Isoorotate Decarboxylase Activity Present in Various Strains of Neurospora

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ABSTRACT

N. crassa is known to utilize a salvage pathway for the production of uracil. This is known as the pyrimidine salvage pathway. This pathway consists of four enzymatic steps to convert thymidine to uracil. The final enzyme is isoorotate decarboxylase (IDCase), which forms uracil from isoorotate through a decarboxylation reaction. An in vitro assay for IDCase activity has been developed, which allowed the determination of specific activity of the enzyme in various strains of Neurospora.

We attempted first to determine if another species of Neurospora contained this pathway by looking for the presence of the IDCase enzyme. *N. africana* was chose because it is a homothallic, aconidiate species unlike *N. crassa* which is heterothallic and conidiated. We were able to determine that *N. africana* did contain this enzyme and with higher activity than in *N. crassa*. We next obtained the FGSC# 2203 triple mutant (pyr-4, uc-1, uc-2) to begin to look at the effect of the mutations on the activity of the IDCase enzyme. We concluded that the pyr-4 mutation has little to no effect on the activity of IDCase based on the fact that a pyr-4 transformant that retained the two uc mutations had relatively the same amount of IDCase activity as the triple mutant.

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Introduction

Kingdom Fungi

Kingdom Fungi is a large and very diverse group. There are approximately 60,000 known species that range from the simple yeasts to the more complex molds. Almost all of the species are heterotrophic, non-motile, eukaryotic organisms. They span from single-celled to multicellular organisms and can reproduce asexually and/or sexually. The molds, which include the *Neurospora* species, are made up of hyphae, long threadlike structures, which may be multinucleate, that form a tangled mass called mycelia. There are 4 main divisions to the fungi kingdom: Division Zygomycota, Division Ascomycota, Division Basidiomycota, and Division Deuteromycota. (Solomon, 1993)

Division Ascomycota

The Ascomycota division is made up of most yeasts, many mold, powdery mildews, morels, and truffles. These organisms can reproduce sexually and asexually. In sexual reproduction, cells are produced which contain the male and female nuclei. Asci are also produced which are small sacs that contain the sexual spores. Asexual reproduction allows rapid propagation of new mycelia. It is the desired form of reproduction for biochemical studies due to the rapid propagation. In asexual reproduction spores are pinched off at the tips of specialized hyphae. These spores are

called conidia. The conidia are responsible for the different colors of the various species. The organisms in the Ascomycota division can be heterothallic, which mean that 2 separate strains are required to accomplish sexual reproduction, or homothallic, which means that a single strain has the ability to self fertilize resulting in sexual reproduction. (Solomon, 1993)

Genus Neurospora

Neurospora is widely used in molecular biology studies. There are 10 known species of Neurospora, *N. crassa* being the best studied. *N. crassa* has served as a model system since before the work of Beadle and Tatum in the one gene, one enzyme discovery, (Russo, 1992). Neurospora has continued to be used for multiple reasons. Neurospora species overall have simple, inexpensive nutritional requirements and a short generation time that allow rapid growth. They are haploid, eukaryotic organisms with a small genome, (Perkins, 1992). Metzenberg (1995) stated in the introduction of his paper "Species of the genus Neurospora have been much studied as model systems for understanding metabolic pathways and their regulation, the processes and consequences of sexuality, and the natural history and evolution of a representative fungal genus." These among other reasons make it an ideal organism for use in research laboratories.

N. crassa is a heterothallic organism that has both asexual and sexual cycles. *N. crassa* vegetative mycelium are haploid which allow recessive mutations to be easily observed. It has simple nutritional requirements that include: a carbon source, biotin, and simple mineral salts. Once *N. crassa* is supplied with these simple requirements it

will grow rapidly which is another positive feature for laboratory studies. It has been determined that *N. crassa* has a small genome, made up of 7 chromosomes, and 47 million nucleotide pairs, (Mishra, 1991).

Another species in the genus Neurospora is *N. africana*. This species is a homothallic organism with the same simple nutritional requirements as *N. crassa*. *N. africana* is inexpensive to grow and maintain but it grows at a slower rate than *N. crassa*. This organism, unlike *N. crassa*, does not produce conidia. Little research has been completed with *N. africana* and therefore little information is available on this organism.

Nucleotide Metabolism

Nucleotides are active in almost every biochemical process; this alone makes them an important part of research studies. There are various types of nucleotides, which can contain one, two, or three phosphate groups; these compounds are called mononucleotides, dinucleotides, and trinucleotides respectively. Trinucleotides contain high-energy phosphate bonds that supply energy needed for many energy dependent reactions. We will mainly focus on the nucleotides used in the synthesis of nucleic acids which are of importance and will be discussed in detail. Nucleotides contain 3 main components. First is the 5-carbon sugar which will be a ribose sugar in the case of RNA or a deoxyribose sugar in the case of DNA. Second is the nitrogenous base which can be either a purine or a pyrimidine base. The last component is the phosphate group, which is generally located on the 5 carbon of the sugar. (Voet, 1995) Once all of these components have come together to form the nucleotide, the triphosphate forms can be

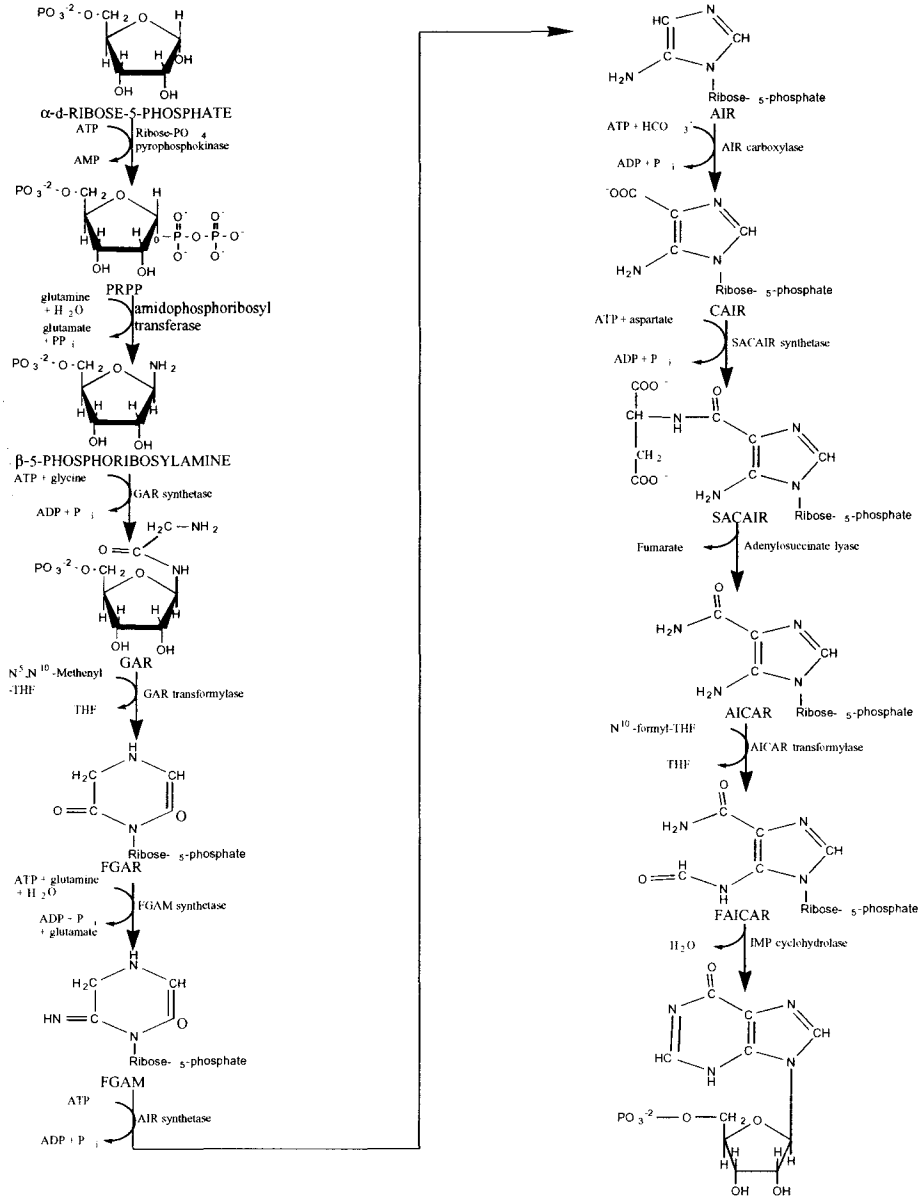
used to synthesis the nucleic acids, which are essential for life. DNA and RNA both contain the purines adenine and guanine and the pyrimidine cytosine. Uracil is a pyrimidine generally found only in RNA whereas thymidine is the pyrimidine found in DNA. One of the main reasons that uracil in not found in DNA is due to the fact that cytosine will spontaneously deaminate into uracil. Since uracil is not found in DNA a mechanism has evolved that allows uracil to be nicked out so cytosine can be replaced. RNA is used to transfer the information found in the DNA out of the nucleus in the cytosol at which time it is translated into a protein. Once the RNA is translated into the desired proteins it is degraded. Specialized pathways accomplish the synthesis of both purines and pyrimidines. The most studied pathways are the *de novo* pathways; these pathways form the nucleotides from scratch. Since the nucleotides are synthesized from small compounds, large energy expenditures are required for the many reactions involved in these *de novo* pathways. There are also many different salvage pathways that have been or are currently being researched. These salvage pathways allow the nucleotides to be reused, thus avoiding the large energy expenditure of the *de novo* pathways. This is believed to be the reason why organisms have developed these salvage mechanisms.

De Novo Purine Pathway

The *de novo* purine pathway is common in many organisms. It is responsible for the production of the purine nucleotides adenine and guanine, which are present in every cell. John Buchanan was responsible for discovering the first clues to the process of the *de novo* purine pathway. He determined through experiments that N1 comes from the

amine group of aspartate, C2 and C8 come from formate, N3 and N9 come from the amine group of glutamine, C4, C5, and N7 are derived from glycine and finally C6 originates from HCO^{3-}. Purines are first formed as a ribonucleotide, Inosine monophosphate (IMP), in an eleven-step pathway (Figure 1). This pathway is entirely cytosolic and requires expenditure of energy, generally in the form of triphosphates (Voet, 1995). This pathway begins with phosphorylation of α-D-ribose-s-phosphate by the enzyme ribose phosphate pyrophosphokinase resulting in the formation of phosphoribosyl pyrophosphate (PRPP). This step requires the expenditure of ATP. A displacement of PRPP's pyrophosphate with glutamine's amide nitrogen by the enzyme amidophosphoribosyl transferase yields phosphoribosylamine. This is the flux-generating step of the pathway. Phosphoribosylamine's amino group forms an amide with glycine's carboxyl group with the assistance of glycinamide ribotide (GAR) synthetase to yield GAR. This is followed by formylation of GAR's free α amino group to yield formylglycinamide ribotide (FGAR). This requires the action of GAR transformylase. The fifth step involves the enzyme FGAM synthetase that transfers the amide amino group from a second glutamine to form formylglycinamidine ribotide (FGAM). AIR synthetase then assists in the formation of the purine imidazole ring to yield 5-aminoimidazole ribotide (AIR). AIR is then transformed to carboxyamino imidazole ribotide (CAIR) by AIR carboxylase. This involves the introduction of C6 from a bicarbonate ion. Aspartate contributes N1 by an amide forming condensation reaction that is dependent on the enzyme SACAIR synthetase as well as ATP. This results in the formation of 5-aminoimidazole-4- (N-succinylocarboxamide) ribotide

Figure 1. *De novo* purine biosynthetic pathway adapted from Biochemistry Second Edition, Voet and Voet, 1995.


