  M  S  P  Y  A  D  S  - 399
```

Fig. 5. Comparative alignment of the nct gene sequence and protein sequence.

Alignment of nickel permeases. Comparison of the amino acid sequences of *Nct* and related proteins from *S.pombe* (*Nic1p*), *Cupriavidus necator* (*HoxN*), *Rhodococcus rhodochrus* (*NhlF*), *Helicobacter pylori* (*NixA*) . Identical residues are marked by astrix, and similar residues are marked by colon. The nickel-binding motif HAXDADH is underlined (Fig. 6).

```
NhlF            MTSTTITPHHIGGAW--------TRTERRRLASVVGAIVILHVLGVALYLGYS
52
NixA            --MKLWFPYFL-------------------------AIVFLHALGLALLF---
28
HoxN            -MFQLLAGVRMN-------------STGRPRAKIILLYALLIAFNIGAWLCAL
46
Nic1p           -MSEYVKPRKNEFLRKFENFYFEIPFLSKLPPKVSVPIFSLISVNIVVWIVAA
59
Nct             -MARLKLP----HLR-------LPPPLSYIPRPALHLITLLILINCLVWAGVG
48
                                                  *  ..

NhlF            G-LAGSGVLAYVLGVRHAFDADHIAAIDDTTRLMLLRGRRPVGVGFFFAMGHS
111
NixA            S-FYAAASMAYMLGAKHAFDADHIACIDNTIRKLTQQGKNAYGVGFYFSMGHS
87
HoxN            V-LLGTALLAYGLGLRHAVDADHLAAIDNVTRKLMQDGRRPITAGLWFSLGHS
105
Nic1p           RSLFLSVLLSWTLGLRHALDADHITAIDNLTRRLLSTDKPMSTVGTWFSIGHS
119
Nct             K-MISPAVLSYTLGLRHALDADHISAIDLMTRRLIASGQRPVAVGTFFSLGHS
107
                  :  .  ::: ** :**.****::.**   * :   .:    .* :*::***

NhlF            LVVALGASALTTTELEGVQEIGGLVATVVAVTFLSIVAGLNSVVLRNLLCLSR
171
NixA            IISAF-AIAWAKEHTPMLEEIGGVVGTLVSGLFLLIIGLLNAIILLDLLKIFK
146
HoxN            VLIAVMATTLQ-ERLDAFHEVGSVIGTLASALFLFAIAAINLVILRSAYRAFR
164
Nic1p           IVVAATSSKFA-DRWNNFQTIGGIIGTSVSMGLLLLLAIGNTVLLVRLSYWLW
178
Nct             IVVAATSGALR-DRFDGFQRVGGIIGTSVSAAFLIILGVGNGWVLYKLVKRLR
166
                :: *  :      .   .. :*.::.* .:  :*  :.  *  :*

NhlF            ITG----DLESRLS-------------ERGLFTRLLGNRWRGLVRSSWHMYPV
214
NixA            LSQQQNEEIERLLT-------------SRGLLNRFFKPLFN-FVSKSWHIYPI
192
HoxN            YVE---EDFDLLFG-------------NRGFLARIFRPLFR-FITRSWHMYPL
207
Nic1p           TK----DEG------------------VTGFLARKMQRLFR-LVDSPWKIYVL
215
Nct             ERRRRVEEGEDLRAEEEQEAMNNLQLEGAGFLARVFRRVFA-IVDRPWKMYPL
225
                       :                     *:: * :   :  ::  .*::* :