α-d-RIBOSE-5-PHOSPHATE
ATP
AMP
Ribose-PO 4 pyrophosphokinase
PRPP
glutamine + H 2O
glutamate + PP i
amidophosphoribosyl transferase
β-5-PHOSPHORIBOSYLAMINE
ATP + glycine
ADP + P i
GAR synthetase
GAR
N5,N10-Methenyl -THF
THF
GAR transformylase
Ribose- 5-phosphate
FGAR
ATP + glutamine + H 2O
ADP + P i + glutamate
FGAM synthetase
FGAM
ATP
ADP + P i
AIR synthetase
Ribose- 5-phosphate
AIR
ATP + HCO 3
ADP + P i
AIR carboxylase
Ribose- 5-phosphate
CAIR
ATP + aspartate
ADP + P i
SACAIR synthetase
SACAIR
Ribose- 5-phosphate
Fumarate
Adenylosuccinate lyase
AICAR
Ribose- 5-phosphate
N10-formyl-THF
THF
AICAR transformylase
FAICAR
Ribose- 5-phosphate
H2O
IMP cyclohydrolase

(SACAIR) from which fumarate is eliminated in the ninth step. Adenylosuccinate lyase is the enzyme responsible for converting SACAIR to 5-aminoimidazole-4-carboxamide ribotide (AICAR). AICAR transformylase is the next enzyme active in this pathway. It is responsible for the addition of the final purine ring atom C2 from the formylation of N^{10}-formyltetrahydrofolate yielding 5-formaminoimidazole-4-carboxamide ribotide (FAICAR). Finally cyclization to form IMP occurs causing the elimination of water. The hydrolysis of ATP is not required for this final step to occur. IMP is quickly converted to AMP or GMP. IMP is converted to AMP in a 2-reaction pathway. First, aspartate's amino group is linked to IMP, which is driven by the hydrolysis of GTP to form adenylosuccinate. This is followed by the elimination of fumarate by adenylosuccinate lyase to result in the formation of AMP. Two reactions are also required to convert IMP to GMP. IMP dehydrogenase oxidizes IMP to introduce oxygen to the compound by way of reduction of NAD+ to form xanthosine monophosphate (XMP). Next, ATP hydrolysis drives the transfer of the glutamine amide nitrogen to XMP to form GMP. Both GMP and AMP are monophosphate and as mentioned earlier they must be converted to triphosphates to be used in the synthesis of nucleic acids. Two subsequent kinase reactions accomplish this. The final products are GTP and ATP. (Voet, 1995)

De Novo Pyrimidine Pathway

The *de novo* pyrimidine pathway is a 6-reaction pathway making it simpler than the 11-reaction de novo purine pathway. This pathway also differs in that the sugar is attached at the end of the pathway not the beginning and the reactions occur in both the

cytosol and mitochondria. These six reactions result in the formation of UMP. The first step is synthesis of carbamyl phosphate (CAP). This is accomplished by the interaction of a bicarbonate ion with glutamine in the presence of two ATP molecules. The enzyme for this interaction is CAP synthetase. Next, CAP reacts with aspartate in the presence of the enzyme aspartate transcarbomylase to yield carbamoyl aspartate. The carbamoyl aspartate is then cyclized into a ring by a condensation reaction that is catalyzed by the enzyme dihydroorotase to result in dihydroorotate. This is followed by oxidation of dihydroorotate by way of the reduction of a quinone, which is driven by dihydroorotate dehydrogenase yielding orotate. Phosphoribosyl-α -pyrophosphate (PRPP) is then introduced and allowed to react with orotate to yield orotidine monophosphate (OMP). This is catalyzed by the enzyme orotate phosphoribosyl transferase, which is pulled forward by the hydrolysis of pyrophosphate. Finally, OMP decarboxylase decarboxylates OMP to result in UMP (Figure 2). Again 2 subsequent kinase reactions are required to form the triphosphate UTP. CTP synthetase catalyzes the addition of an amino group to UTP to yield CTP (Figure 3). (Jones, 1980)

The synthesis of thymidylate from UMP is somewhat more complicated. First UMP must be converted to deoxy-UMP (dUMP). Thymidylate synthetase is the enzyme responsible for the conversion of dUMP to deoxy-TMP (dTMP). In this reaction the methyl donor for the reductive reaction is N^5, N^{10}-methylenetetrahydrofolate. This step is an irreversible methylation of dUMP to dTMP. Again kinase reactions are required to form dTTP for use in nucleic acid synthesis (Figure 4). (Carreras, 1995)

Figure 2. *De novo* pyrimidine biosynthetic pathway adapted from Biochemistry Second Edition, Voet and Voet, 1995.

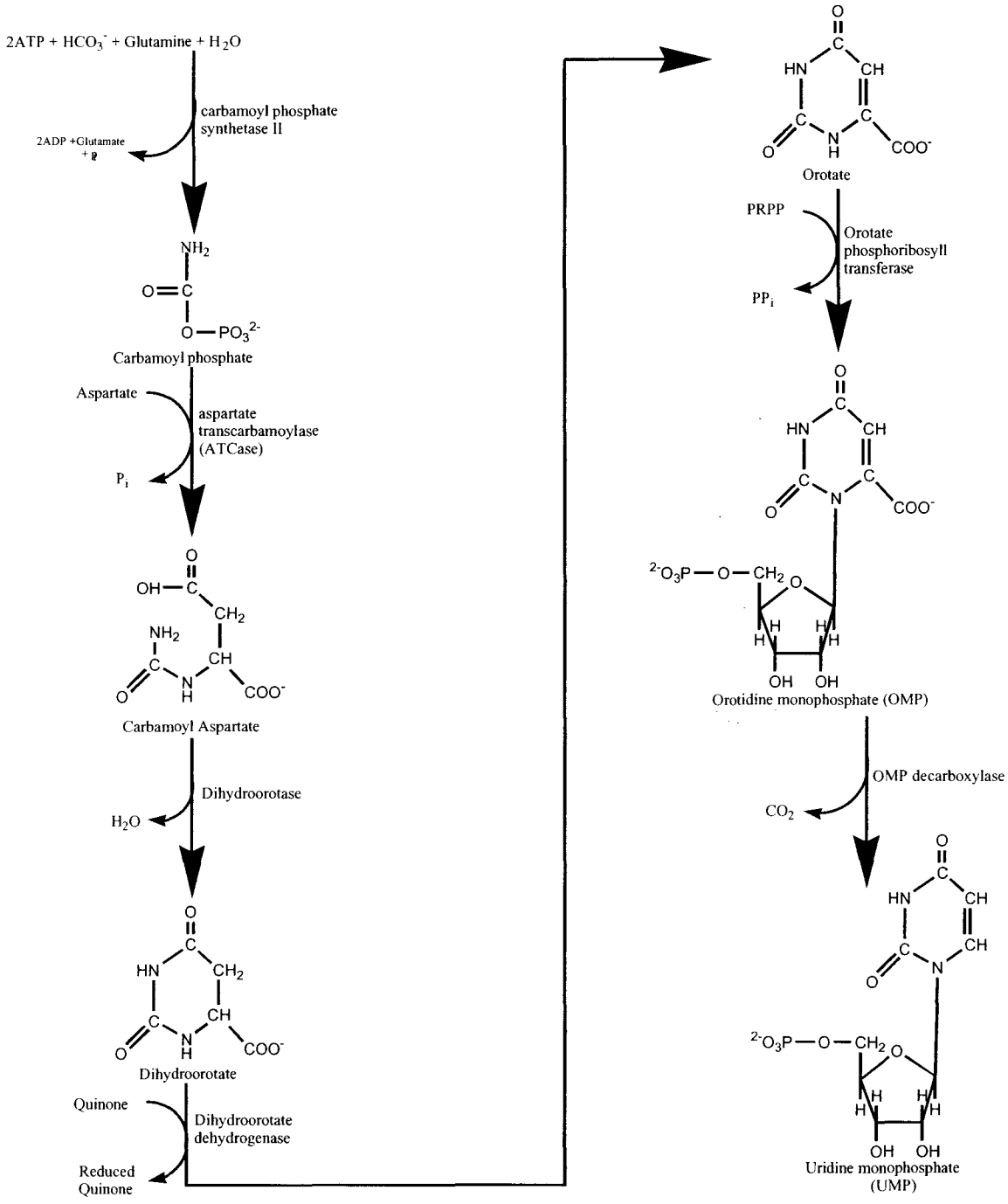
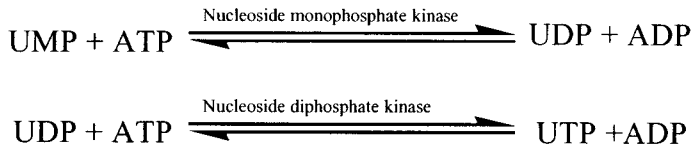
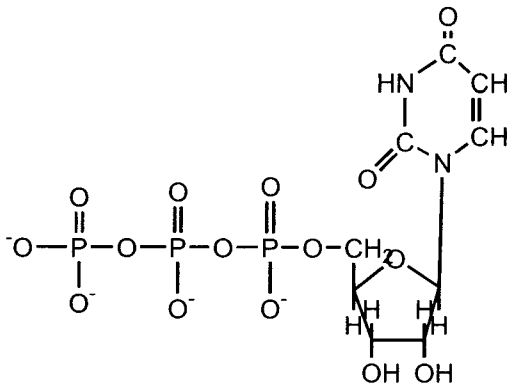
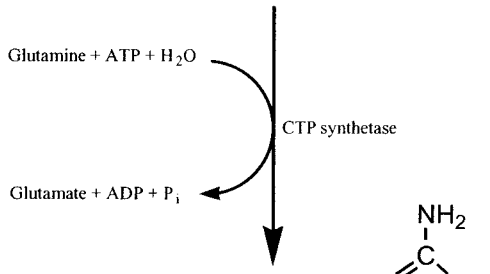

2ATP + HCO3- + Glutamine + H2O
carbamoyl phosphate synthetase II
2ADP +Glutamate + P
Carbamoyl phosphate
Aspartate
aspartate transcarbamoylase (ATCase)
Pi
Carbamoyl Aspartate
Dihydroorotase
H2O
Dihydroorotate
Quinone
Dihydroorotate dehydrogenase
Reduced Quinone
Orotate
PRPP
Orotate phosphoribosyll transferase
PPi
Orotidine monophosphate (OMP)
OMP decarboxylase
CO2
Uridine monophosphate (UMP)

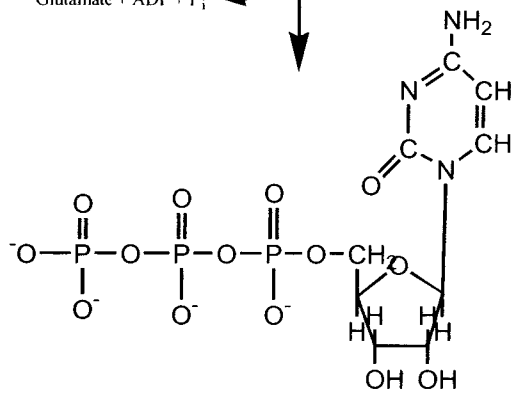
Figure 3. The final step of the *de novo* pyrimidine pathway for the conversion of UMP to CTP.