NhlF            LETASEVTLLTLTASAATGGTLSIAAVLSLPLLFAAGMSTFDTADSLFMTRAY
272
NixA            FDTASEIALLALSSSAIK---VSMVGMLSLPILFAAGMSLFDTLDGAFMLKAY
247
HoxN            FDTATEVALLGISTMEASRG-VPIWSILVFPALFTAGMALIDTIDSILMCGAY
264
Nic1p           FDTSTEVSLLGIATLQALKG-TSIWAILLFPIVF-------------------
248
```

Fig. 6. Multiple sequence alignment (Clustal W)

BLASTP of *N. crassa* NiCoT against fungal orthologs identifies *Fusarium graminearum* as the closest member. *Nct* shows 48% identity to *Nic1p (Schizosaccharomyces Pombe)* and 38% identity to *HoxN (Cupriavidus necator H16)* (Table 1).

Table 1. Best hits of BLASTP of *N. crassa* NiCoT against fungal orthologs

Organism	Identity (%)	E-Value	Score	GeneID/ locus
Neurospora crassa (Nct)	100	0.0	637	NCU08225.3
Fusarium graminearum	76	2e-116	422	FG06513.1
Podospora anserina	72	3e-129	465	Pa_7_3480
Magnaporthe grisea	65	5e-113	411	MG05503.4
Aspergillus niger	54	2e-103	379	gw1.9.206.1
A. fumigatus	54	2e-99	366	Afu2g08830
A. oryzae	50	6e-87	324	AO090011000817
A. terreus	49	3e-77	292	ATEG_09723.1
A. flavus	44	1e-90	328	AFSG002698
A. nidulans	40	1e-55	221	AN6115.2
Ustilago maydis	35	3e-30	136	UM01429.1
Schizosaccharomyces pombe (Nic1p)#	48	2e-41	173	074869074912
Cupriavidus necator H16 (HoxN)#	39	5e-56	565	gi:8575575
Rhodococcus rhodochrous (NhlF)#	33	2e-29	336	gi:1850787
Helicobacter pylori (NixA)#	32	1e-34	380	gi: 2314223

experimentally investigated

The transmembrane domains of the nct gene were identified using the TMpred software (Fig. 7).

The gel extracted PCR product was cloned into the T/Avector (pTAnct-c). Clones containing the insert were sequenced with universal primers for sequence identity.

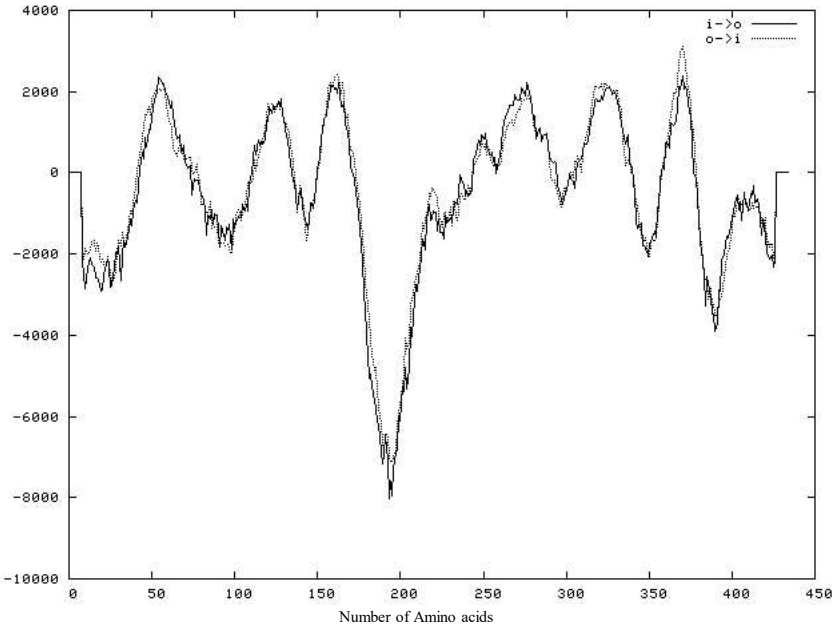
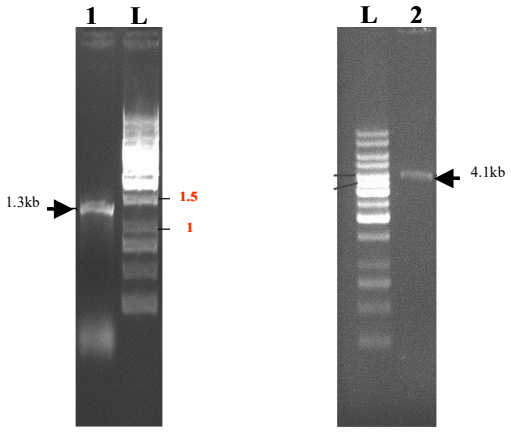


Fig. 7. Output for nct

The PCR product was cloned into TA vector and transformed into DH5á competent cells. The positive clones were selected on Ampicillin containing plates. For further confirmation the plasmids were isolated from the positive colonies and subjected to restriction digestion by BamHI. The appearance of 4.1kb (linearized vector + *nct* gene) fragment indicated the presence of gene (Fig. 8 (ii))



Lane
1: *nct* PCR product
2: pTAnct-g *BamH1* linearised
L: 1 kb ladder

Fig. 8. (i) PCR product of nct gene
(ii) BamHI digestion product
(linearized vector+nct gene)

DISCUSSION

Metal transport, toxicity and resistance in *Neurospora crassa* have been extensively investigated over the last three decades. A number of metal resistant strains have been characterized and concerned gene loci mapped. Despite this progress, cellular functioning of genes involved in transport of important metal ions remains unexplored. With the recent elucidation of *Neurospora* genome sequence in 2003 (Galagan et al, 2003), it is now possible to comprehensively characterize the metal transporter genes. Based on research work in this direction, "Metal Transportome", a complete suite of metal transporting genes in this model organism was defined (Kiranmayi and Maruthi Mohan, Insilico Biology **6**, 0016, 2006). A hypothetical nickel-cobalt permease belonging to NiCoT family was identified in this study is annotated as NCU08225.2. NiCoT is a new transporter gene family for nickel and/or cobalt. The presence of this gene in *Neurospora*, a lower eukaryote, raises fundamental questions regarding the requirements of either Ni or Co, which have not been explored in *N. crassa*.