UMP + ATP
Nucleoside monophosphate kinase
UDP + ADP
UDP + ATP
Nucleoside diphosphate kinase
UTP +ADP



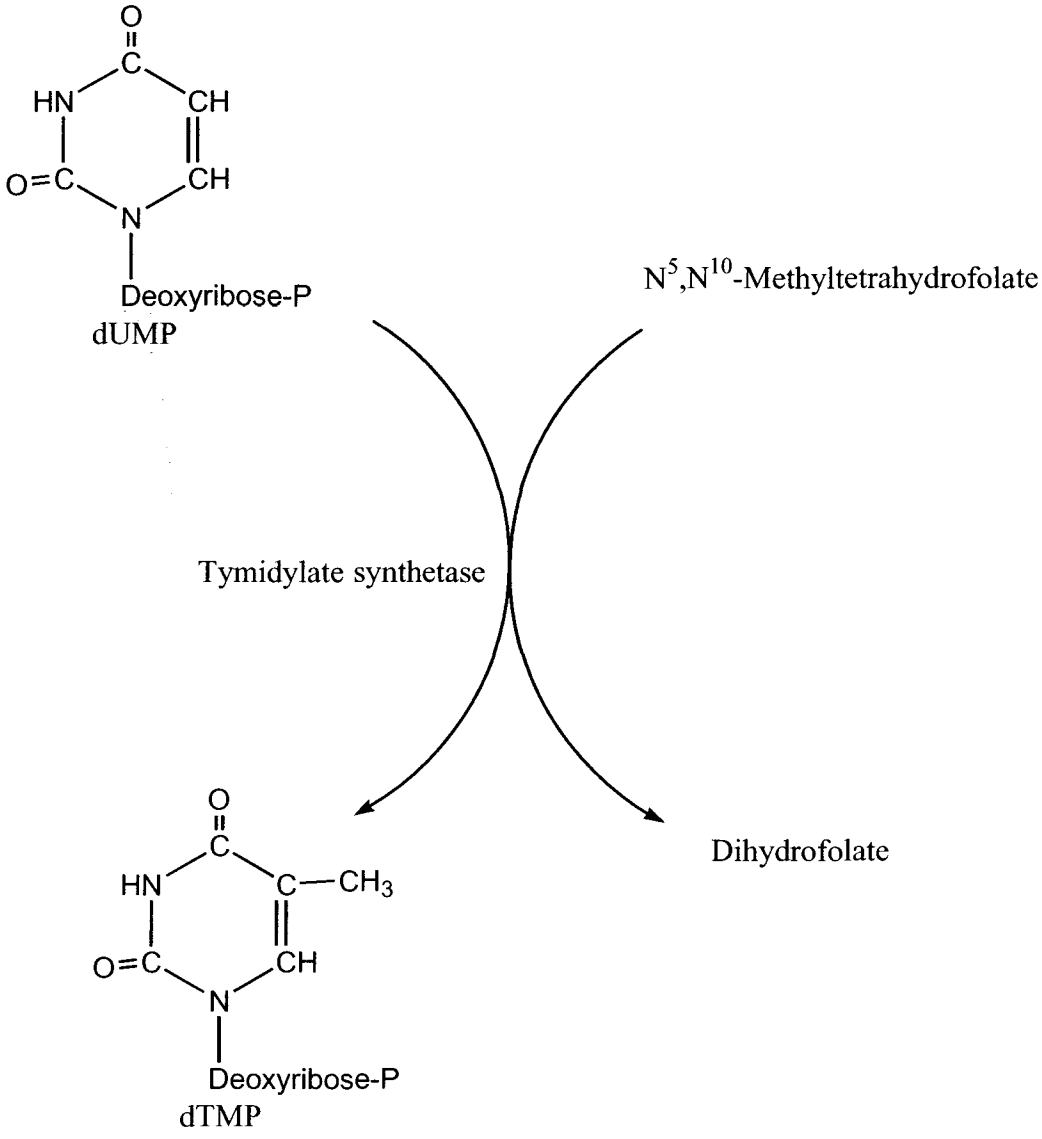
UTP


Glutamine + ATP + H2O
CTP synthetase
Glutamate + ADP + Pi



CTP

Figure 4. Final step of the *de novo* pyrimidine pathway for the conversion of dUMP to dTMP.


O
C
HN
CH
O=C
CH
N
Deoxyribose-P
dUMP
N5,N10-Methyltetrahydrofolate
Tymidylate synthetase
Dihydrofolate
O
C
HN
C—CH3
O=C
CH
N
Deoxyribose-P
dTMP

SALVAGE PATHWAYS

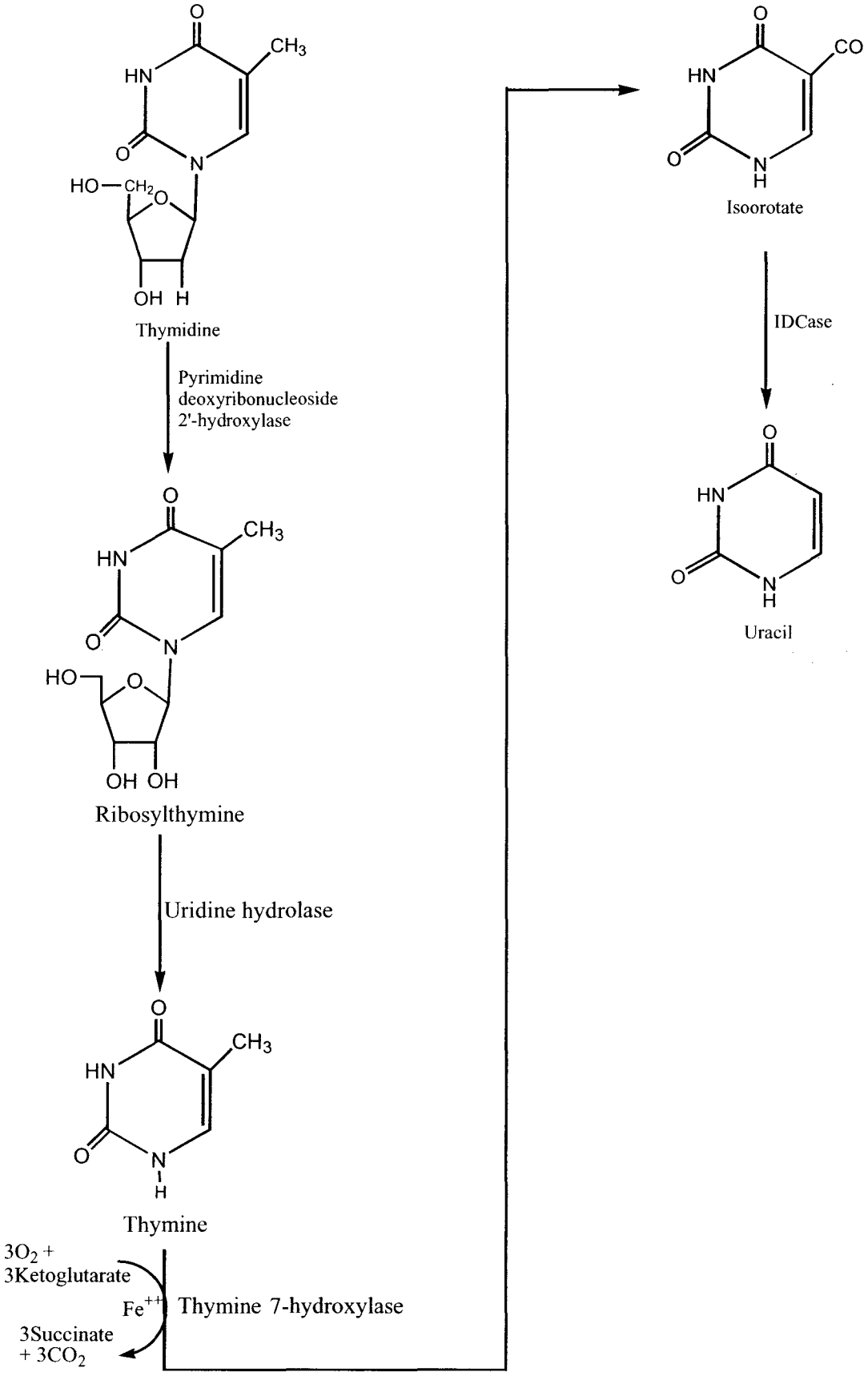
Salvage pathways are diverse in character as well as distribution. That is unlike *de novo* pathways they are not similar in all cells. Salvage pathways have been discovered for both purines and pyrimidines. In particular some species of fungi have a salvage pathway for the conversion of thymidine to uracil; it has been shown that *N. crassa* has such a pathway. This pathway requires 4 enzymatic steps, shown in figure 5. First, oxidation of the deoxyribonucleotide thymidine to thymine ribonucleotide is catalyzed by the enzyme pyrimidine deoxyribonucleoside 2'-hydroxylase. This is followed by hydrolytic cleavage of the glycosidic bond resulting in thymine and ribose that is catalyzed by the enzyme uridine hydrolase. Thymine-7-hydroxylase then causes a 3-step oxidation of thymine to uracil-5-carboxylate (isoorotate). Finally isoorotate is decarboxylated to yield uracil due to the catalytic activity of isoorotate decarboxylase (IDCase). The uracil is converted to UMP by uracil phosphoribosyl transferase; which is common in many organisms. (Neuhard, 1983) This pathway was discovered in *N. crassa*, but it is also believed to be present in *Aspergillus nidulans* and *Rhodotorula glutinis* due to the presence of at least one of the enzymes for the thymidine salvage pathway in each of the organisms. Several research groups have investigated thymidine hydroxylase, which is responsible for the third step of the salvage pathway. Thornburg and Stubbe are responsible for some of the more recent research on this enzyme.

Currently there is not much information available on the final enzyme involved in the thymidine salvage pathway. IDCase has been partially purified and it has been determined that orotic acid is not able to serve as a substrate for IDCase (Palmatier, 1970). Thus far the only compound found that can replace IOA as a substrate for IDCase

is 2-thio-5-carboxyuracil, (Smiley, et al., 1999). The presence of IDCase has been directly demonstrated in *N. crassa* and *Rhodotorula glutinis* however, it is believed that *Aspergillus nidulans* will use IDCase to complete the pathway to uracil due to the presence of thymidine-7-hydroxylase in this organisms. It has been determined that there are no cofactor requirements for the conversion of IOA to uracil and carbon dioxide by IDCase (Palmatier, 1970). It has also been shown that the common strategies for decarboxylation are not the likely mechanism for IDCase due to the molecular structure of the substrate (O'Leary, 1992).

Renita Cannon (1997) showed that IDCase activity is elevated when *N. crassa* is grown in Westergaard's media compared with Vogel's media. The nitrogen source in Vogel's media is ammonia whereas it is nitrate in Westergaard's media. From this increase in enzyme activity it is suggested that nitrogen source must be important in the regulation of the thymidine salvage pathway. Eva Marshall (1999) determined nitrogen source did have an effect on the IDCase activity. She determined this by comparing the IDCase activity in cultures grown in Vogel's only and those shifted from Vogel's to Westergaard's. She showed that more tissue and higher IDCase activity are obtained when cultures are shaken for approximately 18 hours. This study also showed the effect of uracil on the thymidine salvage pathway. It was determined that the addition of uracil depressed the activity of the IDCase enzyme. It was expected that the addition of thymidine would have the opposite effect but for unknown reasons that did not occur. Finally, she determined that the pyr-4 mutant did show only a slight increase in IDCase activity, due to the fact that this strain is mutant in the *de novo* pathway. This increase allowed the production of the needed uracil by the thymidine salvage pathway.

Figure 5. The pyrimidine salvage pathway adapted from Palmatier, 1970.


Thymidine
Pyrimidine
deoxyribonucleoside
2'-hydroxylase
Ribosylthymine
Uridine hydrolase
Thymine
3O2 +
3Ketoglutarate
Fe++
3Succinate
+ 3CO2
Thymine 7-hydroxylase
Isoorotate
IDCase
Uracil

Mutants Affecting the Thymidine Salvage Pathway

Several mutations have been isolated that affect the thymidine salvage pathway. Williams and Mitchell (1969) determined that the mutations affecting pyrimidine salvage are not clustered close together on the genetic map. A mutation in the pyr-4 gene results in a defective *de novo* pathway for the formation of pyrimidines. This mutation results in the loss of the orotidylate decarboxylase (ODCase) enzyme activity. ODCase is the final enzyme of the de novo pyrimidine pathway. Using a strain containing a mutation in the pyr-4 gene, a mutation was isolated that allows use of thymidine, thymine, 5-hydroxymethyl uracil, and 5-formyluracil as pyrimidine sources this mutation was designated uc-1. It was hypothesized that the pyr-4, uc-1 mutants would be useful in the selection of other mutants in the pyrimidine salvage pathway (William and Mitchell, 1969). A mutation designated uc-2 was found to block the second step of the salvage pathway. It forms a defective nucleosidase enzyme that keeps the conversion of thymidine ribonucleoside to thymine from occurring. The uc-3 mutation was found to affect the enzyme responsible for the conversion of 5-hydroxymethyluracil (5-HMU) to 5-formyluracil, (Williams and Mitchell, 1969). This defect causes the inability of the organism to use thymidine, thymine, and 5-hydroxymethyl uracil as pyrimidine sources.

IDCase activity in various Neurospora species is the focus of the research for this paper. *N. crassa* and *R. glutinis* (Yun, 1999) are the only known organisms that IDCase activity has been reported in to date. We feel that it is probably present in other species of Neurospora as well. We will begin by assaying *N. africana* for IDCase activity. This study will also look at the effects of the uc-1 mutation on IDCase activity. To do this we

will use transformation experiments with the pyr-4+ gene to restore the *de novo* pathway in the triple mutant strain (pyr-4, uc-1, uc-2) of *N. crassa*. We will then assay the pyr-4+, uc-1- , uc-2- strain for IDCase activity.