N.crassa has a single member (NCU08225.3) belonging to this family with seven TMDs and a conserved signature sequence (RHALDADHI) that is a characteristic feature of NiCoT family. Multiple sequence alignment showed *N.crassa* member to be a closer homologue to HoxN, a high-affinity nickel uptake permease of *Ralstonia eutropha* (Eitinger *et al*, 2005). The only eukaryotic member studied experimentally is that of Nic1p of *Schizosaccharomyces pombe* (Eitinger *et al*, 2000). Search for NiCoT genes in yeast and fungi showed that most of the filamentous fungi have a single member and surprisingly none was observed in *S.cerevisiae* (Maruthi Mohan *et al*, 2007). Though Ni- specific transporters with respect to NiCoT family have been shown no such members are reported for cobalt till date. Cobalt is now recognized as the trace element for a few specialized bacteria and more than half a dozen enzymes dependent on this cation are known (Kobayashi and Shimizu, 1999). Though cobalt is not a great threat as a pollutant in the environment, the gamma emitting radioisotopes (^{60}Co, ^{58}Co) generated in nuclear reactor effluents are a genuine problem throughout the world. In addition to transporting (inward) these cations there are transporters involved in efflux function conferring resistance to both the toxic ions in bacteria (Rodrigue *et al*, 2005). This type of efflux member was not identified in *N.crassa* till date either through *insilico* approach or wet lab experiments. However till date the importance of either Ni^{2+} or Co^{2+} in *N.crassa* metabolism is not known and there is only one report off a cobalt binding protein being overproduced in a cobalt resistant strain (Sajani and Maruthi Mohan, 1998).

Metal ions must be transported into intracellular organelles where they function as catalytic and structural cofactors for

compartmentalized enzymes. Thus, intracellular transport mechanisms are also present. When present in high levels, metal ions can be toxic, so their uptake and intracellular transport is tightly regulated at both transcriptional and post-transcriptional levels to limit metal ion over accumulation and facilitate storage and sequestration.

Metal ion transporters play a major role in maintaining the necessary concentrations of the various metal ions in the different cellular compartments (Mulrooney, SB, Hausinger, RP, 2003). Recent studies of yeast mutants revealed key elements in metal ion homeostasis, including novel transport systems. Several of the proteins discovered in yeast are highly conserved, and defects in some of the yeast mutants could be complemented by their human homologs. These studies of yeast metal ion transporters helped to unravel the molecular mechanism of macrophage defense against bacterial infection and hereditary diseases.

Most of the control of metal ion concentrations is accomplished by regulating metal ion flow across lipid membranes surrounding the cytoplasm or individual organelles. As the cell becomes deficient in certain metal ions, transporters (importers) can be activated at the transcriptional level to bring more ions across the inner-membrane and into the cell (Degen *et al*, 1999) similarly, when intracellular metal ion concentrations are too high, metal sequestration proteins/molecules or efflux systems are expressed to maintain homeostasis. This regulation provides solution to the changing environment and the potential damage caused by abnormal concentrations.

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