MATERIALS AND METHODS

Strains and Media

Wild-type *Neurospora crassa* (74A), *Neurospora africana* (FGSC#1740), a single mutant (pyr-4) strain of *Neurospora crassa* (FGSC# 4030), and a triple mutant (pyr-4, uc-1, uc-2) strain of *Neurospora crassa* (FGSC#2203) were obtained from The Fungal Genetics Stock Center in Kansas City, Kansas for use in this study (Table 1). [Carboxy-^{14}C]IOA was purchased for this study from Moravek Biochemical.

Several types of media are used for this research. Horowitz (Horowitz, 1947), Vogel's media (Vogel, 1956), and Westergaard's media (Westergaards and Mitchell, 1974) are used throughout the research.

IDCase Assay

Flasks containing Horowitz complete media + agar (0.5%) are inoculated with Neurospora and placed in the 30°C incubator for 2-3 days. These are then removed and placed under a fluorescent light for 3-5 days to allow optimal conidiation. Once the flasks have conidiated well the conidia is harvested and transferred into Vogel's liquid media and shaken in the 30°C incubator at 200 revolutions per minute (rpms) for approximately 12 hours. Mycelia are harvested and the culture is then shifted to

Table 1. Strains of Neurospora

Strains of Neurospora

FGSC#	**Genotype**	**Reference**
2489	*N. crassa* wild type 74A	Dr. Asch's stock collection
4030	pyr-4	Dr. Asch's stock collection
2203	uc-2, uc-1, pyr-4	Williams and Mitchell 1969
1740	*N. africana* wild type	Dr. Asch's stock collection

Westergaard's liquid media for 4 hours. Mycelia are harvested by vacuum filtration and resuspended in lysis buffer (1M Tris pH 8.0, 250mM EDTA). The following protease inhibitors are added, including 0.2mM phenylmethylsulfonyl fluoride (PMSF), 0.7 µg/ml pepstatin A, and 0.3 µg/ml leupeptin. The tissue to buffer ratio is 1 gram tissue to 3 ml buffer. The cultures are prepared for the IDCase assay by use of a tissue tearer (Biospec Products, Inc., Model 985-370). The cultures are homogenized using five high-speed 30-second intervals being iced between cycles. This mixture is then centrifuged at 17,000 rpm at 10°C for 20 minutes to pellet the cell debris. The supernatant is placed in a clean conical tube on ice to be used for the assays.

The desired number of vials are placed in a 30°C water bath and subjected to shaking. A paper wick is then saturated with NaOH and placed in the wick holder suspended from a rubber septum. At this point 50µl of [carboxy- ^{14}C]IOA is added to the vial to which 450µl of supernatant is added. The mixture is shaken in the water bath for 10 minutes before being quenched with 100µl 2M HCl. They are left in the water bath for 1 hour to collect $^{14}CO_2$ on the NaOH-moistened wicks. The wicks are then removed from the wick holders and placed in a tray to go in the oven. The wicks are dried at 70°C for 25-30 minutes. Next the wicks are moved to ScintiSafe Econo 1 scintillation fluid (Fisher). The vials are then placed in the scintillation counter (Packard Brand, model number 102992) to collect the counts per minute (CPMs) from the $^{14}CO2$ that is collected on the paper wicks. CPMs obtained from the scintillation counter are used to calculate specific activity of IDCase as nmol product min^{-1} mg $protein^{-1}$. (Smiley et al, 1999)

Figure 6. Calculations for specific activity of the IDCase enzyme.

Calculations of Enzyme Activity for the radioactive assay of IDCase activity:

counts of 14CO2 (cpm)	/	counting efficiency (cpm/dpm)	=	Radioactivity of 14CO2 (dpm)	
2. Radioactivity of 14CO2 (dpm)	/	specific activity (dpm/nmol)	=	nmol product	
3. nmol enzyme activity product	/	time (minutes)	=	nmol/min	
4. enzyme activity	/	total protein (µg)	=	specific activity (nmol min µg-1)	

ODCase Assay

A quartz cuvette is obtained to read the UV absorbance. The samples are prepared in the same manner as those for the IDCase assay. The sample (100µl) is placed in the cuvette with 850µl water. The spectrophotometer is set at 220-320nm and the individual absorbance is taken at 286nm. The spectrophotometer is blanked with the initial mixture. Then, 50µl of 1mM OMP is added and mixed with a Pasteur pipet. The sample is then scanned and this is recorded as the zero time point. The sample is scanned every 15 seconds for 2 minutes.

Bradford Assay

Protein concentrations are determined using Bradford assays. Bovine Serum albumin (BSA) is used to obtain a standard curve. The BSA (0.5mg/ml) is placed into microcentrifuge tubes using 0, 5, 10, 15, and 20µl bringing each to 100 with 0.15 M NaCl, which makes the standards. Then 900 µl of the Coomassie Brilliant Blue G-250 (Sigma) solution is added to all of the mixtures and the absorbance at 595nm is determined. The samples are then prepared to allow collection of the wavelength. Using the standard curve obtained from the BSA to calculate the protein concentrations of the Neurospora samples follows this. (Bradford, 1976)

Figure 7. Calculations for specific activity of the ODCase enzyme.

Calculation of Enzyme Activity for ODCase:

Change in A286 / 0.00225 = Change in OMP, in nmol

Time necessary for the above change in A286 = minutes

Protein concentration from Bradford assay and volume of protein used gives # μg protein

Specific activity = nmol min-1 μg -1

Preparation of Competent Conidia

Horowitz flasks are inoculated with the pyr-4, uc-1, uc-2 mutant strain of *N. crassa* (FGSC#2203), 1mM uracil must be added to the flasks (25ml media/400µl uracil). The flasks are placed in the 30°C incubator for 2-3 days and moved under fluorescent light for 3-5 days. Conidia are harvested and transferred to Vogel's liquid media. The culture is then placed in the shaker at 37°C and checked for germination. At 75% germination, approximately 2 hours, the culture is transferred to a Sorvall tube and centrifuged for 10-12 minutes at 5000 rpm and 10°C. The pellet is the washed three times with 30 ml 1M sorbitol. At this point 0.025 g of Novoenzyme 234 is added to 10 ml 1M sorbitol and the pellet is transferred to the mixture. This is shaken for 2 hours at 30°C and 1 rpm. After the hour, the mixture is centrifuged at less than 800 rpm for 10 minutes. The pellet is washed with 1M sorbitol followed by a wash with 1M sorbitol/10mM Mops/50mM $CaCl_2$. Finally it is resuspended in 1.2 ml 1M sorbitol/10mM Mops/50mM $CaCl_2$, 15µl DMSO, and 300µl 40% PEG/10mM Mops/50mM $CaCl_2$ and aliquoted 250µl per ependorf tubes. These are placed in the deep freezer at -70°C to be used for transformations. (Akins and Lambowitz, 1985)

Spheroplast Test

A spheroplast test is run to determine successful production of competent conidia. Regeneration agar is made by mixing 1X Vogels, 1 M sorbitol, and 2.8% agar together water is then added to bring the mix to 190ml. This is then autoclaved, followed by the addition of 10ml 2X Figs (40% sorbose, 1% fructose, 1% glucose). Dilutions on the

samples are done in preparation for the test. The spheroplast test requires 50µl of sample being mixed with 450µl 1M sorbitol. A control is done using 450µl of water. The dilutions are done to 1 X 10^{-4}. Next, 10 ml regeneration agar is poured into a conical tube containing 100 µl sample and 40 µl 1 mM uracil. This mixture is then overlaid on Vogel figs plates (4ml Vogels media, 3 grams agar, and 180ml water, autoclave then add 10 ml 2X Figs) that are placed in the 30°C incubator for 2-3 days. There should be colonies from samples diluted in sorbitol and cultures diluted in water should show little growth. If the test is successful a transformation is attempted.

Transformation

Vogel Figs plates and regeneration agar are prepared as before. One hundred microliters of competent conidia is placed in a clean Eppendorf tube to which 60 µl DNA is added. The pyr-4 DNA was obtained from a phage library for wild type *N. crassa* (FGSC# 2489) that is trans selected into a plasmid. The mixture is iced for half an hour at which point 1 ml Peg/Mops/$CaCl_2$ is added, the sample is set at room temperature for 20 minutes. This is followed by the addition of 10 ml regeneration agar to a conical tube containing 100µl transformation mix. This mixture is then poured onto the Vogel figs plated. The plates are then incubated at 30°C for 24 hours. The transformant colonies are picked and placed into Horowitz slants. The slants are incubated for 2-3 days and placed under the light for 2-3 days. They are then streaked for isolation on Vogel Figs plates and picked 2 more times in preparation for the DNA extraction. (Akins and Lambowitz, 1985)

DNA Extraction

The method of Metzenberg and Baisch (1981) and Stevens and Metzenberg (1982) was used for DNA extraction. Vogels liquid media is inoculated with the transformant *N. crassa* strain and allowed to grow for 3-5 days. The pads are harvested by vacuum filtration from the liquid media and wrapped in aluminum foil to be placed in the deep freezer at -70°C. Pads are ground into a fine powder in liquid nitrogen along with a small amount of sand using a motor and pestle. This fine powder is then added to 500 µl EDTA, 0.2%SDS and vortexed to mix. This is placed at 68°C for 20 minutes before being centrifuged for 5 minutes to pellet the debris. Which is followed by the addition of 30 µl of 8M KOAc, pH 4.2 to the supernatant, which is mixed well and iced for 5 minutes. This is centrifuged for 5 minutes to remove the K-SDS precipitate, after which 600 µl isopropanol is added to the supernatant. This is mixed well and centrifuged for 5 minutes to pellet the DNA. The pellet is resuspended in 200 µl 10mM EDTA to which 3 µl RNAse A (10 mg/ml) is added. This is set at room temperature for 15 minutes. Equal amounts of phenol m-cresol is then added and the mixture is vortexed and centrifuged for 5 minutes. The aqueous layer is extracted and placed in a clean tube to which chloroform is added. The mixture is vortexed, then spin for 5 minutes, the organic layer is removed and extraction with chloroform is completed two more times. After the extractions the aqueous layer is transferred to a clean Eppendorf tube and 10 µl 10M LiCl and 500 µl 95% ethanol is added. This is mixed well and centrifuged for 5 minutes. The tubes are inverted to allow the DNA to dry after which the pellets are resuspended in 100 µl TE by heating at 65°C and mixing gently. Next, 5 µl of the sample

is diluted with 10 µl sterile water to which 2 µl tracking dye is added before loading into an 1% agarose gel prepared in 1X Tris phosphate EDTA (TPE). The gel is placed in a bath of 1X TPE where the wells are loaded with the DNA samples. The cover is placed on and the gel is run at 72 volts for approximately 1 hour. The gel is removed from the TPE bath and placed in a pan containing a mixture of 50 µl 10 mg/ml ethidium bromide with sterile water. The gel is allowed to stain for at least 30 minutes followed by washing it with sterile water. The gel is then moved to a transluminator, where it can be visualized and a photograph can be obtained.

Southern Blot

The Southern method (1975) with modifications based on personal communications with Dr. Heather Lorimer was used for the Southern blot. The DNA from the extraction is digested with EcoRI overnight in a mixture containing 50µl DNA, 15µl 10X buffer, 2µl EcoRI, and 83µl water. The following day this mixture is precipitated with 200µl isopropanol by centrifuging for 15 minutes. The liquid is removed and the pellet is dried in the speed vac before being resuspended in 30µl of TE. The DNA samples are run out on a 1% agarose gel at 15 volts overnight. This gel is stained and a photograph is obtained. The gel is placed in 250 ml 0.25 N HCl rocking for 15 minutes after which it is removed and place again in 0.25 N HCl for 15 minutes. This is followed by 2 washes in 0.5 M NaOH, 1M NaCl again both rocking for 15 minutes. The final wash is in 250ml 0.5 M Tris, 3 M NaCl pH 7.4 rocking for 30 minutes. The membrane is then cut to size and the bottom corner is marked for orientation. The

membrane is wet in water followed by wetting in the final high salt buffer (0.5 M Tris , 3 M NaCl pH 7.4) and placed face down over the gel, any bubbles must be removed at this point. Two pieces of wet blotting paper are placed on top of the membrane and paper towels are stacked on to of these. A weight is placed on top and it is allowed to blot overnight. The membrane is cross-linked in the UV cross-linker (Fisher Scientific model # FB-UVXL-1000) the following day.

Isolation of the Fragment for the Probe

The probe for the southern blot is started by mixing 60µl of the pyr-4 DNA, 15µl 10X buffer, 2µl EcoRI, and 73µl water. This is also allowed to digest overnight. The probe is then run on a 1% agarose gel at 15 volts overnight. The band is cut out of the gel and placed in a dialysis bag to which .5X TPE is added. The excess TPE is squeezed out of the dialysis bag and the bag is run in .5X TPE for 15 minutes. At which point the current is reversed for 15 seconds to remove the band from the side of the bag. The fluid is then pipeted out of the bag and placed in phenol/chloroform which is vortexed and centrifuged. The aqueous layer is transferred to a new Eppendorf tube to which 1/10 volume 3 M sodium acetate is added followed by 2 ½ volumes ethanol. This is froze for 15 minutes followed by centrifuging for 20 minutes. The pellet is washed with 75% cold ethanol and placed in the speed vac. The pellet is then resuspended in 25µl TE. A gel check is done to confirm the presence of the pyr-4 probe DNA.

Probe Preparation

The pyr-4 fragment is labeled with ^{32}P by placing 50ng of the specific DNA in 5µl TE and 6µl water. This is placed in the heating block at 90°C for 5 minutes. The New England Nuclear Kit Method is used for the preparation of the probe. Added to the specific DNA mixture is 2.5µl buffer, 6µl nucleotide mix, 2µl 1:100 ATP, 2.5µl [^{32}P]dATP, and 1µl klenow. This is mixed gently and incubated at room temperature overnight. The probe mixture is run through a spin column containing glass wool at the bottom and sphadex G-50 for 5 minutes.

Hybridization of the Membrane

In preparation 20X sodium chloride phosphate (SCP) is made by mixing 7 ml concentrated HCl, 160.75 g $Na_2HPO_4 \bullet 7H_2O$, 100 ml EDTA, and 117 g NaCl, pH 6.8. This is diluted to 6X, 2X, and 0.2 X SCP for use in the experiment. The membrane is placed in a tube to which 30ml 6X SCP with 1% sarkosyl is added. This is incubated rolling for 30 minutes at 60°C. In the meantime, 12ml 6X SCP with 1% sarkosyl and 150 µl 10 mg/ml sheared salmon sperm (SSS) DNA is boiled. The probe is then added to this mixture and boiled for 5 minutes. The prehybridization mix is dumped and the probe mix is added to the tube. This is incubated overnight rolling at 60°C. The probe mix is placed in a tube and saved in the freezer. The tube containing the membrane is filled with 2X SCP with 1% SDS and rotated for 30 minutes at 60°C. The 2X wash is discarded and the membrane is placed in a tray containing 0.2X SCP with 1% SDS

rocking for 15 minutes. The membrane is then wrapped in plastic wrap before being exposed to X-ray film. The film was allowed to expose for 3 days before being developed.

Results

The amount of IDCase activity present in various Neurospora strains was compared by growing the strains in Vogels media for 12 hours with a shift to Westergaards media for 4 hours. The cultures were shaken at 30°C because previous studies have shown that this produces optimal activity at around 18 hours of growth with a drop following this time point (Marshall Masters Thesis, 1999). IDCase levels in wild type *N. crassa* 74A were used as a control to compare the activity of this enzyme present in other strains which included; *N. africana* FGSC# 1740, a pyr-4 mutant of 74A FGSC# 4030, a triple mutant of 74A FGSC# 2203 (uc-1, uc-2, pyr-4), and a pyr-4^{+} transformant of FGSC# 2203.

The first comparison was that of *N. crassa* to the activity present in *N. africana*. The purpose of this comparison was to determine whether IDCase activity would be present in *N. africana*. It is known that IDCase enzyme activity is present in the conidiated, heterothallic *N. crassa* species. Therefore, we decided to determine whether IDCase activity was present in the aconidiate, homothallic species *N. africana.* Initially, the tissue tearer was used to prepare the extracts for testing the IDCase activity of these two strains. We found that the amount of protein in crude extracts from *N. africana* was substantially lower than that of *N. crassa* (Table 2). *N. crassa* had a protein concentration that averaged greater than 1.9 μg/ml whereas *N. africana* had a much lower average at less than 0.16 μg/ml. With the tissue tearer the specific activity of IDCase in *N. africana* averaged 0.4704 μg min^{-1} ml^{-1} which appeared to be higher than that of *N. crassa* average of 0.3431 μg min^{-1} ml^{-1} (Figure 8). The protein concentrations were so

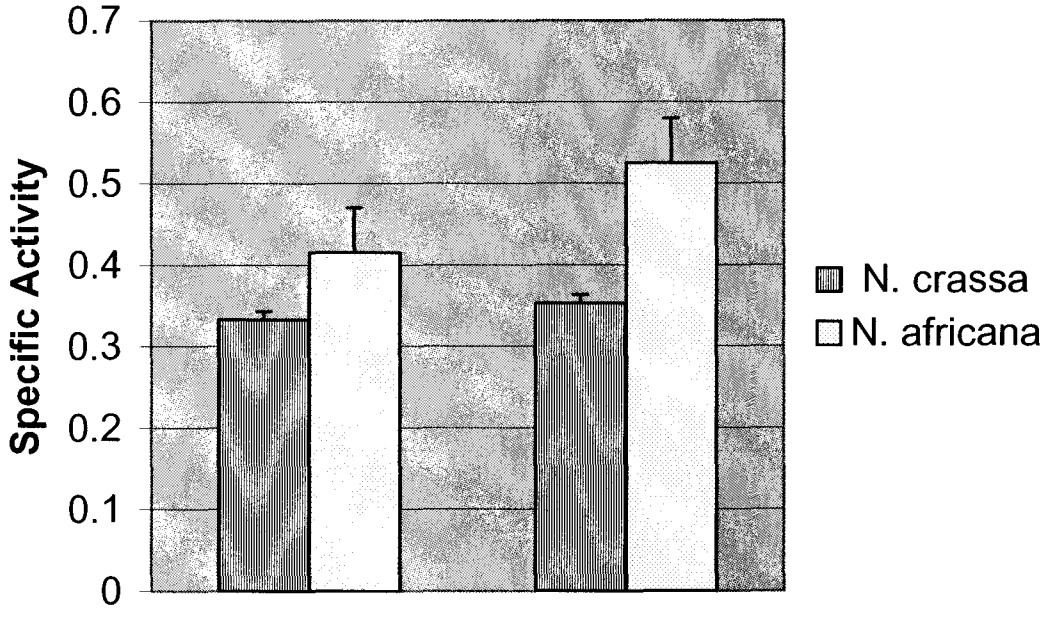
Table 2. Protein concentration and specific activity of the IDCase enzyme in *N. crassa* and *N. africana* when homogenized with the tissue tearer.

Table 2

Species	Protein concentration in µg/ml	Specific activity in nmol min^{-1} μg^{-1}
N. crassa	2.415	0.333
N. crassa	1.567	0.3532
N. africana	0.1097	0.4157
N. africana	0.2110	0.5251

Figure 8. The specific activity of IDCase present in *N. crassa* and *N. africana* when homogenized with the tissue tearer.

Figure 8



variant that the comparison between the two species could not provide experimental evidence of the amount of activity in *N. africana* relative to *N. crassa.* We then attempted to homogenize the mycelial pads with liquid nitrogen in an effort to obtain higher protein concentrations from *N. africana.* We did not obtain higher protein concentrations from *N. africana* using the liquid nitrogen method as expected which then required a dilution of the *N. crassa* protein extract for a better comparison. However, we found that this was a successful way to extract protein from the mycelial pads because an increase in IDCase activity in *N. africana* occurred (Table 3). The specific activity in *N. africana* increased to an average of 0.8687 $\mu g\ min^{-1}\ ml^{-1}$ while the average activity in *N. crassa* remained relatively close to 0.300 $\mu g\ min^{-1}\ ml^{-1}$ at 0.2758 $\mu g\ min^{-1}\ ml^{-1}$. These extracts allowed a better comparison of the IDCase activity present in the two species (Figure 9).

The next comparison was between wild type *N. crassa* 74A and the triple mutant of 74A FGSC# 2203 (uc-1, uc-2, pyr-4). We hypothesized that the uc-1 mutation is responsible for maintaining low levels of IDCase activity and therefore, the triple mutant would have higher specific activity of the IDCase enzyme than wild type *N. crassa.* The amount of protein extracted from the two organisms was relatively equivalent (Table 4) and therefore allowed comparison of the activity present in the two organisms. The average protein concentration in wild type *N. crassa* was 0.211µg/ml and the average in the triple mutant was 0.432 µg/ml. The triple mutant appeared to have higher IDCase activity with an average of 1.078 $\mu g\ min^{-1}\ ml^{-1}$ than wild type at 0.430 $\mu g\ min^{-1}\ ml^{-1}$ as seen in Figure 10. This had also been observed by Cannon (1997) and Marshall (1999).

Table 3. Protein concentration and the specific activity of the IDCase enzyme in *N. crassa* and *N. africana* when homogenized with liquid nitrogen.

Table 3

Species	Protein concentration in μg/ml	Specific activity in nmol min^{-1} μg^{-1}
N. crassa	0.08725	0.2739
N. crassa	0.1689	0.2777
N. africana	0.1354	0.7445
N. africana	0.07643	0.9928

Figure 9. Specific activity of the IDCase enzyme in *N. crassa* and *N. africana* when homogenized with liquid nitrogen.

Figure 9

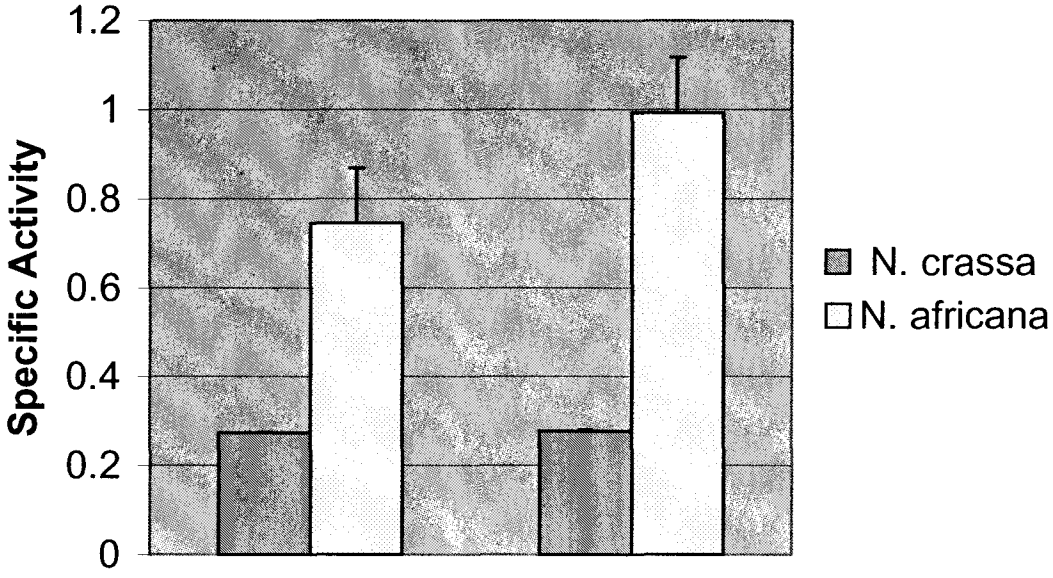


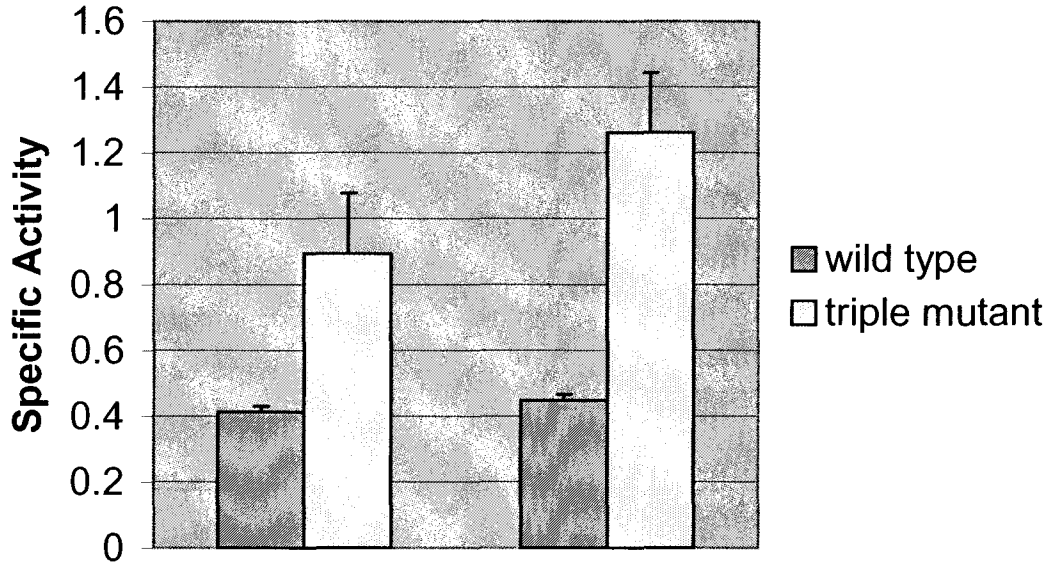
Table 4. Protein concentration and specific activity of IDCase in wild type *N. crassa* and the FGSC# 2203 triple mutant.

Table 4

Species	Protein concentration in μg/ml	Specific activity in nmol min^{-1} μg^{-1}
Wild type *N. crassa*	0.2110	0.4118
Wild type *N. crassa*	0.2110	0.4478
FGSC# 2203 Triple mutant	0.4125	0.8948
FGSC# 2203 Triple mutant	0.4511	1.261

Figure 10. Specific activity of IDCase in wild type *N. crassa* and the FGSC# 2203 triple mutant.

Figure 10


Specific Activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
wild type
triple mutant

There was also a comparison of a pyr-4 mutant FGSC# 4030 to wild type 74A, again the protein concentrations were close and therefore allowed a comparison of the activity present in the two organisms (Table 5). Again the average for *N. crassa* was 0.169 μg/ml with the average for the pyr-4 mutant being 0.167 μg/ml. It was determined that the pyr-4 mutant had higher activity with an average of 1.640 μg min^{-1} ml^{-1} than wild type *N. crassa* with an average activity of 0.268 μg min^{-1} ml^{-1} (Figure 11). Another comparison that can be concluded with these results is that the pyr-4 mutant has higher activity than that of the triple mutant. The triple mutant had an average protein concentration of 0.432 μg/ml while the pyr-4 mutant had an average of 0.167 μg/ml (Table 6). The average specific activity of the IDCase enzyme was 1.08 μg min^{-1} ml^{-1} in the triple mutant and 1.64 μg min^{-1} ml^{-1} in the pyr-4 mutant (Figure 12).

A transformation of FGSC# 2203 the triple mutant to pyr-4^{+} leaving the uc-1 and uc-2 mutations was then completed. The purpose of transforming was to determine the effect of the pyr-4 gene on the specific activity of the IDCase enzyme in the triple mutant. We hypothesized that the absence of the *de novo* pathway does not cause the salvage pathway activity to increase. Therefore, the pyr-4 mutation should have little to no effect on the IDCase enzyme activity This transformant was then tested to determine the amount of IDCase activity present and compared to the triple mutant as well as wild type *N. crassa*. The first step was to transform the FGSC# 2203 triple mutant and select for transformants in the absence of uracil. The transformants were streaked for isolation three times in the absence of uracil. This was done because the pyr-4 mutation requires the addition of uracil to the media for the organisms to grow but because the transformant would be pyr-4^{+} the organism should grow in absence of uracil. Once the transformants

Table 5. Protein concentration and specific activity of IDCase in wild type *N. crassa* and the FGSC# pyr-4 mutant.

Table 5

Species	Protein concentration in µg/ml	Specific activity in nmol min^{-1} μg^{-1}
Wild type *N. crassa*	0.08713	0.3890
Wild type *N. crassa*	0.2501	0.1460
FGSC# 4030 pyr-4 mutant	0.09328	0.9310
FGSC# 4030 pyr-4 mutant	0.2408	2.348

Figure 11. Specific activity of IDCase in wild type *N. crassa* and the FGSC# 4030 pyr-4 mutant.

Figure 11

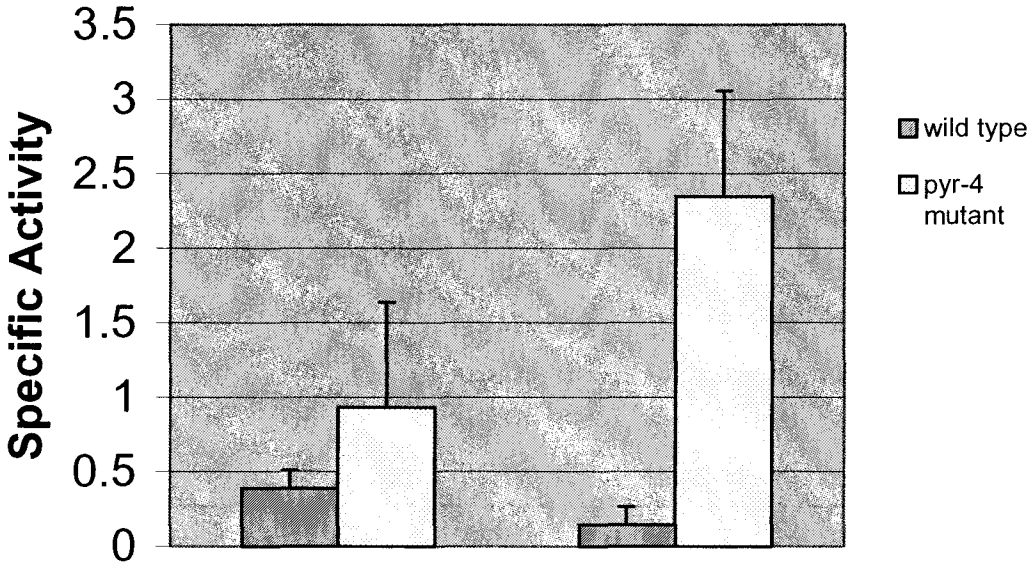


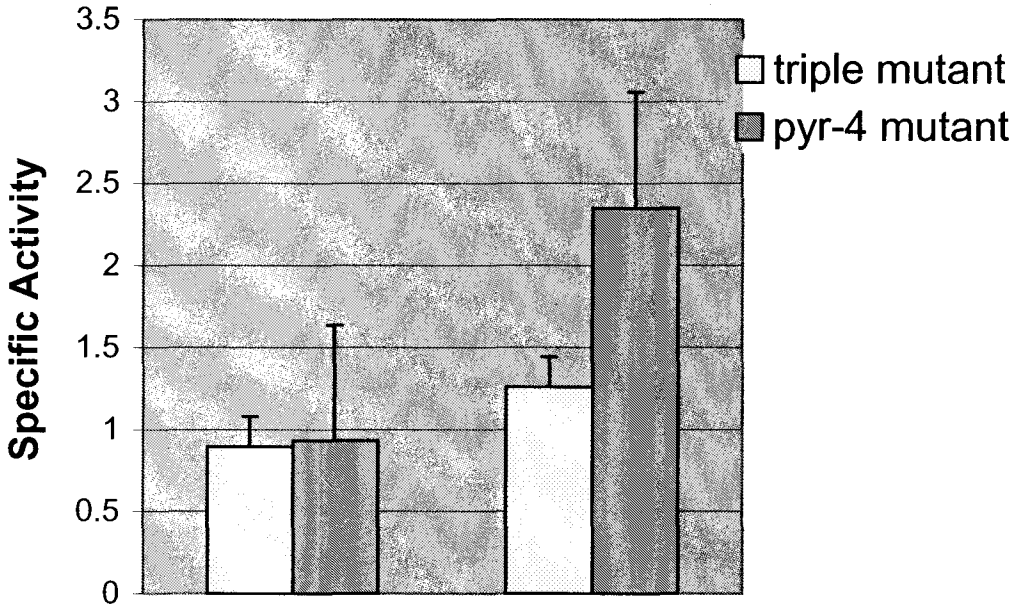
Table 6. Protein concentration and specific activity of IDCase in the FGSC# 2203 triple mutant and the FGSC# 4030 pyr-4 mutant.

Table 6

Species	Protein concentration in μg/ml	Specific activity in nmol min^{-1} μg^{-1}
FGSC# 2203 Triple mutant	0.4125	0.8948
FGSC# 2203 Triple mutant	0.4511	1.261
FGSC# 4030 pyr-4 mutant	0.09328	0.9310
FGSC# 4030 pyr-4 mutant	0.2408	2.348

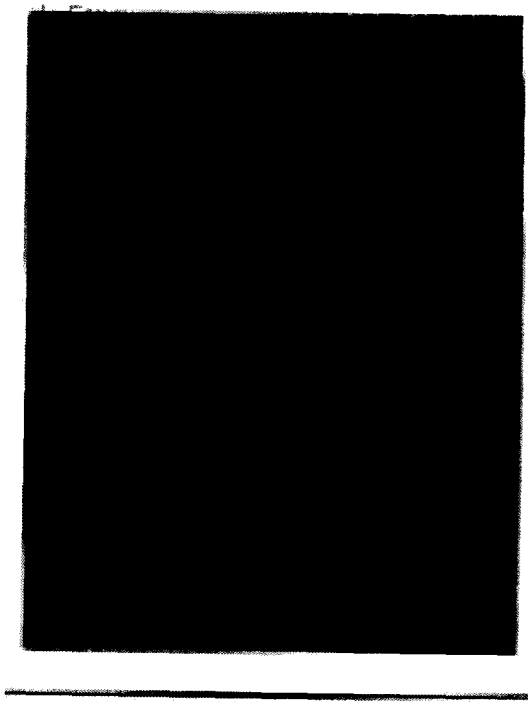
Figure 12. Specific activity of IDCase in the FGSC# 2203 triple mutant and the FGSC# 4030 pyr-4 mutant.

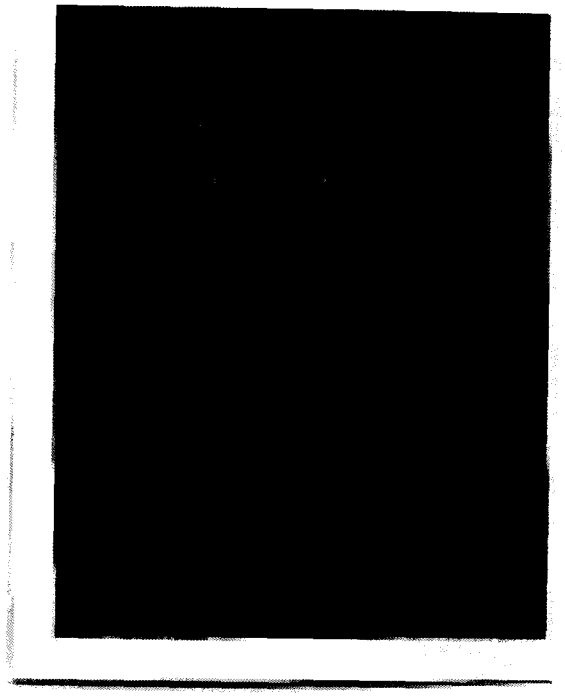
Figure 12


Specific Activity
3.5
3
2.5
2
1.5
1
0.5
0
triple mutant
pyr-4 mutant

were successfully isolated extraction of the chromosomal DNA was attempted. The gels present in Figure 13 are evidence of successful DNA extractions on the transformants. Genomic DNA from the transformants was then digested with EcoRI and then was run out on a large gel in preparation for the Southern blot (Figure 14). The gel was then blotted and probed for the presence of pyr-4 using a fragment containing the cloned pyr-4 gene as a probe. An autoradiograph on X-ray film was obtained from the probe for pyr-4, which is found in Figure 15. We found that all of the transformants showed the wild type fragment, which contained the pyr-4 gene. One transformant (Lane R) displayed the presence of two EcoRI fragments containing the pyr-4 sequences. The IDCase activity present in the transformant found in lane T on the autorad was then compared to the activity of the FGSC# 2203 triple mutant. The average protein concentration was 0.429 μg/ml in the triple mutant and 0.377 μg/ml in the transformant (Table 7). The average specific activity of the IDCase enzyme was 0.560 μg min^{-1} ml^{-1} in the triple mutant while it was 0.625 μg min^{-1} ml^{-1} in the transformant (Figure 16). The specific activity of the IDCase enzyme in the transformant was then compared to that of wild type *N. crassa* as well as to the FGSC# pyr-4 mutant of *N. crassa*. We then compared the transformant to wild type *N. crassa* because it has no mutations and to the pyr-4 mutant because it has only the pyr-4 mutation. We expected the single mutant and wild type to have roughly the same activity, which would support our hypothesis that the presence or absence of the *de novo* pathway does not affect the activity of the salvage pathway. We found that the specific activity of the transformant was higher than wild type *N. crassa* but lower than the pyr-4 mutant. Again the protein concentrations from the various organisms were relatively close. The transformant had an average protein concentration of 0.195 μg/ml

Figure 13. Gels of DNA extraction for transformants A-T and wild type *N. crassa*.





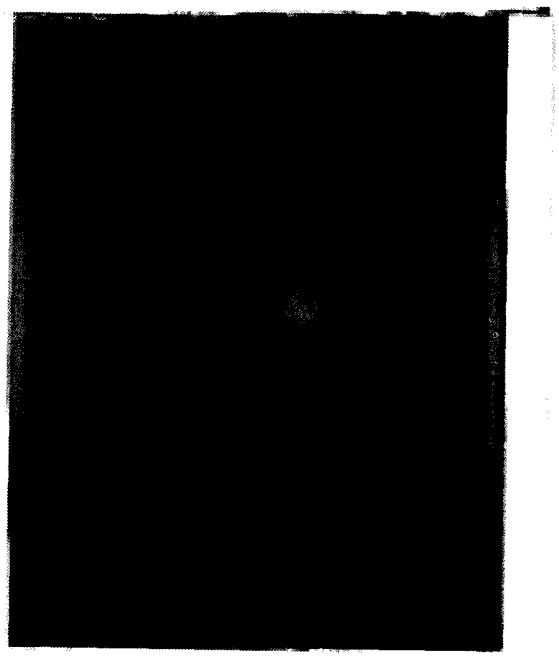


Figure 14. Agarose gel of the transformants used for the Southern Blot.

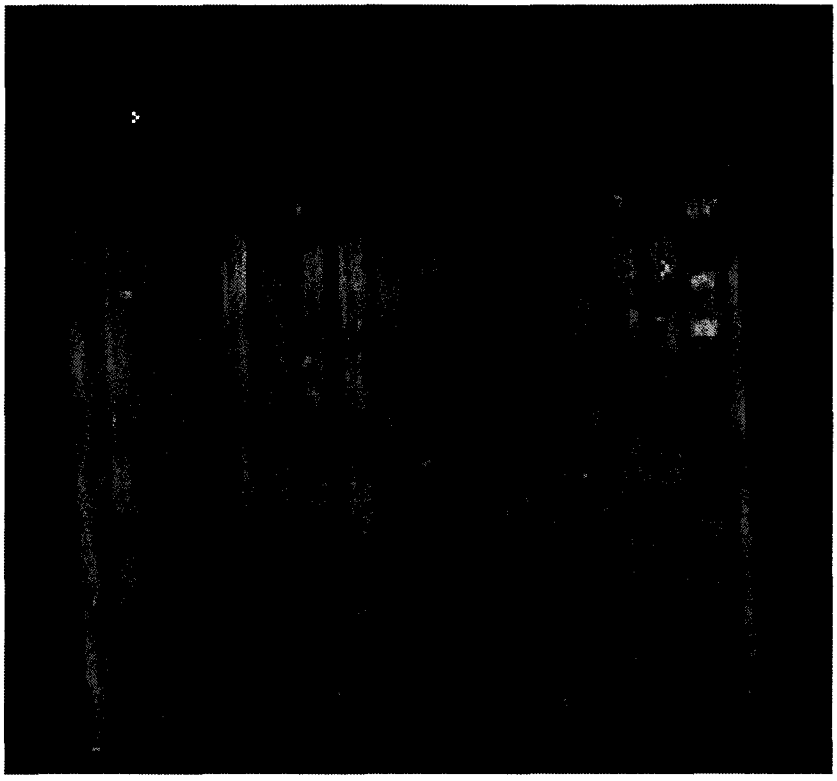


Figure 15. The autoradiograph obtained from the Southern Blot membrane.

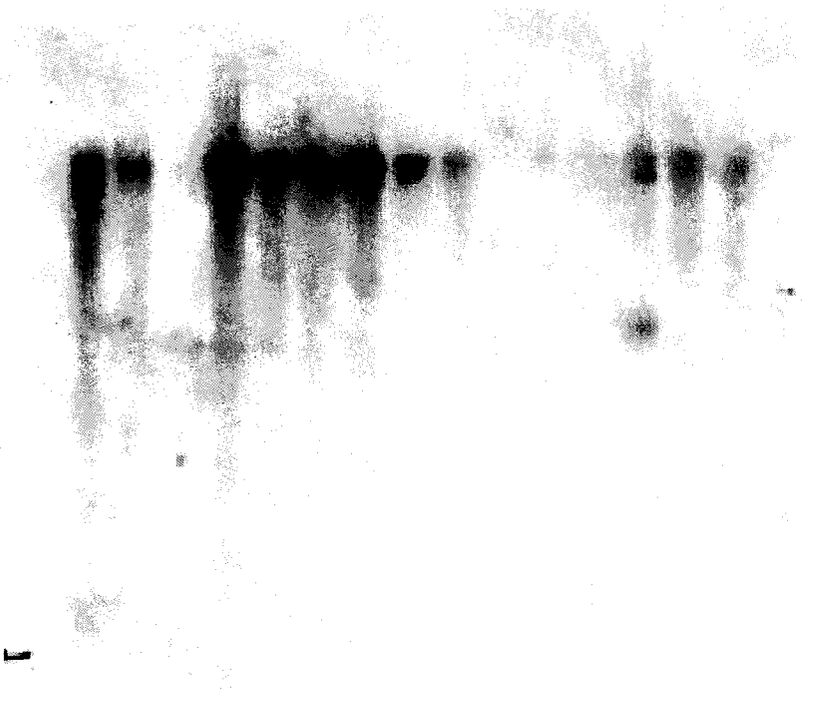


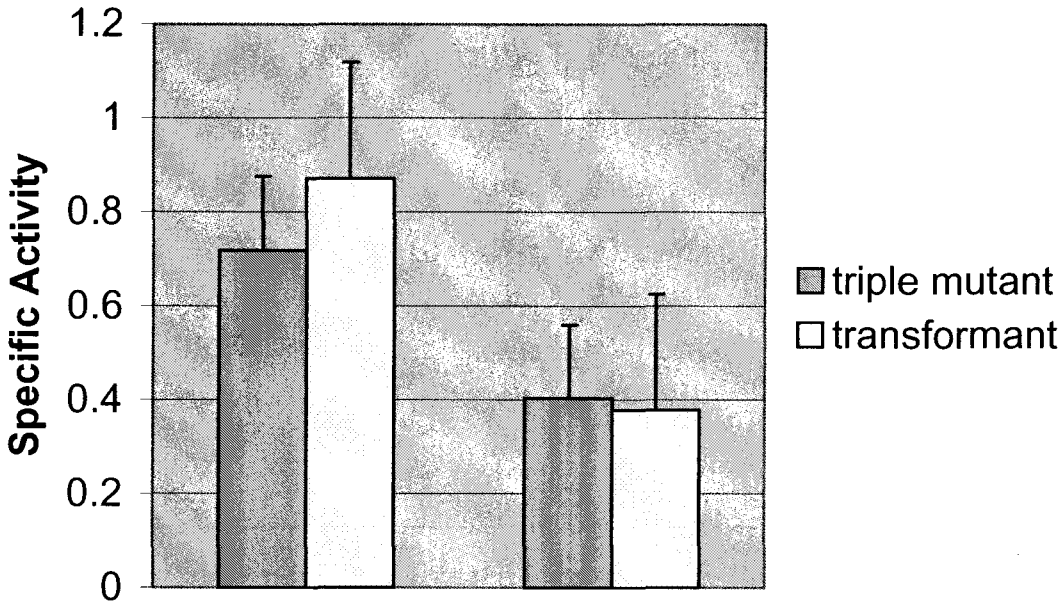
Table 7. Protein concentration and specific activity of IDCase in the FGSC# 2203 triple mutant and the pyr-4^{+} transformant both in the presence of uracil

Table 7

Species	Protein concentration in µg/ml	Specific activity in nmol min^{-1} μg^{-1}
pyr-4 transformant + uracil	0.4125	0.8720
pyr-4 transformant + uracil	0.3414	0.3779
FGSC# 2203 Triple mutant + uracil	0.4345	0.7181
FGSC# 2203 Triple mutant + uracil	0.4232	0.4026

Figure 16. Specific activity of IDCase in the pyr-4^{+} and the FGSC# 2203 triple mutant.

Figure 16


1.2
1
0.8
0.6
0.4
0.2
0
Specific Activity
triple mutant
transformant

while wild type *N. crassa* had an average concentration of 0.169 μg/ml and the pyr-4 mutant had an average of 0.167 μg/ml (Table 8). The average specific activity of the IDCase enzyme was 0.268 μg min^{-1} ml^{-1} for wild type *N. crassa*, 0.826 μg min^{-1} ml^{-1} for the transformant, and 1.64 μg min^{-1} ml^{-1} for the pyr-4 mutant (Figure 17). The transformant was then also subjected to a test for the presence of ODCase, which is the final enzyme in the *de novo* pathway. We would expect that a pyr-4^{+} transformant would have ODCase activity but the pyr-4 mutant would not display ODCase activity. Figure 18 shows that ODCase enzyme activity was present in the transformant based on the decrease in absorbance at 286 nm from 0.12708 to 0.10894. The ODCase activity in wild type *N. crassa* decreased from 0.16051 to 0.15824 whereas the absorbance at 286 nm for the pyr-4 mutant only decreased from 0.13828 to 0.13130, also shown in Figure 18. The ODCase activity present in wild type *N. crassa* which serves as a positive control whereas the ODCase activity present in the pyr-4 mutant which serves as a negative control. When comparing these three graphs side by side it is easy to see that the activity present in the transformant is comparable to that of wild type *N. crassa*. This is also evidence of a successful transformation because the activity of the ODCase enzyme would not be present if the pyr-4 gene had not successfully been transformed.

Table 8. Protein concentration and specific activity of IDCase in wild type *N. crassa*, pyr-4^{+} transformant, and the FGSC# 4030 pyr-4 mutant all in the presence of uracil.

Table 8

Species	Protein concentration in μg/ml	Specific activity in nmol min^{-1} μg^{-1}
N. crassa + uracil	0.08713	0.3890
N. crassa +uracil	0.2501	0.1460
pyr-4 transformant + uracil	0.03734	1.218
pyr-4 transformant + uracil	0.3536	0.433
FGSC# 4030 pyr-4 mutant + uracil	0.09328	0.9310
FGSC# 4030 pyr-4 mutant + uracil	0.2408	2.348

Figure 17. Specific activity of the IDCase enzyme in wild type *N. crassa*, the pyr-4^{+} transformant, and the FGSC# 4030 pyr-4 mutant all in the presence of uracil.

Figure 17

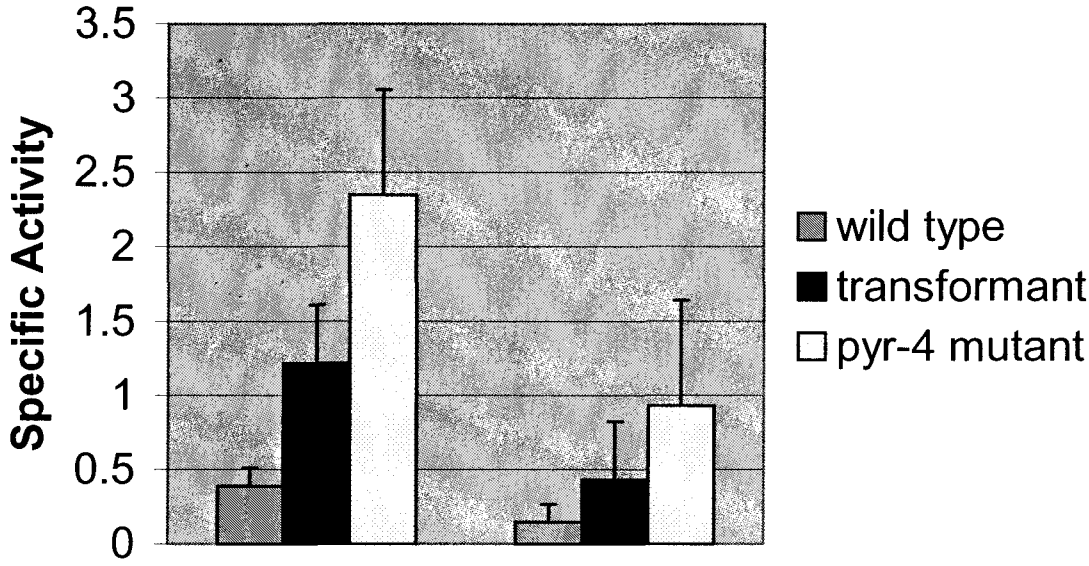
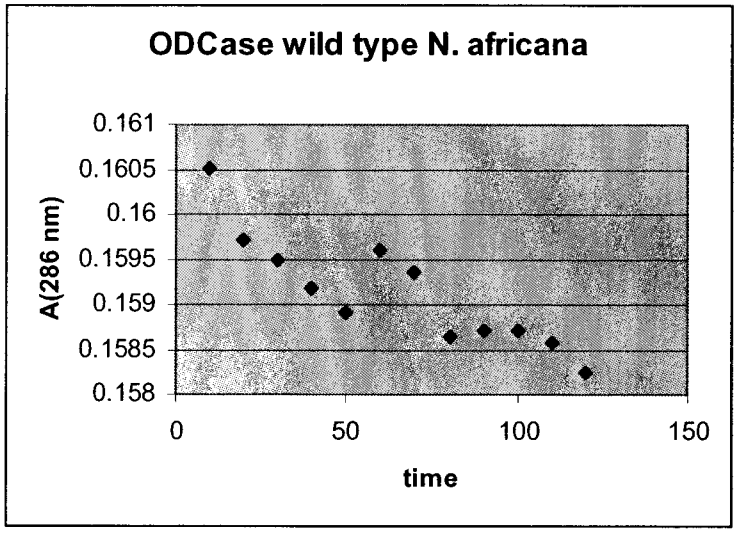
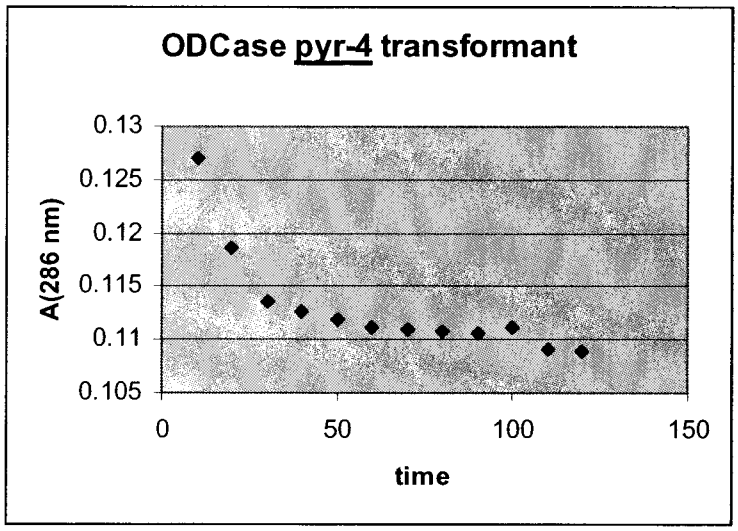
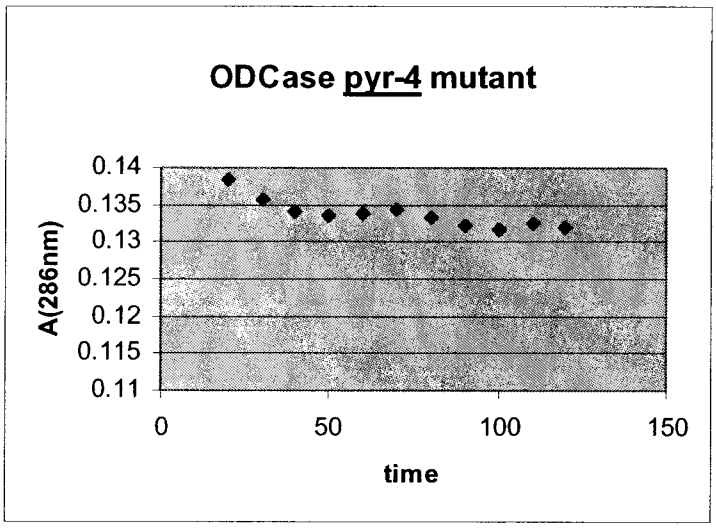

Specific Activity
3.5
3
2.5
2
1.5
1
0.5
0
wild type
transformant
pyr-4 mutant

Figure 18. ODCase graphs from wild type *N. crassa*, the pyr-4^{+} transformant, and the FGSC# pyr-4 mutant shown side by side.


ODCase wild type N. africana
A(286 nm)
0.161
0.1605
0.16
0.1595
0.159
0.1585
0.158
0
50
100
150
time


ODCase pyr-4 transformant
A(286 nm)
0.13
0.125
0.12
0.115
0.11
0.105
0
50
100
150
time


ODCase pyr-4 mutant
A(286nm)
0.14
0.135
0.13
0.125
0.12
0.115
0.11
0
50
100
150
time

Discussion

This work had two main goals; (1) to determine if IDCase activity could be detected in *N. africana* and (2) to transform FGSC# 2203 a triple mutant strain (pyr-4, uc-1, uc-2) to pyr-4^{+} to examine the effect of uc-1 in a pyr-4^{+} background. We began by growing and preparing the various strains in preparation for testing the IDCase enzyme activity. Once the samples were prepared the assay for the activity of the IDCase enzyme was run. The results obtained will be discussed in detail.

It might be hypothesized that all strains of Neurospora will have the thymidine salvage pathway known to be present in wild type *N. crassa*. IDCase is the final enzyme of this pathway and can be readily assayed in wild type *N. crassa*. *N. africana* was chosen to be investigated for the presence of IDCase activity. *N. africana* is a homothallic, aconidiate species whereas *N. crassa* is a heterothallic, conidiate species. This is one of the reasons why we decided to look at the activity present in *N. africana*. We expected to find IDCase activity present in *N. africana* based on our hypothesis that all species of Neurospora will contain the same thymidine salvage pathway present in *N. crassa*. Our study demonstrated significant IDCase activity in *N. africana*. We compared the amount of activity in *N. africana* to wild type *N. crassa* and found that *N. africana* had higher specific activity levels of the IDCase enzyme.

To examine the effect of uc-1 on IDCase activity levels the triple mutant strain FGSC# 2203 (pyr-4, uc-1, uc-2) was transformed to be pyr-4 $^{+}$. We expected that the transformant would not require the addition of uracil because the *de novo* pyrimidine pathway should be producing the required amount of uracil. We found that the

transformant would grow successfully in the absence of uracil. In order to determine a successful transformation a Southern blot was done on the transformant DNA and the membrane was probed for the presence of the pyr-4 gene. This showed that successful transformants had been isolated based on the presence of wild type EcoRI fragments containing the pyr-4 gene as well as the presence of ectopic fragments. The transformant was then assayed for the presence of IDCase activity and compared to the triple mutant strain as well as to wild type *N. crassa*. The comparison between the triple mutant and the transformant had to be completed in the presence of uracil because uracil is required for the triple mutant (FGSC# 2203) to grow. It was expected that the pyr-4^{+} transformant would have the same amount of activity as the triple mutant because it is believed that the uc-1 gene is responsible for maintaining low IDCase levels in wild type *N. crassa*. We hypothesized that restoration of pyr-4 will have no effect on the control mechanism of uc-1. This is based on the idea that the *de novo* pyrimidine pathway is not believed to be affected by the activity of the thymidine salvage pathway. We found that the amount of IDCase activity in the transformant was relatively close to that of the triple mutant and higher than that of wild type *N. crassa*. This was what had been hypothesized and supported the assumption that the uc mutations are having some affect on the activity of the IDCase enzyme.

The pyr-4 single mutant (FGSC# 4030) was also obtained and assayed for activity of the IDCase enzyme. We hypothesized that the activity of the salvage pathway is not affected by the presence or absence of the salvage pathway. Therefore, the pyr-4 mutant should display roughly equal amounts of IDCase activity as wild type *N. crassa*. Eva Marshall (Masters Thesis, 1999) also looked at the activity of IDCase in the pyr-4 mutant

(FGSC# 4030). She showed that the single mutant displayed only slightly higher activity than wild type *N. crassa*. When we looked at the activity in the single mutant we found that there was substantially higher IDCase activity than was present in wild type *N. crassa* . The pyr-4 mutant (FGSC# 4030) was compared to both wild type *N. crassa* and the pyr-4^{+} transformant in the presence of uracil. The hypothesis was that the pyr-4 mutant would have lower activity than the transformant due to the fact that we expected the uc mutations to affect the activity of the IDCase enzyme not the pyr-4 mutation. We hypothesized that the uc-1 mutations is responsible for maintaining low levels of IDCase activity. We found that the pyr-4 mutant had higher activity than both other strains with wild type having the lowest amount of activity. We found that the transformant averaged roughly half the activity of the pyr-4 mutant. The transformant had higher activity than wild type, which is what we had expected to see. We were then able to conclude that the uc mutations were having an affect on the activity of the IDCase enzyme which as we initially hypothesized.

ODCase is the final enzyme of the *de novo* pyrimidine pathway and it is affected by the presence or absence of the pyr-4 gene. ODCase catalyzes a decarboxylation reaction that converts OMP to UMP. The presence of the pyr-4 gene allows the enzyme to function normally. A mutation in the pyr-4 gene results in a defective *de novo* pathway by inactivating the ODCase enzyme. For this reason we tested the transformant for the presence of the ODCase enzyme. The ODCase enzyme was assayed using the UV spectrophotometer. We concluded that the transformation had been successful because the amount of activity of this particular enzyme in both wild type *N. crassa* and the pyr-4 transformant were comparable. Whereas the amount of activity present in the pyr-4

mutant was absent. Allowing the conclusion that the pyr-4 was successfully transformed into the triple mutant. The southern blot had also suggested that pyr-4 had been successfully transformed but the ODCase activity was much stronger evidence to the fact that the organism was a transformant.

Overall, we were able to make several conclusions. The first being that *N. africana* had the IDCase enzyme based on the fact that it displayed higher activity than wild type *N. crassa.* The next conclusion was that the transformation had been successful which allowed a comparison of the activity of both the IDCase enzyme and the ODCase enzyme to the mutant strains as well as wild type *N. crassa.* We were able to determine that the IDCase activity in the transformant was higher than wild type *N. crassa,* which was expected and was lower than the pyr-4 mutant. We were then led to conclude that the hypothesis that the uc mutations were having an affect on the activity of the IDCase enzyme was correct. Finally, we were able to conclude that the activity in the transformant was roughly equal to that of the triple mutant, which suggests that the pyr-4 mutation has little affect on the activity of the IDCase enzyme, which is what we had expected. This is due to the fact that we hypothesized that the presence or absence of the *de novo* pathway does not cause an increase in the activity of the salvage pathway. The fact that both species displayed roughly equal activities led to the conclusion that the *de novo* pathway has little or no affect on the salvage pathway.

Future studies will be to continue to investigate for the presence of IDCase activity in other homothallic and/or heterothallic species. To continue observing the effects of different genes on IDCase levels is also a possibility. Transformations to obtain single mutations in the pyr-4, uc-1, uc-2 mutant strain to determine which

mutation has the most important effect on IDCase activity. Upon determining the gene or genes that affect IDCase activity it may also be possible to sequence the gene or genes.

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